Supporting information

Strains and media

Saccharomyces kudriavzevii CR85 (supplied by Prof. A. Querol, Universitat de València, València, Spain) a wild isolate from oak bark (Ciudad Real, Spain) (1) (Table S5), was grown at 16 ˚C on YPD medium containing (10 $g{\cdot}l^{-1}$ Bacto Yeast extract, 20 $g{\cdot}l^{-1}$ Bacto Peptone and 20 $g{\cdot}l^{-1}$ glucose). All *S. cerevisiae* strains used in this study belong to the CEN.PK family (Table S5) (2-4) and were grown at 30 ˚C. Cultures for transformation were grown in YPD medium. For galactose induction of *SCEI*, overnight cultures were transferred to and grown for 4 h on YPGal medium (containing 20 $g{\cdot}l^{-1}$ galactose instead of glucose) prior to transformation (5). Synthetic media (SM) contained, per liter of demineralized water, 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7{\cdot}H_2O$, and trace elements (6). The pH was set at 6.0 by 1M KOH and filter-sterilized vitamin solution (5) was added after autoclaving the medium at 120 °C for 20 min. Glucose was separately sterilized at 110 °C and added to a final concentration of 20 $g{\cdot}l^{-1}$. Uracil auxotrophic strains were grown on SM supplemented with 150 $mg{\cdot}L^{-1}$ uracil (7). SM without nitrogen source (SMwn) was prepared by replacing $(NH_4)_2SO_4$ with 6.6 $g{\cdot}l^{-1}$ K_2SO_4. SMU, which was used for growth-rate determinations, was prepared by supplementing SMwn with 2.3 $g{\cdot}l^{-1}$ urea (filter sterilized). Use of urea as the nitrogen source prevents the strong acidification which occurs in ammonium-based SM (8). For transformation experiments with the amdSYM marker cassette (7), 1.8 $g{\cdot}l^{-1}$ acetamide was added to SMwn. For counter-selection of the *URA3* and *KlURA3* marker gene, SMwn was supplemented with 3.53 $g{\cdot}l^{-1}$ proline, 0.010 $g{\cdot}l^{-1}$ uracil and 0.20 $g{\cdot}l^{-1}$ 5-fluoroorotic acid (5-FOA) (Sigma Aldrich, St. Louis, MO). For selection of transformants carrying the marker genes kanMX (9), natNT1 or hphNT1 (10), 200 $mg{\cdot}l^{-1}$ G418 (11), 100 $mg{\cdot}l^{-1}$ nourseothricin or 200 $mg{\cdot}l^{-1}$ hygromycin, respectively, were added to complex media. Solid media were prepared by adding 2% (w/v) agar prior to autoclaving.

Plasmid propagation and isolation were performed with chemically competent *Escherichia coli* DH5α (Z-competent transformation kit; Zymo Research, Orange, CA) cultivated in lysogeny broth (LB) medium supplemented with 100 mg liter.1^{-1} ampicillin when required (12, 13). Frozen glycerol stocks were prepared by addition of glycerol (30% v/v) to exponentially growing shake-flask cultures of *S. cerevisiae* and overnight cultures of *E. coli* and stored aseptically in 1 mL aliquots at –80℃.

Molecular Biology techniques

PCR amplification for cloning purposes was performed with Phusion® Hot Start II High Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). To improve PCR efficiency, conditions in the PCR reaction as recommended by the supplier were modified by decreasing the primer concentration from 500 nM to 200 nM and increasing the polymerase concentration from 0.02 U μl^{-1} to 0.03 U μl^{-1}. Diagnostic PCR was performed with the DreamTaq PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's recommendations. Template genomic DNA for amplification of glycoblocks was isolated from *S. cerevisiae* CEN.PK113-7D and *S. kudriavzevii* CR85 using the Qiagen 100/G kit (Qiagen, Hilden, Germany). Genomic DNA for sequencing, PCR or restriction analysis was isolated with the YeaStar kit (Zymo Research, Irvine, CA). Plasmids maintained in *E. coli* DH5α were isolated with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). PCR products were separated in 1% (w/v) agarose (Sigma) gels in 1x TAE (40 mM Tris-acetate, pH 8.0 and 1 mM EDTA) or, when fragments were smaller than 500 bp, in 2% (w/v) agarose in 0.5x TBE (45 mM Tris-borate, pH 8.0, and 1 mM EDTA). Glycoblocks were isolated from gel using the Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA). Prior to transformation, fragments were pooled, maintaining equimolar concentrations (150 fmol per fragment) with the DNA fragments containing the marker (5). Yeast transformation was performed with the

LiAc/ssDNA method (14). Plasmids used in this study and primers used for their construction are described in Tables S6 and S7, respectively.

Construction of glycoblocks and marker cassettes.

The glycolytic gene cassettes flanked by SHR-sequences (glycoblocks) were obtained by extension PCR. Genomic DNA of *S. cerevisiae* CEN.PK113-7D or *S. kudriavzevii* CR85 was used as PCR template for amplification of glycolytic genes, including their native promoter and terminator sequences. Promoters of *S. cerevisiae* glycolytic genes were tentatively defined as the 800-bp sequences upstream of their start codon. When these 800-bp sequence overlapped with another, upstream gene, the promoter size was shortened to eliminate overlap with the coding sequence of the neighboring gene. Sequences of *S. kudriavzevii* CR85 glycolytic genes were kindly provided by Prof. Eladio Barrio (Universitat de València, València, Spain). For *S. kudriavzevii* genes, fragments of ca. 800 bp upstream of the genes were selected as promoter sequence. In all glycoblocks, terminator sequences comprised of the ca. 200 bp downstream of the respective stop codons. PCR primers used to construct the glycoblocks are described in Table S8 and the length of *S. cerevisiae* and *S. kudriavzevii* promoters used in this study are reported in Table S9.

To add extra restriction sites for HO and I-CreI endonucleases, enabling later excision of the single locus glycolysis, the *PDC1* glycoblock was prepared differently. *PDC1* was obtained by PCR amplification from CEN.PK113-7D genomic DNA using primers PDC1 Fw+RES and PDC1 Rv+M (Table S8). The fragment SYN2 was obtained by fusion PCR of oligonucleotides Syn2 Fw and Syn2 Rv using primers FUS2 Fw and FUS2 Rv (Fig. S8). The resulting product was cloned in a pCR™4Blunt-TOPO® vector and verified by restriction/digestion, resulting in pUD336. The glycoblock PDC1-SYN$_{MF}$, was obtained from pUD336, using primers FUS2 Fw and FUS2 Rv.

Coding sequences for *Homo sapiens* genes *TPI1* (muscle, splicing variant 1) and *PGK1* (muscle, splicing variant 1) were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) (Table S3), codon optimized (15) and chemically synthesized and cloned in plasmids pSYN-TPI1 and pSYN-PGK1 (GeneArt, Life Technologies, Table S4). These plasmids were used as templates for PCR amplification of the codon-optimized ORFs with specific primers (Table S7). Promoters and terminators of the corresponding *S. cerevisiae* orthologues were amplified from CEN.PK113-7D genomic DNA using primers that added overlapping sequences to the codon-optimized human ORFs (Table S7). The promoter, ORF and terminator fragments were mixed in equimolar amounts, normalized to 100 ng of the ORF, and stitched by fusion-PCR. The resulting products were cloned in pCR™4Blunt- TOPO®vectors and verified by restriction/digestion, yielding pUD329 (p*TPI1-HsTPI1-tTPI1*) and pUD331 (p*PGK1-HsPGK1-tPGK1*). Plasmids pUD329 and pUD331 were used as templates for the human *TPI1* and *PGK1* glycoblocks, respectively. The amdSYM and kanMX marker cassettes were obtained by PCR with pUGamdSYM (16) and pUG6 (17) as templates, respectively, using specific oligonucleotide primers (Table S8). All cassettes were gel-purified prior to transformation and DNA concentrations were measured with a NanoDrop 2000 spectrophotometer (wavelenght 260 nm) (Thermo Fisher Scientific).

Construction of deletion cassettes and CRISPR-Cas9 plasmids

Native *S. cerevisiae PYK1, PGI1, TPI1, TDH3* and *PGK1* genes were deleted using standard techniques and deletion cassettes were obtained as previously described (18) using the pDS-plasmid series (Table S6). Primers used for construction of deletion cassettes are given in Table S10. Cassettes were gel-purified and 500 ng of each cassette was used for yeast transformation. To enable CRISPR-Cas9 mediated genome editing, the gene encoding Cas9 (19, 20), driven by the constitutive *TEF1* promoter, was integrated in the genome of strain IMX511. Two fragments were constructed to replace the native locus of the deleted *PFK2* gene with *cas9* (Fig.

S4). A cassette containing *cas9* was obtained by PCR with p414-TEF1p-cas9-CYC1t (19) as template and primers CAS9 Fw+pfk2 and CAS9 Rv+link (Table S10). A second cassette containing the natNT1 marker gene was obtained by PCR on plasmid pUGnatNT1 with primers nat Fw+link and nat Rv+Rpt+pfk2 (Table S10). Both cassettes were gel purified and pooled in equimolar amounts. 500 ng of this mixture were used to transform strain IMX511, yielding strain IMX535 constitutively expressing Cas9. CRISPR-Cas9 editing was subsequently used to delete *HXK2*, *FBA1*, *ENO2*, *GPM1*, *PFK1*, *PDC1* and *ADH1* (20) (Fig. S2 and Table S5). To rescue the double strand DNA break (DSB) introduced by Cas9, 120 bp marker-free deletion cassettes (repair fragments) were used. These dsDNA repair fragments were constructed by annealing two complementary single-stranded oligonucleotides listed in Table S10 (20). Expression cassettes for the guide RNAs (gRNAs) used to target Cas9 to *HXK2*, *FBA1*, *ENO2*, *GPM1* and *PFK1*, flanked by SHR-sequences (21), were chemically synthesized (GeneArt). Plasmids containing the synthesized gDNAs, as supplied by the manufacturer, were used as templates for construction of the gRNA expression cassettes including the SHR-sequences by PCR. Primers are given in (Table S7). To incorporate the gRNA cassettes in a yeast expression vector, p426-GPD (18) was linearized by PCR with primers adding SHR-sequences corresponding to the SHR-sequences of the gRNA cassettes (Table S7). The gRNA cassettes were assembled into the p426 backbone by Gibson assembly (New England Biolabs, Ipswich, MA) following the manufacturer's recommendations (Fig. S9a). Each plasmid contained a single gRNA. For each deletion 100 ng of the appropriate CRISPR-plasmid was co-transformed with 1.5 µg of the corresponding repair fragment. In its native locus, the *S. cerevisiae ENO2* gene is closely flanked by other genes. To avoid interference with the expression of these adjacent genes, deletion sites were chosen that were also present in the *ENO2* glycoblock. To prevent deletion of the *ENO2* gene on the single locus glycolysis, two different repair fragments were used (Fig. S10).

PDC1 and *ADH1* were simultaneously deleted using Cas9. The two plasmids carrying the gRNAs targeting *PDC1* and *ADH1* were constructed using *in vivo* assembly. Plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t (19) was linearized with primers p426-crispr Fw and p426-crispr Rv. The 120 bp targeting fragments (*crPDC1* and *crADH1*) were obtained by annealing complementary oligonucleotides as previously described (20) (Table S7). A mix consisting of 100 ng of the linearized CRISPR-backbone, 300 ng of *crPDC1*, 300 ng of *crADH1* and 1.8 µg of each of the appropriate repair fragments (obtained as described above, Table S10) was used for transformation.

Two additional CRISPR-plasmids, targeting the amdSYM cassette and the flanking regions of the single-locus glycolysis gene cluster, respectively, were constructed. Plasmids were designed as previously described (20) (Fig. S9b). As described above, the linearized plasmid backbone was obtained with primers p426-crispr Fw and p426-crispr Rv from p426-SNR52p-gRNA.CAN1.Y-SUP4t (19) and the 120 bp targeting fragments (cramdSYM and crRECYCLE) were obtained by annealing complementing 120 bp oligo's (Table S7). The backbone and the desired targeting fragments were assembled into the CRISPR plasmids by Gibson assembly resulting in pUDE337 carrying cramdSYM and pUDE342 carrying crRECYCLE (Fig. S9b).

Construction of the SwYG strain

Fig. S2 provides an overview of the construction of the SwYG strain. A locus for chromosomal integration of the glycolytic gene cluster was prepared by introduction of an I-SceI restriction site at the *SGA1* locus on chromosome IX. Expression of *SGA1*, encoding a sporulation-specific glucoamylase, is induced by Ime1p in diploid cells during late sporulation but is repressed by Rme1p during vegetative growth of *S. cerevisiae* (22, 23). The cassette carrying the I-SceI recognition site targeted to *SGA1* also carried the *SCEI* gene which encodes an intron-encoded homing endonuclease, under the control of the galactose inducible promoter *GAL1* (5), and the

selection marker *KlURA3*. First the *SCEI/KlURA3* cassette was obtained by PCR using IMX221 genomic DNA as template (5) and the primers Tag G Fw and SGA1 Rv (Table S8, Fig. S11a). Fragment SYN1 (Fig. S11b), was obtained by mixing the oligonucleotides Syn1 Fw and Syn1 Rv. The resulting fragment SYN1 and the *SCEI/KlURA3* cassette were gel-purified and fused by fusion-PCR (18) using primers FUS1 Fw and FUS1 Rv (Table S8, Fig. S11b and S11c). The resulting product was cloned in a pCR™4Blunt-TOPO®vector (Invitrogen, Life Technologies), resulting in pUD335, which was checked by restriction analysis. The *KlURA3-SCEI* cassette was obtained by PCR from pUD335 using primers FUS1 Fw and FUS1 Rv (Table S8). *S. cerevisiae* strain IMX370 (24), which carries a minimal set of 13 glycolytic genes, was transformed with 100 ng of the *KlURA3-SCEI* cassette (Fig. S1a), resulting in IMX377. IMX377 also harbored additional restriction sites, recognized by the HO and I-CreI endonucleases, in its integrated *KlURA3-SCEI* cassette, and homologous flanking regions to promote recombination upon excision of the endogenous Single Locus Glycolysis (SinLoG) cassette (Fig. S1a).

The endogenous SinLoG cassette was assembled and integrated in IMX377 using the Combined *in vivo* Assembly and Targeted chromosomal Integration (CATI) approach (5). IMX377 was transformed with a mix consisting of the *S. cerevisiae* glycoblocks and the *amdS* marker cassette (*FBA1*$_{GH}$, *TPI1*$_{HP}$, *PGK1*$_{PQ}$, *ADH1*$_{QN}$, *PYK1*$_{NO}$, *TDH3*$_{OA}$, amdSYM$_{AB}$, *HXK2*$_{BC}$, *PGI*$_{CD}$, *PFK1*$_{DJ}$, *PFK2*$_{JK}$, *ENO2*$_{KL}$, *GPM1*$_{LM}$, *PDC1-SYN*$_{MF}$) (subscript letters indicate the SHR-sequences, Fig. S1b). The molar ratio of transformed fragments was 1:1 normalized to 150 ng of the amdSYM$_{AB}$ cassette. Transformants were selected on medium containing acetamide as sole nitrogen source. Clones were analysed for presence of all junctions between glycoblocks and selection markers with primers given in (Table S11). One of the colonies that showed correct PCR patterns was selected and named IMX382. This strain was further analyzed by sequencing a set of 14 PCR products obtained with primer pairs 1 to 14 (Table S11 and Fig.

S1d). All PCR products were pooled in a molar ratio of 1:1. From this set of 14 products a library of 300 bp insert was constructed and paired end sequenced (100bp paired end reads) using an Illumina HiSeq 2500 sequencer (BaseClear, Leiden, The Netherlands). Sequence reads were mapped onto the glycolytic genes cluster using Burrows-Wheeler Alignment tool (using "BWA mem" command; version 0.7.10-r789) and the resulting Alignment file (BAM file) was further processed by Pilon (version 1.10; using "--vcf --fix all,breaks" parameter (25)) for variant detection which were stored in VCF (Variant Call Format) file.

To construct the SwYG strain, the 13 genes made redundant by the newly added glycolytic cluster were removed from IMX382 in the following order: *PYK1, PGI1, TPI1, TDH3, PFK2, PGK1, GPM1, FBA1, HXK2, PFK1, ADH1, PDC1, ENO2* (Fig. S2). The natNT1, kanMX and hphNT1 marker cassettes were used for the deletion of *PYK1, PGI1* and *TPI1* respectively. These marker cassettes were excised using I-SceI as previously described (18) by transforming strain IMX493 with plasmid pUDC073 carrying *SCEI* (Fig. S3). *PFK2* was deleted by a cassette containing *cas9* and the natNT1 marker cassette (Fig. S4). The *KlURA3* and kanMX markers used for the subsequent deletion of *TDH3* and *PGK1* were recycled by the same I-SceI facilitated marker removal, by transforming the *SCEI* expressing plasmid pUDE206 to IMX557. Deletion of *GPM1, FBA1, HXK2* and *PFK1* was performed by the CRISPR/Cas9 system by transforming the appropriate CRISPR-plasmid and accompanying repair fragment. *ADH1* and *PDC1* were simultaneously deleted using the CRISPR/Cas9 cloning-free deletion method(20). Transformants were selected on SM and the CRISPR-plasmids were recycled by growing transformants overnight on YPD medium followed by plating on SM medium with 5-FOA. In order to restore a functional *ENO2* glycoblock to the single locus glycolysis, a glycoblock containing *ENO2* with a longer promoter sequence ($ENO2\text{-}LONG_{AB}$) was introduced to the SinLoG-IX cluster by replacing the $amdSYM_{AB}$ marker cassette in IMX583 resulting in strain IMX586 (Fig. S10). This was achieved by transforming CRISPR-plasmid

pUDE337 together with the *ENO2-LONG*$_{AB}$ glycoblock. Transformants were selected on SM. Subsequently, the endogenous *ENO2* gene could be deleted by co-transforming the CRISPR-plasmid pUDE326 and the corresponding repair fragments in IMX586 resulting in IMX587. Transformants were selected on SM. Finally the dysfunctional glycoblock ENO2$_{KL}$ was replaced by transforming 500 ng of marker cassette amdSYM$_{KL}$. Transformants were selected on SMwn with acetamide and one clone displaying the correct PCR profile was plated on medium with 5-FOA to recycle the CRISPR-plasmid and stocked as IMX589 (auxotrophic SwYG strain). To be able to perform growth experiments on SM, the uracil auxotrophy was repaired by transforming IMX589 with pUDE325, resulting in the prototrophic SwYG strain IMX606.

Construction of glycolytic gene clusters in the *CAN1* locus

The SinLoG clusters introduced in the *CAN1* locus were obtained by transforming IMX589 with a mix of glycoblocks for assembly and targeted integration of the desired glycolytic design. To facilitate the targeted integration into the genome, a similar approach to the CATI approach was chosen, but the CRISPR/Cas9 system was used instead of I-SceI to promote the formation of a double strand break and therefore integration of the SinLoG clusters at the targeted locus. Therefore 300 ng of p426-SNR52p-gRNA.CAN1.Y-SUP4t plasmid coding for the gRNA targeting the *CAN1* locus(19) was co-transformed with the glycoblocks and with a cassette carrying the kanMX selection marker. The glycoblocks for the native SinLoG$_V$ were: *FBA1*$_{can1H}$, *TPI1*$_{HP}$, *PGK1*$_{PQ}$, *ADH1*$_{QN}$, *PYK1*$_{NO}$, *TDH3*$_{OA}$, *ENO2*$_{AB}$, *HXK2*$_{BC}$, *PGI*$_{CD}$, *PFK1*$_{DJ}$, *PFK2*$_{JK}$, *GPM1*$_{LM}$, *PDC1*$_{Mcan1}$. For the *S.k.* SinLoG were used: *skFBA1*$_{can1H}$, *skTPI1*$_{HP}$, *skPGK1*$_{PQ}$, *skADH1*$_{QN}$, *skPYK1*$_{NO}$, *skTDH1*$_{OA}$, *skHXK2*$_{BC}$, *skPGI*$_{CD}$, *skPFK1*$_{DJ}$, *skPFK2*$_{JK}$, *skENO2*$_{KL}$, *skGPM1*$_{LM}$, *skPDC1*$_{Mcan1}$. For the mosaic SinLoG the following mixture was transformed: *scFBA1*$_{can1H}$, *hsTPI1*$_{HP}$, *hsPGK1*$_{PQ}$, *skADH1*$_{QN}$, *scPYK1*$_{NO}$, *scTDH3*$_{OA}$, *scHXK2*$_{BC}$, *skPGI*$_{CD}$, *skPFK1*$_{DJ}$, *skPFK2*$_{JK}$, *skENO2*$_{KL}$, *skGPM1*$_{LM}$, *scPDC1*$_{Mcan1}$. Cassettes

were mixed in a 1:1 molar ratio normalized to 140 ng of the kanMX cassette. Selection was on SM for presence of the CRISPR-plasmid, which contained the *URA3* marker. For each transformation eight clones were plated to medium selective for kanMX. Resistant clones were analyzed by PCR for presence of the full SinLoG clusters with primers given in Table S12. For each glycolytic variant, a correctly assembled strain was grown on complex medium and plated on SM proline with 5-FOA and uracil to recycle the CRISPR-plasmid. The resulting strains were stocked on SM acetamide supplemented with uracil (IMX591, IMX607, IMX633).

Excision of the native SinLoG cassette from chromosome IX

The native SinLoG, integrated at the *SGA1* locus, was removed from strains IMX591, IMX607 and IMX633 using CRISPR-Cas9. To this end, 100 ng of the CRISPR-plasmid pUDE342 was transformed into these strains, together with 1.5 µg of the recycle repair fragment (Table S10 and Fig. S12). Transformants were selection on SM glucose plates and analyzed for correct removal of the endogenous SinLoG by PCR with primers SGA1 Fw and SGA1 Rv (Table S12).

skTDH1 overexpression in IMX637.

A plasmid backbone, PCR amplified with primers p426-rv+O and p426-fw+A (Table S5) and plasmid p426-GPD as the template, and the *skTDH1* glycoblock were assembled *in vitro* using Gibson assembly (New England Biolabs, Beverly, MA, USA), resulting in the plasmid pUDESkTDH1 (Table S4). IMX637 was plated on complex medium with 5-FOA to counterselect the pUDE342 plasmid. A selected colony was then transformed with 100 ng of the pUDEskTDH1 plasmid and transformants were selected on SM. One transformant was stocked as IMX652.

Sequencing

Genomic DNA of strains IMX589 (auxotrophic SwYG strain), IMX605 (endogenous SinLoG on chromosome V), IMX637 (*Sk*SinLoG) and IMX645 (mosaic SinLoG) was sequenced.

Ilumina Nextera libraries (300-bp insert size) were constructed and paired-end sequenced (100 bp reads) using an Illumina HISeq 2500 sequencer at Baseclear BV (Leiden, The Netherlands). A minimum quantity of 750 Mb was generated, representing a minimum 60-fold coverage. Genome sequences were *de novo* assembled using the gsAssembler (version 2.6) software package, also known as the Newbler software package (454 Life Sciences, Branford, CT). To verify deletions in IMX589, all contigs were mapped to the *in silico* design after gene removal using Clustal X in Clone Manager 9 (Sci-Ed Software, Cary, NC). The sequences are accessible at NCBI (http://www.ncbi.nlm.nih.gov/) under the bioproject number PRJNA317665.

To verify correct integration of the different glycolytic gene clusters, contigs were mapped to the *in silico* design. To exclude possible duplications of glycolytic genes, a copy number variation analysis was performed with the Magnolya algorithm (26) (Fig. S6).

To identify any unintended changes at the nucleotide level, all sequence libraries of samples IMX372 (24), IMX589, IMX605, IMX637 and IMX645 were processed by an in-house pipeline hosted in Galaxy (https://galaxyproject.org/). Sequence data were mapped to the genome of *S. cerevisiae* CEN.PK113-7D (2) for whole genome comparison, as well as to the *in silico* design of the SinLoG present each sequenced strain. The Burrows-Wheeler Alignment tool (BWA, version 0.7.10-r789) was used and the resulting binary alignment file (BAM file) was further processed using SAMtoolsmpileup (version 0.1.18) and bcftools (from the SAMtools package) to compute the genotype likelihood and stores these likelihoods in Binary variant call format (BCF). The script vcfutils.pl was used, with parameter varFilter and maximum read depth 400, to filter and convert to variant call format (VCF). The resulting VCF files were annotated and effects of variants on genes were predicted by the snpEff package (version 3.4). To compare IMX589 to IMX372, the called and annotated variants in both IMX589 and IMX372 samples were subtracted from sample IMX589 with the "subtract whole

dataset from another dataset" tool in Galaxy. The same procedure was followed to compare IMX605, IMX637 and IMX645 to IMX589.

Determination of specific growth rates in shake-flask cultures

Glycerol stocks from strains IMX372 (MG), IMX606 (prototrophic SwYG strain), IMX605 (endogenous SinLoGv), IMX637 (*Sk*SinLoG), IMX652 (*Sk*SinLoG with *SkTDH1* overexpression), IMX645 (mosaic SinLoG) were inoculated in 100 ml SM urea + 2% glucose (w/v) in 500 ml shake flasks and grown to late exponential phase. Cells were harvested and immediately transferred to pre-warmed 500 ml flasks containing the same medium at an OD660 of 0.2. Biomass formation was followed by measuring OD660. Concentrations of extracellular metabolites in culture supernatants were measured by HPLC using a Aminex HPX-87H ion exchange column operated at 60 ˚C with 5 mM H_2SO_4 as mobile phase at an isocratic flow rate of 0.6 ml·min^{-1}. Data reported in the results section are calculated based on at least two independent culture replicates.

Determination of *in vitro* enzyme activities

Cell extracts were prepared as previously described (27) from culture samples (ca. 60 mg biomass dry weight) taken from mid-exponential phase shake-flask cultures. Spectrophotometric assays of glycolytic enzyme activities were done as previously described (28), except for phosphofructokinase, whose activity was determined as described by Cruz and co-workers (29). Enzyme activities are expressed as µmol substrate converted (mg protein)$^{-1}$ h^{-1}. Protein concentrations in the cell extracts were determined as described by Lowry and co-workers(30) with bovine serum albumin as a standard. Reported enzyme activities are based on measurements on at least two independent culture replicates, with at least two analytical replicates for each assay.

Quantitative physiological analyses of SwYG strains in aerobic batch cultures

For analysis of quantitative physiology, SwYG strains IMX605, IMX606, IMX645 and IMX652 were grown in duplicate aerobic batch cultures in bioreactors. SM was supplemented with 20 $g.L^{-1}$ glucose as sole carbon-source and 0.2 $g.L^{-1}$ antifoam Emulsion C (Sigma, St. Louise, USA). Batch cultures were inoculated at an initial OD660 of 0.3 with in water resuspended cells obtained from exponentially growing cultures on identical medium. Aerobic batch cultures were performed at a working volume of 1.4 L in 2 L bioreactors (Applikon, Schiedam, The Netherlands). Culture conditions were a temperature of 30 °C, constant agitation at 800 rpm, sparging of 700 $mL·min^{-1}$ dried, compressed air (Linde Gas Benelux, The Netherlands) and a pH of 5.0, maintained by automatic addition of 2 M KOH and 2 M H_2SO_4. Biomass concentrations as culture dry biomass were measured by filtering samples of 10 mL culture through pre-dried filters (pore-size 0.45um, Whatman / GE Healthcare Life Sciences, United Kingdom) and drying in a microwave oven at 360W for 20 minutes, as adapted from (27). Additionally biomass concentrations were more frequently determined by measuring the optical density at 660 nm (OD660). Biomass concentrations measured as OD660 and culture dry weight were linearly correlated (coefficients of determination were at least 0.997). Based on these experimentally determined linear correlations, dry biomass concentrations were calculated using OD660 measurements and were used to estimate specific growth rates by simple linear regression of the natural logarithm of dry biomass concentrations as function of culture age.

Extracellular glucose, ethanol, glycerol and acetate concentrations were determined by high-performance liquid chromatography (HPLC) analysis of culture supernatants obtained by centrifugation of samples (3 min. at 20.000 g). HPLC analysis was performed using a Agilent HPLC equipped with a Bio-Rad Aminex HPX-87H column at 60 °C and 5 mM H_2SO_4 as a

mobile phase at a flow rate of 0.6 $mL\cdot min^{-1}$ using, coupled to a UV and RI detector (Agilent, Santa Clara, USA).

CO_2 and O_2 concentrations in the exhaust gas were analysed using a Rosemount NGA 2000 analyser (Baar, Switzerland), after cooling by means of a condenser (2 °C) and drying using a PermaPure Dryer (model MD 110-8P-4; Inacom Instruments, Veenendaal, the Netherlands) of the gas.

Independent culture duplicates were performed for each tested strain. Carbon balances for all cultures closed within 5%.

Previously published data were used for the MG strain (31). The aerobic batch cultures of the MG strain were performed in conditions scrupulously identical to those described above.

Supporting Table S1. Amino acid substitutions identified in the proteins encoded by the SinLoG genes of the constructed strains as compared to the *in silico* design

Systematic name	Name	Type	Amino acid change
SwYG strain (IMX589)			
YOL086C	*ADH1*	Synonymous	A180A
SinLoG-V strain (IMX605)			
YOL086C	*ADH1*	Non-synonymous	R212G
YGR240C	*PFK1*	Non-synonymous	T118A
***Sk*-SinLoG-V strain (IMX637)**			
No syst. name	*SkPYK1*	Synonymous	A167G
Mosaic-SinLoG-V strain (IMX645)			
None detected			

Supporting Table S2. Amino acid substitutions identified in the constructed strains as compared to the most relevant parental strains

Systematic name	Name	Type	Amino acid change
SwYG strain (IMX589) vs. Minimal Glycolysis strain (IMX372)			
YBR079W	*VPS15*	Non-synonymous	E474K
YJL212C	*OPT1*	Non-synonymous	I463T
YNL245C	*CWC25*	Non-synonymous	P62L
YDL079C	*MRK1*	Synonymous	I190I
YLR180W	*SAM1*	Synonymous	V217V
YNL262W	*POL2*	Synonymous	F1536F
SinLoG-V strain (IMX605) vs SwYGstrain (IMX589)			
YNL215W	*IES2*	Non-synonymous	E160G
***Sk*-SinLoG-V strain (IMX637) vs SwYG strain (IMX589)**			
YGL195W	*GCN1*	Non-synonymous	G427C
Mosaic-SinLoG-V strain (IMX645) vs SwYG strain (IMX589)			
YDR539W	*FDC1*	Non-synonymous	P117S

Supporting Table S3. Comparison between *S. cerevisiae* and *S. kudriavzevii or H. sapiens* of the DNA and protein sequence of the glycolytic genes used in the SinLoG gene cluster.

Prefix *Sc*, *Sk* and *Hs* indicate the gene origin, i.e. *S. cerevisiae*, *S. kudriavzevii* and human respectively. *S. cerevisiae* CEN.PK 113-7D sequences were compared to *S. kudriavzevii* IFO1802 (http://sss.genetics.wisc.edu/cgi-bin/s3.cgi) and *H. sapiens* (http://www.ncbi.nlm.nih.gov/, accession number NP_000356.1 for *HsTP1* and NP_000282.1 for *HsPGK1*) sequences by BLASTN and BLASTX analysis according to (32).

Gene		% sequence identity	
		Gene	Protein
S. kudravzevii vs S. cerevisiae			
ScHXK2	*SkHXK2*	90	96
ScPGI1	*SkPGI1*	91	98
ScPFK1	*SkPFK1*	89	98
ScPFK2	*SkPFK2*	90	98
ScFBA1	*SkFBA1*	95	95
ScTPI1	*SkTPI1*	95	97
ScTDH3	*SkTDH1*	88	89
ScPGK1	*SkPGK1*	97	99
ScGPM1	*SkGPM1*	96	97
ScENO2	*SkENO2*	97	98
ScPYK1	*SkPYK1*	95	97
ScPDC1	*SkPDC1*	95	98
ScADH1	*SkADH1*	95	96
H. sapiens vs S. cerevisiae			
ScTPI1	*HsTP1*	- [a]	53
ScPGK1	*HsPGK1*	- [a]	66

[a] The DNA sequence was not compared because the human gene was codon-optimized.

Supporting Table S4. Sequence of synthetic DNA constructs

Bold and underlined sequences represent the protospacer region of CRISPR-Cas9 single guide RNAs

Description	Sequence
Homo sapiens TPI1, muscle, splicing variant 1, codon-optimized	ATGGCTCCATCTAGAAAGTTCTTCGTCGGTGGTAACTGGAAGATGAACGGTAGAAAGCAATCT TTAGGTGAATTGATCGGTACCTTGAACGCTGCTAAGGTCCCAGCTGACACCGAAGTTGTCTGT GCTCCACCAACCGCTTACATCGACTTCGCTAGACAAAAGTTGGACCCAAAGATCGCTGTCGCT GCTCAAAACTGCTACAAGGTCACCAACGGTGCTTTCACCGGTGAAATCTCTCCAGGTATGATT AAGGACTGCGGTGCTACCTGGGTCGTTTTGGGTCACTCTGAAAGAAGACACGTCTTCGGTGAA TCTGACGAATTGATCGGTCAAAAGGTCGCTCACGCTTTGGCTGAAGGTTTGGGTGTCATTGCT TGTATCGGTGAAAAGTTGGACGAAAGAGAAGCTGGTATCACCGAAAAGGTCGTCTTCGAACAA ACCAAGGTCATTGCTGACAACGTCAAGGACTGGTCTAAGGTCGTCTTGGCTTACGAACCAGTC TGGGCTATTGGTACCGGTAAGACCGCTACCCCACAACAAGCTCAAGAAGTCCACGAAAAGTTG AGAGGTTGGTTGAAGTCCAACGTCTCTGACGCTGTCGCTCAATCCACCAGAATCATTTACGGT GGTTCTGTCACCGGTGCTACCTGCAAGGAATTGGCTTCTCAACCAGACGTCGACGGTTTCTTG GTCGGTGGTGCTTCTTTGAAGCCAGAATTCGTCGACATCATTAACGCTAAGCAATAA
Homo sapiens PGK1, muscle, splicing variant 1, codon-optimized	ATGTCTTTGTCTAACAAGTTGACTTTGGACAAGTTGGACGTTAAGGGTAAGAGAGTCGTCATG AGAGTTGACTTCAACGTTCCAATGAAGAACAACCAAATTACCAACAACCAAAGAATTAAGGCT GCTGTCCCATCTATTAAGTTCTGTTTGGACAACGGTGCTAAGTCTGTCGTCTTGATGTCTCAC TTGGGTAGACCAGACGGTGTTCCAATGCCAGACAAGTACTCCTTGGAACCAGTTGCTGTTGAA TTGAAGTCTTTGTTGGGTAAGGACGTCTTGTTCTTGAAGGACTGCGTCGGTCCAGAAGTCGAA AAGGCTTGTGCTAACCCAGCTGCTGGTTCCGTCATTTTGTTGGAAAACTTGAGATTCCACGTC GAAGAAGAAGGTAAGGGTAAGGACGCTTCTGGTAACAAGGTTAAGGCTGAACCAGCTAAGATT GAAGCTTTCAGAGCTTCTTTGTCTAAGTTGGGTGACGTTTACGTCAACGACGCTTTCGGTACC GCTCACAGAGCTCACTCTTCTATGGTTGGTGTTAACTTGCCACAAAAGGCTGGTGGTTTCTTG ATGAAGAAGGAATTGAACTACTTCGCTAAGGCTTTGGAATCTCCAGAAAGACCATTCTTGGCT ATCTTGGGCGGTGCTAAGGTCGCTGACAAGATTCAATTGATCAACAACATGTTGGACAAGGTC AACGAAATGATTATTGGTGGTGGTATGGCTTTCACCTTCTTGAAGGTCTTGAACAACATGGAA ATTGGTACCTCCTTGTTCGACGAAGAAGGTGCTAAGATCGTCAAGGACTTGATGTCTAAGGCT GAAAAGAACGGTGTTAAGATCACCTTGCCAGTTGACTTCGTCACCGCTGACAAGTTCGACGAA AACGCTAAGACCGGTCAAGCTACCGTCGCTTCCGGTATTCCAGCTGGTTGGATGGGTTTGGAC TGTGGTCCAGAATCTTCTAAGAAGTACGCTGAAGCTGTCACCAGAGCTAAGCAAATTGTCTGG AACGGTCCAGTCGGTGTCTTCGAATGGGAAGCTTTCGCTAGAGGTACCAAGGCTTTGATGGAC GAAGTCGTCAAGGCTACCTCTAGAGGTTGTATTACCATTATTGGTGGTGGTGACACCGCTACC TGTTGTGCTAAGTGGAACACCGAAGACAAGGTTTCCCACGTCTCTACCGGTGGTGGTGCTTCT TTGGAATTGTTAGAAGGTAAGGTCTTGCCAGGTGTCGACGCTTTGTCTAACATTTAA
FBA1 gRNA + SHR	ATTGCGTCAGAGCCCTCGATGAGACTAAGTTCGGAGAATCTATGGCAGCCTC AGCTTGTG TCTTTGAAAAGATAATGTATGATTATGCTTTCACTCATATTTATACAGAAAC TTGATGTT TTCTTTCGAGTATATACAAGGTGATTACATGTACGTTTGAAGTACAACTCTA GATTTTGT AGTGCCCTCTTGGGCTAGCGGTAAAGGTGCGCATTTTTTCACACCCTACAAT GTTCTGTT CAAAAGATTTTGGTCAAACGCTGTAGAAGTGAAAGTTGGTGCGCATGTTTCG GCGTTCGA AACTTCTCCGCAGTGAAAGATAAATGATC**AATTAACGGCCAGTCCACTG**GTT TTAGAGCT AGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGC ACCGAGTC

	GGTGGTGCTTTTTTTGTTTTTTATGTCTTCATTAGCCCGCAGTGTGTGGTCC TCCAGAAC TCAAATGAAGGCATACTACACAGATTCC
ENO2 gRNA + SHR	TCGAAGCAGTGTTAATTCCGTGCCCGACAGAACACATGGAAAGGACACTTCC CTAACTAGAGACATAAAAAACAAAAAAAGCACCACCGACTCGGTGCCACTTT TTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAA AAC**CTTCCAGTGCATTATGCAAT**GATCATTTATCTTTCACTGCGGAGAAGTT TCGAACGCCGAAACATGCGCACCAACTTTCACTTCTACAGCGTTTGACCAAA ATCTTTTGAACAGAACATTGTAGGGTGTGAAAAAATGCGCACCTTTACCGCT AGCCCAAGAGGGCACTACAAAATCTAGAGTTGTACTTCAAACGTACATGTAA TCACCTTGTATATACTCGAAAGAAAACATCAAGTTTCTGTATAAATATGAGT GAAAGCATAATCATACATTATCTTTTCAAAGATCTGGCAGTCCATTGGCATG CCAGCCCTGCGATTATTTGTTCATACCGGCCAGTAGGATG
HXK2 gRNA + SHR	TCATTAGCCCGCAGTGTGTGGTCCTCCAGAACTCAAATGAAGGCATACTACA CAGATTCCAGACATAAAAAACAAAAAAAGCACCACCGACTCGGTGCCACTTT TTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAA AAC**GCATGATAGCCATTTCTAGG**GATCATTTATCTTTCACTGCGGAGAAGTT TCGAACGCCGAAACATGCGCACCAACTTTCACTTCTACAGCGTTTGACCAAA ATCTTTTGAACAGAACATTGTAGGGTGTGAAAAAATGCGCACCTTTACCGCT AGCCCAAGAGGGCACTACAAAATCTAGAGTTGTACTTCAAACGTACATGTAA TCACCTTGTATATACTCGAAAGAAAACATCAAGTTTCTGTATAAATATGAGT GAAAGCATAATCATACATTATCTTTTCAAAGAGCATCTCTATAACTGGTGTC GCTGAACTACCATGTACTGCCCATGCGGCAAATGAATCCA
GPM1 gRNA + SHR	GCATCTCTATAACTGGTGTCGCTGAACTACCATGTACTGCCCATGCGGCAAA TGAATCCATCTTTGAAAAGATAATGTATGATTATGCTTTCACTCATATTTAT ACAGAAACTTGATGTTTTCTTTCGAGTATATACAAGGTGATTACATGTACGT TTGAAGTACAACTCTAGATTTTGTAGTGCCCTCTTGGGCTAGCGGTAAAGGT GCGCATTTTTTCACACCCTACAATGTTCTGTTCAAAAGATTTTGGTCAAACG CTGTAGAAGTGAAAGTTGGTGCGCATGTTTCGGCGTTCGAAACTTCTCCGCA GTGAAAGATAAATGATC**TGATGTCTAAGTAACCTTTA**GTTTTAGAGCTAGAA ATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCG AGTCGGTGGTGCTTTTTTTGTTTTTTATGTCTTCGAAGCAGTGTTAATTCCG TGCCCGACAGAACACATGGAAAGGACACTTCCCTAACTAG
PFK1 gRNA + SHR	ATCGACCCTGCATCGTGTTGCGATTCTACGGACGCTGAGTGGCCAGCCATTC ATATAACGAGACATAAAAAACAAAAAAAGCACCACCGACTCGGTGCCACTTT TTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAA AAC**CATCATAGTGAGGCGCGCTA**GATCATTTATCTTTCACTGCGGAGAAGTT TCGAACGCCGAAACATGCGCACCAACTTTCACTTCTACAGCGTTTGACCAAA ATCTTTTGAACAGAACATTGTAGGGTGTGAAAAAATGCGCACCTTTACCGCT AGCCCAAGAGGGCACTACAAAATCTAGAGTTGTACTTCAAACGTACATGTAA TCACCTTGTATATACTCGAAAGAAAACATCAAGTTTCTGTATAAATATGAGT GAAAGCATAATCATACATTATCTTTTCAAAGAATTGCGTCAGAGCCCTCGAT GAGACTAAGTTCGGAGAATCTATGGCAGCCTCAGCTTGTG

Supporting Table S5. Strains used in this study

Strain	Relevant genotype	Source
IMX372 (Minimal Glycolysis, prototrophic MG)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxk1::KlLEU2 tdh1::KlURA3 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4*	(33)
IMX221	*MATa ura3-52 MAL2-8c SUC2 spr3::(TagG-KlURA3-* P_{GAL1}*-SCEI-*T_{cyc1}*-TagF)*	(5)
S. kudriavzevii CR85	*Wild isolate*	(34)
IMX370 (auxotrophic MG)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4*	(33)
IMX377	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(TagG-KlURA3-* P_{GAL1}*-SCEI-*T_{cyc1}*-TagF)*	This study
IMX382	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ amdSYM$_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ *PDC1-SYN*$_{MF}$*)*	This study
IMX457	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ amdSYM$_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ *PDC1-SYN*$_{MF}$*), pyk1::*natNT1	This study
IMX492	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc, pdc, adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ amdSYM$_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ *PDC1-SYN*$_{MF}$*) pyk1::*NatNT1 *pgi1::*kanMX	This study

IMX493	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(FBA1*$_{GH}$ *TPI1*$_{HP}$ *PGK1*$_{PQ}$ *ADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ amdSYM$_{AB}$ *HXK2*$_{BC}$ *PGI1*$_{CD}$ *PFK1*$_{DJ}$ *PFK2*$_{JK}$ *ENO2*$_{KL}$ *GPM1*$_{LM}$ *PDC1-SYN*$_{MF}$*), pyk1::*NatNT1 *pgi1::*kanMX *tpi1::*hphNT1	This study
IMX509	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(FBA1*$_{GH}$ *TPI1*$_{HP}$ *PGK1*$_{PQ}$ *ADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ amdSYM$_{AB}$ *HXK2*$_{BC}$ *PGI1*$_{CD}$ *PFK1*$_{DJ}$ *PFK2*$_{JK}$ *ENO2*$_{KL}$ *GPM1*$_{LM}$ *PDC1-SYN*$_{MF}$*) pyk1::*NatNT1*, pgi1::*kanMX*, tpi1::*hphNT1 pUDC073*(CEN6/ARS4 ori URA3 GAL1pr-SCEI-CYC1ter)*	This study
IMX510	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(FBA1*$_{GH}$ *TPI1*$_{HP}$ *PGK1*$_{PQ}$ *ADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ amdSYM$_{AB}$ *HXK2*$_{BC}$ *PGI1*$_{CD}$ *PFK1*$_{DJ}$ *PFK2*$_{JK}$ *ENO2*$_{KL}$ *GPM1*$_{LM}$ *PDC1-SYN*$_{MF}$*) pyk1 pgi1 tpi1*	This study
IMX511	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2, tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(FBA1*$_{GH}$ *TPI1*$_{HP}$ *PGK1*$_{PQ}$ *ADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ amdSYM$_{AB}$ *HXK2*$_{BC}$ *PGI1*$_{CD}$ *PFK1*$_{DJ}$ *PFK2*$_{JK}$ *ENO2*$_{KL}$ *GPM1*$_{LM}$ *PDC1*$_{MF}$*) pyk1 pgi1 tpi1 tdh3::*kanMX	This study
IMX535	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(FBA1*$_{GH}$ *TPI1*$_{HP}$ *PGK1*$_{PQ}$ *ADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ amdSYM$_{AB}$ *HXK2*$_{BC}$ *PGI1*$_{CD}$ *PFK1*$_{DJ}$ *PFK2*$_{JK}$ *ENO2*$_{KL}$ *GPM1*$_{LM}$ *PDC1-SYN*$_{MF}$*) pyk1 pgi1 tpi1 tdh3::*kanMX *pfk2::(pTEF-cas9-tCYC1* natNT1*)*	This study
IMX557	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(FBA1*$_{GH}$ *TPI1*$_{HP}$ *PGK1*$_{PQ}$ *ADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ amdSYM$_{AB}$ *HXK2*$_{BC}$ *PGI1*$_{CD}$ *PFK1*$_{DJ}$ *PFK2*$_{JK}$ *ENO2*$_{KL}$ *GPM1*$_{LM}$ *PDC1-SYN*$_{MF}$*) pyk1 pgi1 tpi1 tdh3::*kanMX *pfk2::(pTEF-cas9-tCYC1*natNT1*) pgk1::KlURA3*	This study

IMX561	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ amdSYM$_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ PDC1-SYN$_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1*	This study
IMX566	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ amdSYM$_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ PDC1-SYN$_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1 gpm1*	This study
IMX568	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ amdSYM$_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ PDC1-SYN$_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEFcas9-tCYC1* natNT1*) pgk1 gpm1 fba1*	This study
IMX570	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ amdSYM$_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ PDC1-SYN$_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2*	This study
IMX571	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ amdSYM$_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ PDC1-SYN$_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1*	This study
IMX583	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ amdSYM$_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ PDC1-SYN$_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1*	This study

IMX586	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ $ENO2_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ $PDC1\text{-}SYN_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1*	This study
IMX587	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6, adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ $ENO2_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $ENO2_{KL}$ $GPM1_{LM}$ $PDC1\text{-}SYN_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2*	This study
IMX589 (auxotrophic SwYG)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ $ENO2_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $AmdSYM_{KL}$ $GPM1_{LM}$ $PDC1\text{-}SYN_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2*	This study
IMX606 (prototrophic SwYG)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ $ENO2_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $AmdSYM_{KL}$ $GPM1_{LM}$ $PDC1\text{-}SYN_{MF}$*)pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2* pUDE325	This study
IMX591	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(*$FBA1_{GH}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ $ENO2_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $AmdSYM_{KL}$ $GPM1_{LM}$ $PDC1\text{-}SYN_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2 can1::(*$FBA1_{can1H}$ $TPI1_{HP}$ $PGK1_{PQ}$ $ADH1_{QN}$ $PYK1_{NO}$ $TDH3_{OA}$ $ENO2_{AB}$ $HXK2_{BC}$ $PGI1_{CD}$ $PFK1_{DJ}$ $PFK2_{JK}$ $KanMX_{KL}$ $GPM1_{LM}$ $PDC1_{Mcan1}$*)*	This study

IMX607	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(FBA1*$_{GH}$ *TPI1*$_{HP}$ *PGK1*$_{PQ}$ *ADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ *ENO2*$_{AB}$ *HXK2*$_{BC}$ *PGI1*$_{CD}$ *PFK1*$_{DJ}$ *PFK2*$_{JK}$ *AmdSYM*$_{KL}$ *GPM1*$_{LM}$ *PDC1-SYN*$_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2 can1::(SkFBA1*$_{can1H}$ *SkTPI1*$_{HP}$ *SkPGK1*$_{PQ}$ *SkADH1*$_{QN}$ *SkPYK1*$_{NO}$ *SkTDH1*$_{OA}$ *KanMX*$_{AB}$ *SkHXK2*$_{BC}$ *SkPGI1*$_{CD}$ *SkPFK1*$_{DJ}$ *SkPFK2*$_{JK}$ *SkENO2*$_{KL}$ *SkGPM1*$_{LM}$ *SkPDC1*$_{Mcan1}$*)*	This study
IMX633	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(FBA1*$_{GH}$ *TPI1*$_{HP}$ *PGK1*$_{PQ}$ *ADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ *ENO2*$_{AB}$ *HXK2*$_{BC}$ *PGI1*$_{CD}$ *PFK1*$_{DJ}$ *PFK2*$_{JK}$ *AmdSYM*$_{KL}$ *GPM1*$_{LM}$ *PDC1-SYN*$_{MF}$*) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2 can1::(FBA1*$_{can1H}$ *pTPI1-HsTPI1-tTPI1*$_{HP}$ *pPGK1-HsPGK1-tPGK1*$_{PQ}$*, SkADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ *KanMX*$_{AB}$ *HXK2*$_{BC}$ *SkPGI1*$_{CD}$ *SkPFK1*$_{DJ}$ *SkPFK2*$_{JK}$ *SkENO2*$_{KL}$ *SkGPM1*$_{LM}$ *PDC1*$_{Mcan1}$*)*	This study
IMX605	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1 pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2 can1::(FBA1*$_{can1H}$ *TPI1*$_{HP}$ *PGK1*$_{PQ}$ *ADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ *ENO2*$_{AB}$ *HXK2*$_{BC}$ *PGI1*$_{CD}$ *PFK1*$_{DJ}$ *PFK2*$_{JK}$ *KanMX*$_{KL}$ *GPM1*$_{LM}$ *PDC1*$_{Mcan1}$*)* pUDE342	This study
IMX637	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1 pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*), pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2 can1::(SkFBA1*$_{can1H}$ *SkTPI1*$_{HP}$ *SkPGK1*$_{PQ}$ *SkADH1*$_{QN}$ *SkPYK1*$_{NO}$ *SkTDH1*$_{OA}$ *KanMX*$_{AB}$ *SkHXK2*$_{BC}$ *SkPGI1*$_{CD}$ *SkPFK1*$_{DJ}$ *SkPFK2*$_{JK}$ *SkENO2*$_{KL}$ *SkGPM1*$_{LM}$ *SkPDC1*$_{Mcan1}$*) pUDE342*	This study
IMX645	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1 pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2, can1::(FBA1*$_{can1H}$ *pTPI1-HsTPI1-tTPI1*$_{HP}$ *pPGK1-HsPGK1-tPGK1*$_{PQ}$*, SkADH1*$_{QN}$ *PYK1*$_{NO}$ *TDH3*$_{OA}$ *KanMX*$_{AB}$ *HXK2*$_{BC}$ *SkPGI1*$_{CD}$ *SkPFK1*$_{DJ}$ *SkPFK2*$_{JK}$ *SkENO2*$_{KL}$ *SkGPM1*$_{LM}$ *PDC1*$_{Mcan1}$*)* pUDE342	This study
IMX652	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1 pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1* natNT1*) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2 can1::(SkFBA1*$_{can1H}$ *SkTPI1*$_{HP}$ *SkPGK1*$_{PQ}$ *SkADH1*$_{QN}$ *SkPYK1*$_{NO}$ *SkTDH1*$_{OA}$ *KanMX*$_{AB}$ *SkHXK2*$_{BC}$ *SkPGI1*$_{CD}$ *SkPFK1*$_{DJ}$ *SkPFK2*$_{JK}$ *SkENO2*$_{KL}$ *SkGPM1*$_{LM}$ *SkPDC1*$_{Mcan1}$*)* pUDESkTDH1	This study

Supporting Table S6. Plasmids used in this study

Plasmid	Characteristic	Source
For construction of KO cassettes:		
pDS1	*I-SceI*$_{rec}$-*Ag*$_{p}$*TEF2-kanMX-Ag* $_{t}$*TEF2-I-SceI*$_{rec}$	(18)
pDS3	*I-SceI*$_{rec}$-*KlURA3-I-SceI*$_{rec}$	(18)
pDS6	*I-SceI*$_{rec}$-*natNT1-SceI*$_{rec}$	(18)
pDS7	*I-SceI*$_{rec}$-*hphNT1--I-SceI*$_{rec}$	(18)
pUGamdSYM	*amdSYM*	(35)
pUG6	*kanMX*	(36)
pUGnatNT1	*natNT1*	(36)
For construction of assembly cassettes:		
pSYN-TPI1	Human codon optimized *TPI1* in pMA-T vector	this study
pSYN-PGK1	Human codon optimized *PGK1* in pMK-RQ vector	this study
pUD335	pCR™4BluntTOPO + TagG-*SCEI/KLURA3*-TagF	this study
pUD336	pCR™4BluntTOPO + *PDC1*-SYN$_{MF}$	this study
pUD331	pCR™4BluntTOPO + *HsPGK1*	this study
pUD329	pCR™4BluntTOPO + *HsTPI1*	this study
For CRISPR/Cas9:		
p414-TEF1p-cas9-CYC1t	$_{p}$*TEF1-cas9-*$_{t}$*CYC1*	(19)
p426-SNR52p-gRNA.CAN1.Y-SUP4t	$_{p}$*SNR52-gRNA.CAN1.Y-*$_{t}$*SUP4*	(19)
P426-GPD	Episomal plasmid	(37)
pUDE324	$_{p}$*SNR52*-gRNA.*GPM1-*$_{t}$*SUP4*	this study
pUDE325	*SNR52p-gRNA.FBA1-SUP4t*	this study
pUDE326	*SNR52p-gRNA.ENO2-SUP4t*	this study

pUDE327	*SNR52p-gRNA.HXK2-SUP4t*	this study
pUDE342	*SNR52p-gRNA.SGA1-SUP4t* RECYCLE SinLoG	this study
pUDE337	*SNR52p-gRNA.AmdSYM-SUP4t*	this study
pUDE329	*SNR52p-gRNA.PFK1-SUP4t*	this study
For Marker recycling		
pUDC073	*pGAL1-SCEI-tCYC1 CEN6/ARS4 URA3*	(5)
PUDE206	*TPI1p-SCEI-TEF1*, episomal, *AgTEF2-hphNT1-CYC1t*	this study
For overexpression of *SkTDH1*		
pUDESkTDH1	*SkTDH1*, episomal, *URA3*	this study

Supporting Table S7: Primers used to construct plasmids.

Plasmid	Name	Sequence 5'--> 3'
pUDE327	p426 Fw + V	GCATCTCTATAACTGGTGTCGCTGAACTACCATGTACTGCCCATGCGGCAAATGAATCCAGCATCTGTGCGGTATTTCACACC
	p426 Rv + U	GGAATCTGTGTAGTATGCCTTCATTTGAGTTCTGGAGGACCACACACTGCGGGCTAATGACTCAAAGGCGGTAATACGGTTATCC
	U Fw	TCATTAGCCCGCAGTGTGTGGTCC
	V Rv	TGGATTCATTTGCCGCATGGGC
pUDE325	p426 Fw + U	TCATTAGCCCGCAGTGTGTGGTCCTCCAGAACTCAAATGAAGGCATACTACACAGATTCCGCATCTGTGCGGTATTTCACACC
	p426 Rv + S	CACAAGCTGAGGCTGCCATAGATTCTCCGAACTTAGTCTCATCGAGGGCTCTGACGCAATCTCAAAGGCGGTAATACGGTTATCC
	S Fw	ATTGCGTCAGAGCCCTCGATGAGAC
	U Rv	GGAATCTGTGTAGTATGCCTTCATTTG
pUDE326	p426 Fw + X	TCTGGCAGTCCATTGGCATGCCAGCCCTGCGATTATTTGTTCATACCGGCCAGTAGGATGGCATCTGTGCGGTATTTCACACC
	p426 Rv + W	CTAGTTAGGGAAGTGTCCTTTCCATGTGTTCTGTCGGGCACGGAATTAACACTGCTTCGACTCAAAGGCGGTAATACGGTTATCC
	W Fw	TCGAAGCAGTGTTAATTCCGTGC
	X Rv	CATCCTACTGGCCGGTATGAAC
pUDE324	p426 Fw + W	TCGAAGCAGTGTTAATTCCGTGCCCGACAGAACACATGGAAAGGACACTTCCCTAACTAGGCATCTGTGCGGTATTTCACACC
	p426 Rv + V	TGGATTCATTTGCCGCATGGGCAGTACATGGTAGTTCAGCGACACCAGTTATAGAGATGCCTCAAAGGCGGTAATACGGTTATCC
	V Fw	GCATCTCTATAACTGGTGTCGCTGAAC
	W Rv	CTAGTTAGGGAAGTGTCCTTTCCATG
pUDE329	p426 Fw + S	ATTGCGTCAGAGCCCTCGATGAGACTAAGTTCGGAGAATCTATGGCAGCCTCAGCTTGTGGCATCTGTGCGGTATTTCACACC
	p426 Rv + R	CGTTATATGAATGGCTGGCCACTCAGCGTCCGTAGAATCGCAACACGATGCAGGGTCGATCTCAAAGGCGGTAATACGGTTATCC
	R Fw	ATCGACCCTGCATCGTGTTG
	S rv	CACAAGCTGAGGCTGCCATAG
p426 CRISPR-backbone	p426-crispr Fw	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC
	p426-crispr Rv	GATCATTTATCTTTCACTGCGGAGAAG
crADH1	crADH1 Fw	GAGTTAGCATATCTACAATTGGGTGAAATGGGGAGCGATTTGCAGGCATTTGCTCGGCATGCTCTATTGTTCGCACCACCGGCAAACTCGCGTCTCGCAAGTCTTGGCTCATTCTTCTAG
	crADH1 Rv	CTAGAAGAATGAGCCAAGACTTGCGAGACGCGAGTTTGCCGGTGGTGCGAACAATAGAGCATGCCGAGCAAATGCCTGCAAATCGCTCCCCATTTCACCCAATTGTAGATATGCTAACTC
crPDC1	crPDC1 Fw	ATCGAGGTGTCTAGTCTTCTATTACGCTAATGCAGTTTCAGGGTTTTGGAAACCACACTGTCAAGTTGAAGACTATATATTTTATTGAGTTTATGTTATGGGGAGGCTACCCTTTACGTC

	crPDC1 Rv	GACGTAAAGGGTAGCCTCCCCATAACATAAACTCAATAAAATATATAGTCTTCAACTTGACAGTGTGGTTTCCAAAACCCTGAAACTGCATTAGCGTAATAGAAGACTAGACACCTCGAT
crAmdSYM	crAmdSYM Fw	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCTGGTTGAACAAGTACGACGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
	crAmdSYM Rv	GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCGTCGTACTTGTTCAACCAGATCATTTATCTTTCACTGCGGAGAAGTTTCGAACGCCGAAACATGCGCA
crRECYCLE	crRECYCLE Fw	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCTTACAATATAGTGATAATCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
	crRECYCLE Rv	GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCGATTATCACTATATTGTAAGATCATTTATCTTTCACTGCGGAGAAGTTTCGAACGCCGAAACATGCGCA
pUD329	pTPI1 Fw	TAGTGTGAGCGGGATTTAAACTGTG
	pTPI1 Rv + link	TCCAGTTACCACCGACGAAGAACTTTCTAGATGGAGCCATTTTTAGTTTATGTATGTGTTTTTTGTAGTTATAGATTTAAGC
	tTPI1 Fw + link	GAAGCCAGAATTCGTCGACATCATTAACGCTAAGCAATAAGATTAATATAATTATATAAAAATATTATCTTCTTTTCTTTATATCTAGTG
	tTPI1 Rv	GCGAAAATGACGCTTGCAGTG
	TPI1 Fw	ATGGCTCCATCTAGAAAGTTCTTCG
	TPI1 Rv	TTATTGCTTAGCGTTAATGATGTCG
pUD331	pPGK1 Fw	CCTGCATTTAAAGATGCCGATTTGG
	pPGK1 Rv + link	CGTCCAACTTGTCCAAAGTCAACTTGTTAGACAAAGACATTGTTTTATATTTGTTGTAAAAAGTAGATAATTACTTC
	tPGK1 Fw + link	TAAGGTCTTGCCAGGTGTCGACGCTTTGTCTAACATTTAAATTGAATTGAATTGAAATCGATAGATCAATTTTTTTC
	tPGK1 Rv	ATTTTAGCGTAAAGGATGGG
	PGK1 Fw	TAAATGTTAGACAAAGCGTCGACACC
	PGK1 Rv	ATGTCTTTGTCTAACAAGTTGACTTTGG
p426SkTDH1 backbone	p426 rv + O	ATACTCCCTGCACAGATGAGTCAAGCTATTGAACACCGAGAACGCGCTGAACGATCATTCCTCAAAGGCGGTAATACGGTTATCC
	p426 fw + A	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGCCAGATCATCAATAGGCACGCATCTGTGCGGTATTTCACACC

Supporting Table S8. Primers used to construct the glycolytic gene cassettes and the *SCEI* cassette.

Product	Name	Sequence 5'--> 3'
***SCEI* cassette**		
	Syn1 Fw	ACATTTACAATATAGTGATAATCGTGGACTAGAGCAAGATTTCAAATAAGTAACAGCAGCAGTTTCA GCTTTCCGCAACAGTATAATTTCAAAACGTCGTAC
	Syn1 Rv	AAGGGCCATGACCACCTGATGCACCAATTAGGTAGGTCTGGCTATGTCTATACCTCTGGCTCAAAAC GTCGTACGACGTTTTGAAATTATACTGTTGCGGAAAGCTGAAACTGCTGC
	Tag G Fw	GCCAGAGGTATAGACATAGCCAGAC
	SGA1 Rv	TCTACAAACTCTGTAAAACTTCTTGTCTTATTTGATAGGCATCCCAGAATGAAGTATAGGGCCGAAC TTTCCCTGTATGAAGC
	FUS1 Fw	TTACAATATAGTGATAATCGTGGACTAGAG
	FUS1 Rv	CAAACTCTGTAAAACTTCTTGTCTTATTTG
Assembly: Marker cassettes		
$AmdSYM_{AB}$	AmdSYM Fw + A	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGCCAGATCATCAATAGGCACGCGACAT GGAGGCCCAGAATACC
	AmdSYM Rv + B	GTTGAACATTCTTAGGCTGGTCGAATCATTTAGACACGGGCATCGTCCTCTCGAAAGGTGAGTATAG CGACCAGCATTCACATACG
$AmdSYM_{KL}$	AmdSYM Fw + K	AAGATAGTCGCCGAACTCGCAAGAGTCATTAACACCTCGCAATTGATGGGAAGTCCTCGCGCGACAT GGAGGCCCAGAATACC
	AmdSYM Rv + L	GCCGTAGCTTCCGCAAGTATGCCGTAGTTGAAGAGCATTTGCCGTCGGTTCAGGTCATATAGTATAG CGACCAGCATTCACATACG
$kanMX_{AB}$	kanMX Fw + A	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGCCAGATCATCAATAGGCACGCGACAT GGAGGCCCAGAATACC
	kanMX Rv + B	GTTGAACATTCTTAGGCTGGTCGAATCATTTAGACACGGGCATCGTCCTCTCGAAAGGTGAGTATAG CGACCAGCATTCACATACG
$kanMX_{KL}$	kanMX Fw + K	AAGATAGTCGCCGAACTCGCAAGAGTCATTAACACCTCGCAATTGATGGGAAGTCCTCGCGCGACAT GGAGGCCCAGAATACC
	kanMX Rv + L	GCCGTAGCTTCCGCAAGTATGCCGTAGTTGAAGAGCATTTGCCGTCGGTTCAGGTCATATAGTATAG CGACCAGCATTCACATACG

Assembly: *Saccharomyces cerevisiae* cassettes:		
TPI1$_{HP}$	TPI Fw + P	CTGATAGTGCTGTAAGTCGCCTCCATCTTAGCAGAGCTGTCCCTGAATGCGTACTCGTGAGCGAAAATGACGCTTGCAGTG
	TPI Rv + H	AGATTACTCTAACGCCTCAGCCATCATCGGTAATAGCTCGAATTGCTGAGAACCCGTGACTAGTGTGAGCGGGATTTAAACTGTG
PGK1$_{QP}$	PGK1 Fw + Q	GAGCTGAATGTATATGCTGCGGGATCATTGCACAGCTCTGAGAGCCCTGCAACGCGATATCCTGCATTTAAAGATGCCGATTTGG
	PGK1 Rv + P	TCACGAGTACGCATTCAGGGACAGCTCTGCTAAGATGGAGGCGACTTACAGCACTATCAGATTTTAGCGTAAAGGATGGGGAAAGAG
PYK1$_{NO}$	PYK1 Fw + N	GATCAGCAGCCACGATTGAGTCCTAACGAAGATATGTGGACCTTGCATCAAAGCCTAGAAAAATAGCCGCCATGACCTCG
	PYK1 Rv + O	ATACTCCCTGCACAGATGAGTCAAGCTATTGAACACCGAGAACGCGCTGAACGATCATTCTGCATTTATGTACCCATGTATAACCTTCC
ADH1$_{QN}$	ADH1 Fw + Q	ATATCGCGTTGCAGGGCTCTCAGAGCTGTGCAATGATCCCGCAGCATATACATTCAGCTCGCCGGTAGAGGTGTGGTCAATAAG
	ADH1 Rv + N	TTCTAGGCTTTGATGCAAGGTCCACATATCTTCGTTAGGACTCAATCGTGGCTGCTGATCGAGGAAACAGCAATAGGGTTGCTAC
TDH3$_{OA}$	TDH3 Fw + O	GAATGATCGTTCAGCGCGTTCTCGGTGTTCAATAGCTTGACTCATCTGTGCAGGGAGTATAAATTTCACTCAGCATCCACAATGTATCAG
	TDH3 Rv + A	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCATAGCCATGCCTTCACATATAGTGAATACGTAAATAATTAATAGTAGTGATTTTCCTAAC
HXK2$_{BC}$	HXK2 Fw + B	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATTCGACCAGCCTAAGAATGTTCAACGACGGCACCGGGAAATAAACC
	HXK2 Rv + C	CTAGCGTGTCCTCGCATAGTTCTTAGATTGTCGCTACGGCATATACGATCCGTGAGACGTGCAAGAGAAAAAAACGAGCAATTGTTAAAAG
PGI1$_{CD}$	PGI1 Fw + C	ACGTCTCACGGATCGTATATGCCGTAGCGACAATCTAAGAACTATGCGAGGACACGCTAGTTCGCGACACAATAAAGTCTTCACG
	PGI1 Rv + D	AATCACTCTCCATACAGGGTTTCATACATTTCTCCACGGGACCCACAGTCGTAGATGCGTCTGAAGAAGGCATACTACGCCAAG
FBA1$_{GH}$	FBA1 Fw + G	GCCAGAGGTATAGACATAGCCAGACCTACCTAATTGGTGCATCAGGTGGTCATGGCCCTTAGTGCATGACAAAAGATGAGCTAGG

	FBA1 Rv + H	GTCACGGGTTCTCAGCAATTCGAGCTATTACCGATGATGGCTGAGGCGTTAGAGTAATCTAAAATCTCAAAAATGTGTGGGTCATTACG
FBA1$_{can1H}$	FBA1 Fw + can1	GGTGTATGACTTATGAGGGTGAGAATGCGAAATGGCGTGGGAATGTGATTAAAGGTAATAAGTGCATGACAAAAGATGAGCTAGG
	FBA1 Rv + H	GTCACGGGTTCTCAGCAATTCGAGCTATTACCGATGATGGCTGAGGCGTTAGAGTAATCTAAAATCTCAAAAATGTGTGGGTCATTACG
PFK1$_{DJ}$	PFK1 Fw + J	CGACGAGATGCTCAGACTATGTGTTCTACCTGCTTGGACATCTTCGCGTATATGACGGCCTGTCGTCTTCGTGAACCATTGTC
	PFK1 Rv + D	ACGCATCTACGACTGTGGGTCCCGTGGAGAAATGTATGAAACCCTGTATGGAGAGTGATTTCGAGATTCCTCAATCCATACACCATTATAG
PFK2$_{JK}$	PFK2 Fw + J	GGCCGTCATATACGCGAAGATGTCCAAGCAGGTAGAACACATAGTCTGAGCATCTCGTCGGAGATCCGAGGGACGTTTATTGG
	PFK2 Rv + K	GCGAGGACTTCCCATCAATTGCGAGGTGTTAATGACTCTTGCGAGTTCGGCGACTATCTTATAGCCATTCTCTGCTGCTTTGTTG
ENO2$_{KL}$	ENO2 Fw + K	AAGATAGTCGCCGAACTCGCAAGAGTCATTAACACCTCGCAATTGATGGGAAGTCCTCGCGAAGCCCACTTTCGTGGACTTTG
	ENO2 Rv + L	GCCGTAGCTTCCGCAAGTATGCCGTAGTTGAAGAGCATTTGCCGTCGGTTCAGGTCATATCCTTCCAGTGCATTATGCAATAGACAG
ENO2-LONG$_{AB}$	ENO2 Fw + A	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGCCAGATCATCAATAGGCACAAGTGCTACAGAAATCCTACTCTTGCC
	ENO2 Rv + B	GTTGAACATTCTTAGGCTGGTCGAATCATTTAGACACGGGCATCGTCCTCTCGAAAGGTGCCAGCTGATTGAAGGTTCTCAAAGTGAC
GPM1$_{LM}$	GPM1 Fw + L	ATATGACCTGAACCGACGGCAAATGCTCTTCAACTACGGCATACTTGCGGAAGCTACGGCCATGTCATGTCACCATTAATTACCACTC
	GPM1 Rv + M	ACGAGAGATGAAGGCTCACCGATGGACTTAGTATGATGCCATGCTGGAAGCTCCGGTCATGGTATATTTCTTAATGTGGAAAGATACTAGCG
PDC1$_{Mcan1}$	PDC1 Fw + can1	GATGAGAAAAGTAAAGAATTGTATCCATTGCGCTCTTTCCCGACGAGAGTAAATGGCGAGTTTAAACAGTGTTCCTTAATCAAGGATAC
	PDC1 Rv + M	ATGACCGGAGCTTCCAGCATGGCATCATACTAAGTCCATCGGTGAGCCTTCATCTCTCGTGCCGAAATGCATGCAAGTAACC
PDC1-SYN$_{MF}$	PDC1 Fw + RES	CGATTTCTTGTGTAACAGAAGTTTCAGCTTTCCGCAACAGTATAATTTCAAAACGTCGTACGACGTTTTGATTTAAACAGTGTTCCTTAATCAAGGATAC

	PDC1 Rv + M	ATGACCGGAGCTTCCAGCATGGCATCATACTAAGTCCATCGGTGAGCCTTCATCTCTCGTGCCGAAATGCATGCAAGTAACC
	Syn2 Fw	TGCCGAACTTTCCCTGTATGAAGCGATCTGACCAATCCTTTGCCGTAGTTTCAACGTATGGCTGCTGTTACTTATTTGAAATCTTGCTCTAGTCCACGATTATCACTATATTGTAAATG
	Syn2 Rv	TCAAAACGTCGTACGACGTTTTGAAATTATACTGTTGCGGAAAGCTGAAACTTCTGTTACACAAGAAATCGTACATTTACAATATAGTGATAATCGTGGACTAGAGCAAGATTTC
	FUS2 Fw	ATGACCGGAGCTTCCAGCATG
	FUS2 Rv	TGCCGAACTTTCCCTGTATGAAGC
***Saccharomyces kudriavzevii* cassettes:**		
skTPI1$_{HP}$	skTPI Fw + P	CTGATAGTGCTGTAAGTCGCCTCCATCTTAGCAGAGCTGTCCCTGAATGCGTACTCGTGACAAATCCCAATTTTTCACGGACGGTAATC
	skTPI Rv + H	AGATTACTCTAACGCCTCAGCCATCATCGGTAATAGCTCGAATTGCTGAGAACCCGTGACGACAAGAGAGAAGACCCAGGGATG
skPGK1$_{QP}$	skPGK1 Fw + Q	GAGCTGAATGTATATGCTGCGGGATCATTGCACAGCTCTGAGAGCCCTGCAACGCGATATGGATCTTAGCTTCAACTCAAGATGTACAG
	skPGK1 Rv + P	TCACGAGTACGCATTCAGGGACAGCTCTGCTAAGATGGAGGCGACTTACAGCACTATCAGGCCTAAATAAATGAAGTAAATGCGAGGTAAGC
skPYK1$_{NO}$	skPYK1 Fw + O	ATACTCCCTGCACAGATGAGTCAAGCTATTGAACACCGAGAACGCGCTGAACGATCATTCTAGCATAAGATGCTACATCTTAGGATTCTG
	skPYK1 Rv + N	GATCAGCAGCCACGATTGAGTCCTAACGAAGATATGTGGACCTTGCATCAAAGCCTAGAACCTTATGTTATGGATATTCTTCTTCTTGCG
skADH1$_{QN}$	skADH1 Fw + Q	ATATCGCGTTGCAGGGCTCTCAGAGCTGTGCAATGATCCCGCAGCATATACATTCAGCTCAACTCGTTGCTGGAGCTAGCATAC
	skADH1 Rv + N	TTCTAGGCTTTGATGCAAGGTCCACATATCTTCGTTAGGACTCAATCGTGGCTGCTGATCGCATAACCGGTAGAGTACTTTGGAGTC
skTDH3$_{OA}$	skTDH1 Fw + O	GAATGATCGTTCAGCGCGTTCTCGGTGTTCAATAGCTTGACTCATCTGTGCAGGGAGTATTGGAAAAGAGGATAGGAAGGAGGAGAAG
	skTDH1 Rv + A	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCATAGCCATGCCTTCACATATAGTTAAAGCACATTTAACCTTTCTCGCTACC
skHXK2$_{BC}$	skHXK2 Fw + B	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATTCGACCAGCCTAAGAATGTTCAACGACCTTAGTCCATTGACGTCTGTATTTG

	skHXK2 Rv + C	CTAGCGTGTCCTCGCATAGTTCTTAGATTGTCGCTACGGCATATACGATCCGTGAGACGTGAGGTCAATCATACACCGGAAGAAAG
$skPGI1_{CD}$	skPGI1 Fw + C	ACGTCTCACGGATCGTATATGCCGTAGCGACAATCTAAGAACTATGCGAGGACACGCTAGTGTTCCAAGACACCAAGAATGTCATAC
	skPGI1 Rv + D	AATCACTCTCCATACAGGGTTTCATACATTTCTCCACGGGACCCACAGTCGTAGATGCGTGCTTGATAATCAAAGCAGCGCACAG
$skFBA1_{can1H}$	skFBA1 Fw + can1	GGTGTATGACTTATGAGGGTGAGAATGCGAAATGGCGTGGGAATGTGATTAAAGGTAATAATGCCGACACGCGTTATGCAAAG
	skFBA1 Rv + H	GTCACGGGTTCTCAGCAATTCGAGCTATTACCGATGATGGCTGAGGCGTTAGAGTAATCTACGGCTTGAACAACAATGCCAACC
$skPFK1_{DJ}$	skPFK1 Fw + J	CGACGAGATGCTCAGACTATGTGTTCTACCTGCTTGGACATCTTCGCGTATATGACGGCCTTCATTGCTCATTGTTATGTGTATCATATCG
	skPFK1 Rv + D	ACGCATCTACGACTGTGGGTCCCGTGGAGAAATGTATGAAACCCTGTATGGAGAGTGATTCCTTTATATTTTATGACACCATCTTCCGTACAC
$skPFK2_{JK}$	skPFK2 Fw + J	GGCCGTCATATACGCGAAGATGTCCAAGCAGGTAGAACACATAGTCTGAGCATCTCGTCGGATTCGAAGGACGTTTATTGGGAATATC
	skPFK2 Rv + K	GCGAGGACTTCCCATCAATTGCGAGGTGTTAATGACTCTTGCGAGTTCGGCGACTATCTTCTTCGAATGCACGGCAATAATGATACG
$skENO2_{KL}$	skENO2 Fw + K	AAGATAGTCGCCGAACTCGCAAGAGTCATTAACACCTCGCAATTGATGGGAAGTCCTCGCGTCATCTGGATCCCATACTTTACGAGAAAC
	skENO2 Rv + L	GCCGTAGCTTCCGCAAGTATGCCGTAGTTGAAGAGCATTTGCCGTCGGTTCAGGTCATATACTTCAGGAAGTCCCGCCGTGTG
$skGPM1_{LM}$	skGPM1 Fw + L	ATATGACCTGAACCGACGGCAAATGCTCTTCAACTACGGCATACTTGCGGAAGCTACGGCGCAAGGAGTCCCAGGCCTTAATTTTC
	skGPM1 Rv + M	ACGAGAGATGAAGGCTCACCGATGGACTTAGTATGATGCCATGCTGGAAGCTCCGGTCATGGACACTTTAACTGGGGCCATATC
$skPDC1_{Mcan1}$	skPDC1 Fw + can1	GATGAGAAAAGTAAAGAATTGTATCCATTGCGCTCTTTCCCGACGAGAGTAAATGGCGAGTGCAGCTATCAGGTTTTGCTTTACAATTG
	skPDC1 Rv + M	ATGACCGGAGCTTCCAGCATGGCATCATACTAAGTCCATCGGTGAGCCTTCATCTCTCGTTCCAGACGGAAAAACCGCACGAG

***Homo sapiens* cassettes:**

hsTPI1$_{HP}$	hsTPI Fw + H	AGATTACTCTAACGCCTCAGCCATCATCGGTAATAGCTCGAATTGCTGAGAACCCGTGACTAGTGTG AGCGGGATTTAAACTGTG
	hsTPI Rv + P	CTGATAGTGCTGTAAGTCGCCTCCATCTTAGCAGAGCTGTCCCTGAATGCGTACTCGTGAGCGAAAA TGACGCTTGCAGTG
hsPGK1$_{QP}$	hsPGK1 Fw + P	TCACGAGTACGCATTCAGGGACAGCTCTGCTAAGATGGAGGCGACTTACAGCACTATCAGATTTTAG CGTAAAGGATGGGG
	hsPGK1 Rv + Q	GAGCTGAATGTATATGCTGCGGGATCATTGCACAGCTCTGAGAGCCCTGCAACGCGATATCCTGCAT TTAAAGATGCCGATTTGG

Supporting Table S9. Length of *S. cerevisiae* and *S. kudriavzevii* promoters used in this study.

Gene	*S. cerevisiae* (bp)	*S. kudriavzevii* (bp)
FBA1	517	808
TPI1	513	819
PGK1	667	769
ADH1	964	992
PYK1	860	1099
TDH1		948
TDH3	632	
ENO2	(411[a]) 1012	840
HXK2	479	843
PGI1	697	850
PFK1	904	1028
PFK2	804	1026
GPM1	431	769
PDC1	864	908

[a] This promoter size did not result in functional expression of *ENO2*.

Supporting Table S10. Primers used to construct the deletion cassettes.

Product	Name	Sequence 5'--> 3'
Deletion cassettes		
PYK1 KO	PYK1 KO Fw	TTTCCCCCCTTATTTTTTTTTTTGTTAGAATTGATCCAAATGTAAATAAACAATCACAACGGTCGTAATTGAGTCAGTTACGCTAGG
	PYK1 KO Rv	GATTAAACCACCAAACGAAGGCCAGAAGCTGAACATAGTTCACTGGCATCCGTTGTGATTGTTTATTTACATTTGGATCAATTCTAACAACGTGCGTAGAATGAAGAACCTATATTACC
PGI1 KO	PGI KO Fw	ACAGTTGATGAGAACCTTTTTCGCAAGTTCAAGGTGCTCTAATTTTTAAAATTTTTACTTGTCGTAATTGAGTCAGTTACGCTAGG
	PGI KO Rv	TTAACTTACTTAGAATAATGCCATTTTTTTGAGTTATAATAATCCTACGTTTTGAGAAGATGTTCTTATTCAAATTTCAACTGTTATATACGTGCGTAGAATGAAGAACCTATATTACC
TPI1 KO	TPI KO Fw	TTGGCAATTTTTTGCTCTTCTATATAACAGTTGAAATTTGAATAAGAACATCTTCTCAAAGTCGTAATTGAGTCAGTTACGCTAGG
	TPI KO Rv	TTAACTTACTTAGAATAATGCCATTTTTTTGAGTTATAATAATCCTACGTTTTGAGAAGATGTTCTTATTCAAATTTCAACTGTTATATACGTGCGTAGAATGAAGAACCTATATTACC
TDH3 KO	TDH3 KO Fw	AACTTTAAAAAAAAAAGCCAATATCCCCAAAATTATTAAGAGCGCCTCCATTATTAACTAGTCGTAATTGAGTCAGTTACGCTAGG
	TDH3 KO Rv	AACACGCTTTTTCAGTTCGAGTTTATCATTATCAATACTGCCATTTCAAATAGTTAATAATGGAGGCGCTCTTAATAATTTTGGGGATATCGTGCGTAGAATGAAGAACCTATATTACC
PGK1 KO	PGK1 KO Fw	CATAATAGGCATTTGCAAGAATTACTCGTGAGTAAGGAAAGAGTGAGGAACTATCGCATAGTCGTAATTGAGTCAGTTACGCTAGG
	PGK1 KO Rv	TATATATACGTATATAAATAAAAAATATTCAAAAAATAAAATAAACTATTTATGCGATAGTTCCTCACTCTTTCCTTACTCACGAGTAATCGTGCGTAGAATGAAGAACCTATATTACC
pfk2::CAS9/natNT1		
CAS9/nat	CAS9 Fw + pfk2	TGTATAAATATTGTATTAAAAGGGTACCTTTATAAATATGAGATCCGAGGCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTC
	CAS9 Rv + link	CGAAGTTATATTAAGGGTTGTCGACCTGCAGCGTACGAAGCTTCAGCTGGGTACCGGCCGCAAATTAAAGCCTTCG
	nat Fw + link	TGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCGGCCGGTACCCAGCTGAAGCTTCGTACGCTGCAG
	nat Rv + Rpt +pfk2	CAGCATTGTAAGTACTACCATCTATATAGATCGAATATCCTACTAATATATGTGCGTTAAATACTTTGTTCTCTTATTATCTATGAACTTGCATAGGCCACTAGTGGATCTGATATCAC
Repair fragments		
GPM1	GPM1 repair Fw	CACATGCAGTGATGCACGCGCGATGGTGCTAAGTTACATATATATATATATATATATATATTATAGCAATAACCTTGATGTTTACATCGTAGTTTAATGTACACCCCGCGAATTCGTTCA

	GPM1 repair Rv	TGAACGAATTCGCGGGGTGTACATTAAACTACGATGTAAACATCAAGGTTATTGCTATAATATATATATATATATATATATATGTAACTTAGCACCATCGCGCGTGCATCACTGCATGTG
HXK2	HXK2 repair Fw	TTTCTAATGCCTTTTCCATCATGTTACTACGAGTTTTCTGAACCTCCTCGCACATTGGTAGCTTAATTTTAAATTTTTTTGGTAGTAAAAGATGCTTATATAAGGATTTCGTATTTATTG
	HXK2 repair Rv	CAATAAATACGAAATCCTTATATAAGCATCTTTTACTACCAAAAAAATTTAAAATTAAGCTACCAATGTGCGAGGAGGTTCAGAAAACTCGTAGTAACATGATGGAAAAGGCATTAGAAA
PFK1	PFK1 repair Fw	AGGCCGACAAATAAACCAAACGGTATTCGTAGACCGATGACAATACGACTACAATTAAGGCATGTTTTTCCATCGTTTTCAACGATGACTGTAACCCGTAGATTGAACCAGGCATGCCAA
	PFK1 repair Rv	TTGGCATGCCTGGTTCAATCTACGGGTTACAGTCATCGTTGAAAACGATGGAAAAACATGCCTTAATTGTAGTCGTATTGTCATCGGTCTACGAATACCGTTTGGTTTATTTGTCGGCCT
FBA1	FBA1 repair Fw	ACTCCAAAATGAGCTATCAAAAACGATAGATCGATTAGGATGACTTTGAAATGACTCCGCAACTATTACGTATTACGATAATCCTGCTGTCATTATCATTATTATCTATATCGACGTAT
	FBA1 repair Rv	ATACGTCGATATAGATAATAATGATAATGACAGCAGGATTATCGTAATACGTAATAGTTGCGGAGTCATTTCAAAGTCATCCTAATCGATCTATCGTTTTTGATAGCTCATTTTGGAGT
ADH1	ADH1 repair Fw	GAGTTAGCATATCTACAATTGGGTGAAATGGGGAGCGATTTGCAGGCATTTGCTCGGCATGCTCTATTGTTCGCACCACCGGCAAACTCGCGTCTCGCAAGTCTTGGCTCATTCTTCTAG
	ADH1 repair Rv	CTAGAAGAATGAGCCAAGACTTGCGAGACGCGAGTTTGCCGGTGGTGCGAACAATAGAGCATGCCGAGCAAATGCCTGCAAATCGCTCCCCATTTCACCCAATTGTAGATATGCTAACTC
PDC1	PDC1 repair Fw	ATCGAGGTGTCTAGTCTTCTATTACGCTAATGCAGTTTCAGGGTTTTGGAAACCACACTGTCAAGTTGAAGACTATATATTTTATTGAGTTTATGTTATGGGGAGGCTACCCTTTACGTC
	PDC1 repair Rv	GACGTAAAGGGTAGCCTCCCCATAACATAAACTCAATAAAATATATAGTCTTCAACTTGACAGTGTGGTTTCCAAAACCCTGAAACTGCATTAGCGTAATAGAAGACTAGACACCTCGAT
ENO2-A	ENO2-A repair Fw	CCAAAACTGGCATCCACTAATTGATACATCTACACACCGCACGCCTTTTTTCTGAAGCCCGGAAAAAAAAGGTGCACACGCGTGGCTTTTTCTTGAATTTGCAGTTTGAAAAATAACTAC
	ENO2-A repair Rv	GTAGTTATTTTTCAAACTGCAAATTCAAGAAAAAGCCACGCGTGTGCACCTTTTTTTTCCGGGCTTCAGAAAAAAGGCGTGCGGTGTGTAGATGTATCAATTAGTGGATGCCAGTTTTGG
ENO2-B	ENO2-B repair Fw	ATTTAGGTTTAAAAATTGATACAGTTTTATAAGTTACTTTTTCAAAGACTCGTGCTGTCTCACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATTCGACCAGCCTAAGAATGTTCAAC
	ENO2-B repair Rv	GTTGAACATTCTTAGGCTGGTCGAATCATTTAGACACGGGCATCGTCCTCTCGAAAGGTGAGACAGCACGAGTCTTTGAAAAAGTAACTTATAAAACTGTATCAATTTTTAAACCTAAAT
RECYCLE	RECYCLE Fw	TTTTTCTCATCTCTTGGCTCTGGATCCGTTATCTGTTCTGTTACACAAGAAATCGTACATACTAGAGCAAGATTTCAAATAAGTAACAGCAGCCATACGTTGAAACTACGGCAAAGGATT
	RECYCLE Rv	AATCCTTTGCCGTAGTTTCAACGTATGGCTGCTGTTACTTATTTGAAATCTTGCTCTAGTATGTACGATTTCTTGTGTAACAGAACAGATAACGGATCCAGAGCCAAGAGATGAGAAAAA

Supporting Table S11. Primers used to check the integration of the minimal set of endogenous glycolytic genes (SinLoG-IX) in the *SGA1* locus on chromosome IX.

Amplicon	primers	Sequence 5'--> 3'
G	G Fw	CTTGGCTCTGGATCCGTTATCTG
	G Rv	GCTCTTTTCTTCTGAAGGTCAATG
H	H Fw	GTTACGTGCTCAGTTGTTAGATATG
	H Rv	GCAGAAGTGTCTGAATGTATTAAGG
P	P Fw	TGAGCCACTTAAATTTCGTGAATG
	P Rv	TTTCTCTTTCCCCATCCTTTACG
Q	Q Fw	GCCCAAATCGGCATCTTTAAATG
	Q Rv	GTCAGGTTGCTTTCTCAGGTATAG
N	N Fw	AGTGTTGTATGTACCTGTCTATTTATACTG
	N Rv	GTCATGGCGGCTATTTTTCTAGG
O	O Fw	TTCCCAAGAACTAACTTGGAAGG
	O Rv	CTCTACCAGAGTTGTCGACTTG
A	A Fw	CCAGGCAGGTTGCATCACTC
	A Rv	CGCACGTCAAGACTGTCAAG
B	B Fw	TCGTATGTGAATGCTGGTCG
	B Rv	ACGGAATAGAACACGATATTTGC
C	C Fw	TCACGGGATTTATTCGTGACG
	C Rv	GCGTCCAAGTAACTACATTATGTG
D	D Fw	ACTCGCCTCTAACCCCACG
	D Rv	ACGGACTATAATGGTGTATGGATTG
J	J Fw	GCTTAATCTGCGTTGACAATGG
	J Rv	CAATAAACGTCCCTCGGATCTC
K	K Fw	GACGCCATTTGGAACGAAAAAAAG
	K Rv	TATGCTGACTTGGTATCACACTTC
L	L Fw	CAAAGACTCGTGCTGTCTATTGC
	L Rv	AATGAGTGGTAATTAATGGTGACATGAC
M	M Fw	ACGGAAAGTGGAATCCCATTTAG
	M Rv	ACCCTCATGAAACATGTATGAGATATTAC
F	F Fw	AAGCTAAGTTGACTGCTGCTACC
	F Rv	TTGGGCTGGACGTTCCGACATAG

Sequencing fragment:		Primers used to obtain fragment:
1	A Fw	CCAGGCAGGTTGCATCACTC
	C Rv	GCGTCCAAGTAACTACATTATGTG
2	N Fw	AGTGTTGTATGTACCTGTCTATTTATACTG

	A Rv	CGCACGTCAAGACTGTCAAG
3	L Fw SGA1 2 Rv	CAAAGACTCGTGCTGTCTATTGC TGGTCGACAGATACAATCCTGG
4	D 2 Fw J Rv	ACGCTGGCACAACATAGTTC CAATAAACGTCCCTCGGATCTC
5	P Fw N Rv	TGAGCCACTTAAATTTCGTGAATG GTCATGGCGGCTATTTTTCTAGG
6	O Fw B Rv	TTCCCAAGAACTAACTTGGAAGG ACGGAATAGAACACGATATTTGC
7	K Fw M Rv	TGTCTTACCCTGGACGGTATC ACCCTCATGAAACATGTATGAGATATTAC
8	H Fw Q Rv	GTTACGTGCTCAGTTGTTAGATATG GTCAGGTTGCTTTCTCAGGTATAG
9	M Fw SGA1 2 Rv	ACGGAAAGTGGAATCCCATTTAG TGGTCGACAGATACAATCCTGG
10	C 2 Fw J Rv	TCACGGGATTTATTCGTGACG CAATAAACGTCCCTCGGATCTC
11	Q Fw O Rv	GCCCAAATCGGCATCTTTAAATG CTCTACCAGAGTTGTCGACTTG
12	J Fw L Rv	GCTTAATCTGCGTTGACAATGG AATGAGTGGTAATTAATGGTGACATGAC
13	SGA1 2 Fw P Rv	ACTCGTACAAGGTGCTTTTAACTTG TTTCTCTTTCCCCATCCTTTACG
14	B Fw D 2 Rv	TCGTATGTGAATGCTGGTCG AATCATGTTGATGACGACAATGG

Supporting Table S12. Primers used to check the integration of the SinLoG in the *CAN1* locus on chromosome V.

Amplicon	primers	Sequence 5'--> 3'
SinLoG-V, *S. cerevisiae* SinLoG in *CAN1*:		
1	CAN1 Fw SC1 Rv	TCGGGAGCAAGATTGTTGTG TTTCTCTTTCCCCATCCTTTACG
2	SC2 Fw SC2 Rv	GCCCAAATCGGCATCTTTAAATG GTAGTTATTTTTCAAACTGCAAATTCAAG
3	SC3 Fw SC3 Rv	GGTGCACACGCGTGGCTTTTTCTTGAATTTGC AATCATGTTGATGACGACAATGG
4	SC4 Fw SC4 Rv	GCTTAATCTGCGTTGACAATGG AAACTCACCGAGGCAGTTCCATAGG
5	SC5 Fw CAN1 Rv	TCGTATGTGAATGCTGGTCG AGAAGAGTGGTTGCGAACAGAG
Sk*-SinLoG-V and Mosaic-SinLoG-V, *S. kudriavzevii* & Mosaic SinLoG in *CAN1:		
1	CAN1 Fw SK1 Rv	TCGGGAGCAAGATTGTTGTG GTTCGGCAAATGCCTGCAAATC
2	SK2 Fw SK2 Rv	CGTTTACCATGGCCTATGTAGC CGCACGTCAAGACTGTCAAG
3	SK3 Fw SK3 Rv	ATGGGAAGCCCGATGCGCCAGAG TGACAATATGCGCCTTGGCGATTTC
4	SK4 Fw SK4 Rv	AGCTGAAGTGGCCGCTTCAACCACC CATCGTTGCTTGCAGGATGTTC
5	SK5 Fw CAN1 Rv	TTAATCGATGACAGCGTAGGG AGAAGAGTGGTTGCGAACAGAG
Removal of the SinLoG-IX from the *SGA1* locus on chromosome IX		
F	SGA1 Fw SGA1 Rv	ACTCGTACAAGGTGCTTTTAACTTG TTGGGCTGGACGTTCCGACATAG

Supporting Figure legends

Supporting Figure S1

Two-step construction and verification of IMX382 carrying the endogenous SinLoG. Introduction of the *SCEI/KlURA3* cassette in the *SGA1* locus of IMX370 to produce IMX377 (a). One-step *in vivo* assembly and integration of the endogenous SinLoG at the *SGA1* locus on chromosome IX (SinLoG-IX) (b). PCR confirmation of the correct assembly and integration of the complete SinLoG-IX cluster at the *SGA1* locus. PCRs were designed to produce amplicons covering the junctions of the assembly cassettes (c). To identify potential mutations within the SinLoG-IX cluster, 14 overlapping fragments were amplified by PCR, pooled in a molar ratio of 1:1 and sequenced by next-generation sequencing (d).

Supporting Figure S2

Workflow for the construction of the SwYG strain. The SwYG strain was constructed from the Minimal Glycolysis IMX370 strain, uracil auxotroph, in 19 rounds of modifications. First the minimal set of native glycolytic genes was introduced as a cluster at the *SGA1* locus on chromosome IX (SinLoG-IX cluster) using the CATI approach (21), leading to strain IMX382. The endogenous glycolytic genes *PYK1*, *PGI1*, *TPI1*, *TDH3* and *PGK1* were deleted using specific marker cassettes, which were recycled using the I-SceI facilitated marker recycling method (38). *PFK2* was deleted by a 'classical' knockout cassette containing cas9 and the marker gene natNT1, leading to strain IMX535. The genes *GPM1*, *FBA1*, *HXK2*, *PFK1*, *PDC1* and *ADH1* were deleted using CRISPR/Cas9 (strain IMX583). As the *ENO2* gene carried by the clustered native glycolysis on chromosome IX proved to be dysfunctional, an additional *ENO2* variant with longer promoter region was introduced into this SinLoG-IX cluster, resulting in strain IMX586. The native *ENO2* was subsequently removed from its original locus using the CRISPR/Cas9 system, resulting in strain IMX587. Finally, the dysfunctional copy of *ENO2* was replaced by amdSYM, leading to IMX589, a yeast platform for pathway swapping, named SwYG. Two versions of SwYG are available, the prototrophic IMX606 and the uracil auxotroph IMX589 (Table S5).

Supporting Figure S3

Schematic overview of deletions with recyclable marker cassettes. Each deletion cassette was targeted to sequences not present on the native SinLoG-IX cluster and contained two

recognition sequences for I-SceI surrounding the marker and a 40-nucleotides sequence homologous to the DNA surrounding the deletion locus (repeat) (a). After three successive deletions, selection markers were removed by expression of I-SceI from a plasmid. The gap resulting from I-SceI restriction was repaired by homologous recombination of the repeats (b).

Supporting Figure S4

Integration of *cas9* in the *PFK2* locus. A cassette containing *cas9,* obtained by PCR from p414-TEF1p-cas9-CYC1t, and a second cassette containing the *natNT1* marker gene, obtained by PCR from plasmid pUGnatNT1, were co-transformed to IMX511 for assembly and integration in the *PFK2* locus. Analysis with different primer sets, resulting in amplicons covering the complete construct, demonstrated the successful replacement of *pfk2* by *cas9/natNT1*.

Supporting Figure S5

Confirmation of deletion of glycolytic genes from their native loci in the auxotrophic SwYG strain IMX589 based on whole genome sequencing. The *in silico* designs of the seamless deletions were blasted against the assembled contigs of the SwYG strain. For *tpi, fba1, gpm1, hxk2, pfk1, pdc1, pyk1, pgk1, pgi1* and *tdh3* the predicted *in silico* sequences were present on single contigs, thereby confirming their removal from the native loci. The loci of *eno2* and *adh1* were not present on a single contig due to the presence of the *adh1* terminator in the *cas9* cassette and the double presence of part of the *ENO2* promoter in the genome as visualized in Figure S10. The deletion of *pfk2* was not included in this analysis, since this gene was removed by replacing it with the *cas9* cassette as represented in Fig. S4.

Supporting Figure S6

Copy number variation within the SinLoG clusters of the engineered strains. The graphs represent the copy number variation as generated by the Magnolya algorithm (26) of contigs that were *de novo* assembled by Newbler (www.454.com) and aligned to the *in silico* designs of the SinLoG clusters.

Supporting Figure S7

Schematic overview of the pathway swapping approach. The new glycolytic gene clusters were introduced into chromosome V in a single step via *in vivo* assembly and targeted integration with the use of CRISPR/Cas9. The endogenous glycolytic cassette was subsequently removed using the CRISPR/Cas9 system in combination with a repair fragment of 120 bp.

Supporting Figure S8

Construction scheme of the *SYN-PDC1*$_{MF}$ glycoblock. The SYN2 fragment was obtained by fusing oligo's Syn2-Fw and Syn2-Rv in an independent PCR (a). *PDC1* from *S. cerevisiae*, including its own promoter and terminator, and SYN2 were fused in a fusion-PCR using primers FUS2-Fw and FUS2-Rv, resulting in the *SYN-PDC1*$_{MF}$ cassette (b).

Supporting Figure S9

Overview of the construction scheme of the CRISPR-plasmids. Gibson-assembly based construction of the CRISPR-plasmids carrying the gRNA targeting *GPM1* (pUDE324), *FBA1* (pUDE325), *ENO2* (pUDE326), *HXK2* (pUDE327) and *PFK1* (pUDE329) (a). Gibson assembly of the CRISPR-plasmids carrying the amdsYM cassette (pUDE337) and the SinLoG-IX flanking regions (pUDE342) (b). *In vivo* assembly of CRISPR-plasmids for the simultaneous double deletion of *ADH1* and *PDC1* (c).

Supporting Figure S10

Overview of the *ENO2* deletion scheme from its native locus. The target sequence of the *ENO2* gRNA was present in the SinLoG-IX cluster and on the native chromosomal locus of *ENO2*. Co-transformation of two different repair fragments targeting the two loci enabled to specifically delete *ENO2* from its native locus, but not from the SinLoG-IX.

Supporting Figure S11

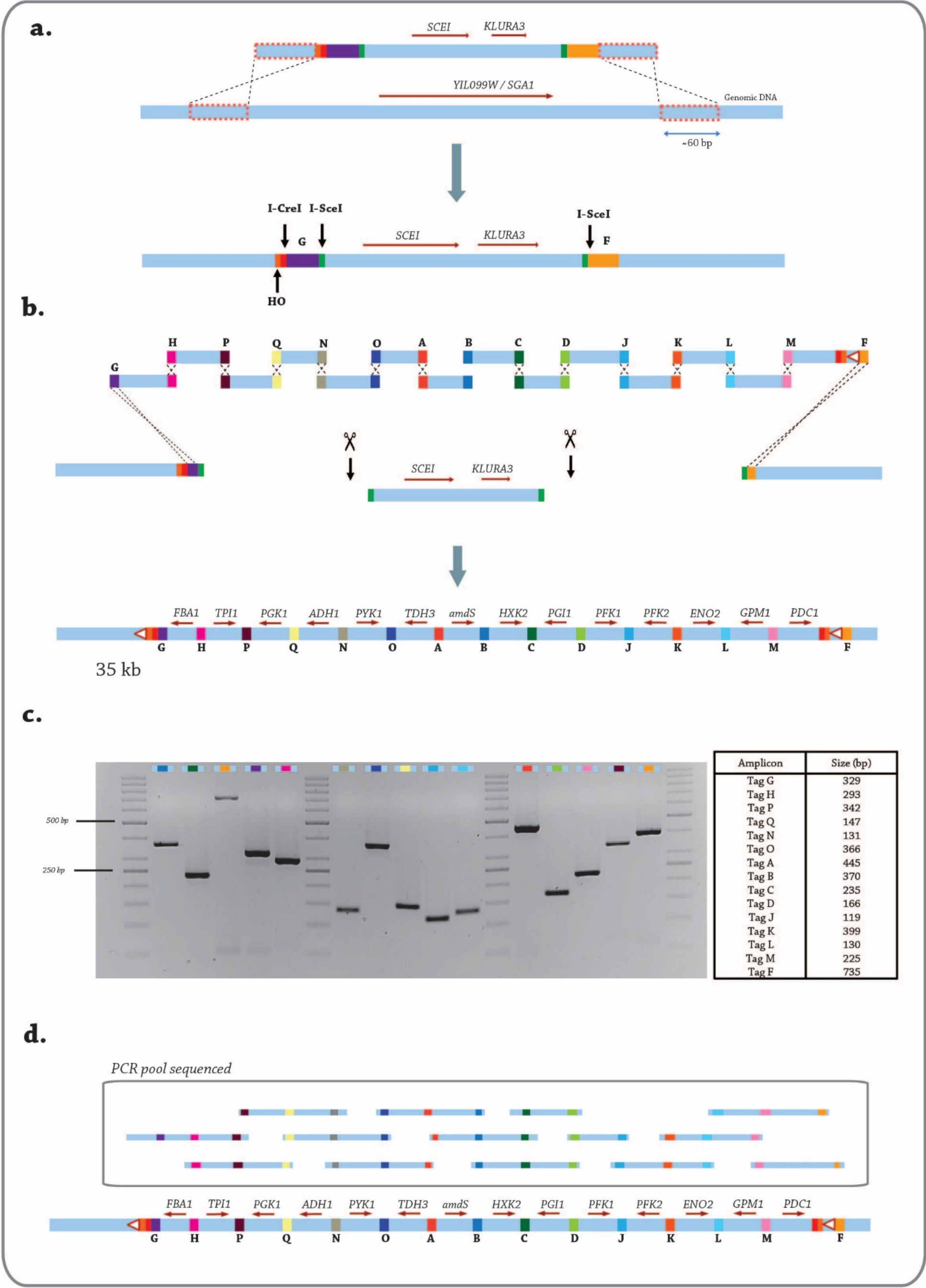
Construction scheme of the *SCEI/KlURA3* cassette for combined *in vivo* assembly and integration (CATI). PCR amplification of the *SCEI/KlURA3* cassette from IMX221 (a). Addition of extra restriction sites and of the flanking regions targeting the *SGA1* locus (b). Final *SCEI/KlURA3* cassette used to promote the integration of the SinLoG-IX cluster in IMX370, resulting in IMX377 (c).

Supporting Figure S12

Schematic overview of the deletion of the SinLoG-IX cluster from the *SGA1* locus. Co-transformation of strains IMX591, IMX607 and IMX633 with the CRISPR plasmid pUDE342 targeting the SinLoG-IX cluster with the corresponding repair fragment resulted in excision of the SinLoG-IX cluster from their genome. PCR analysis and sequencing of the resulting strains

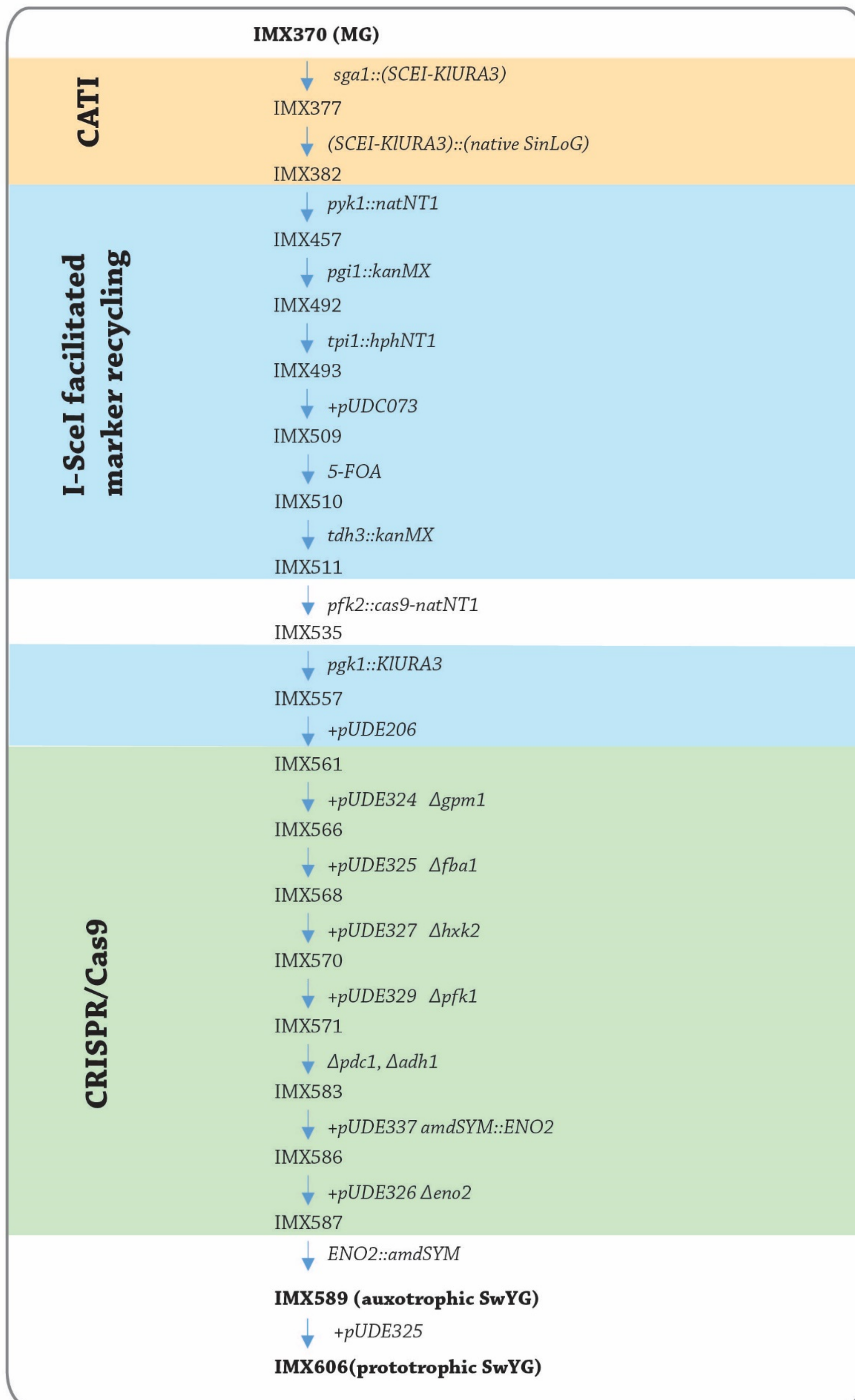
(IMX605, IMX637 and IMX645 respectively) demonstrated the absence of the SinLoG-IX cluster at the *SGA1* locus.

Supporting Figure S1

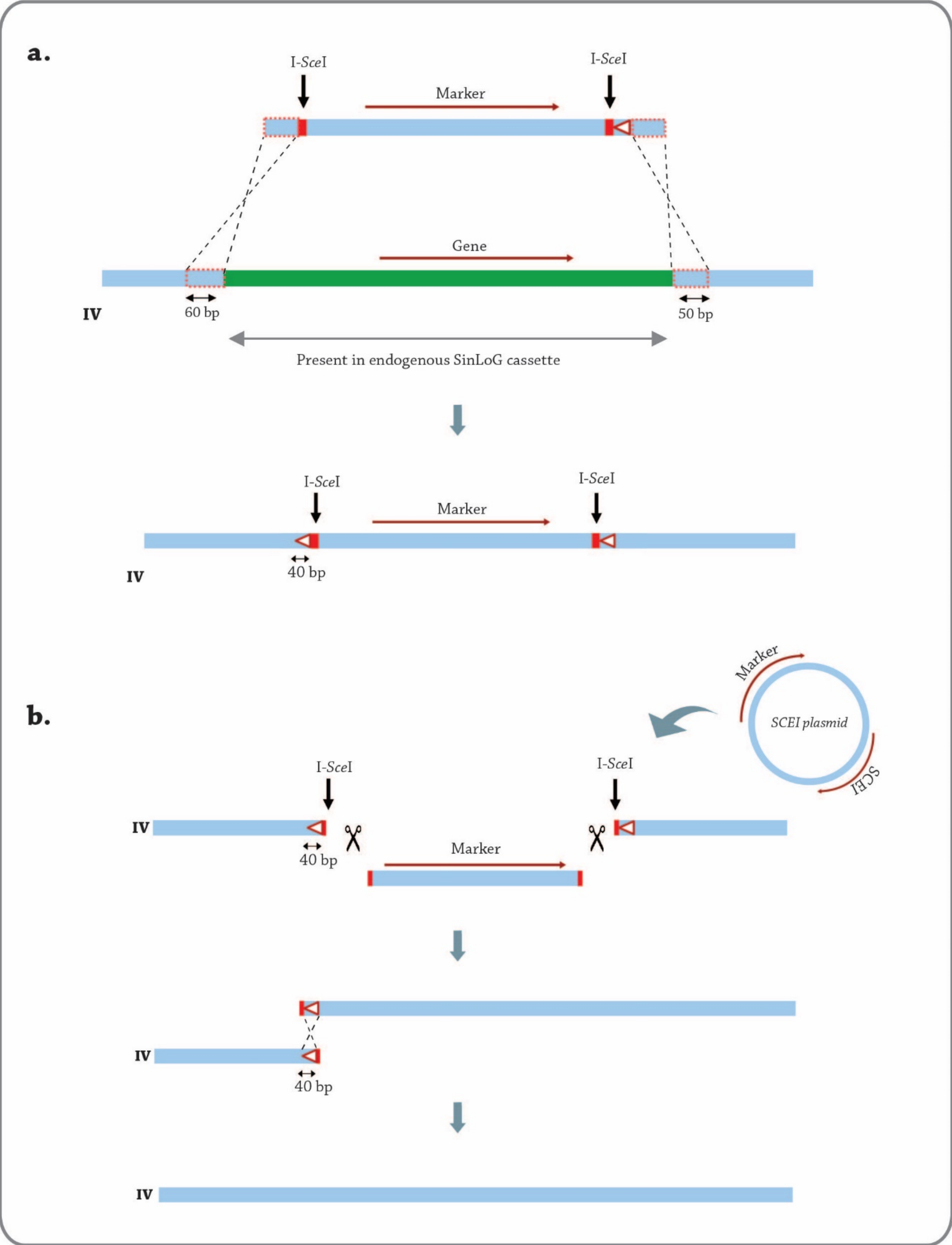


Amplicon	Size (bp)
Tag G	329
Tag H	293
Tag P	342
Tag Q	147
Tag N	131
Tag O	366
Tag A	445
Tag B	370
Tag C	235
Tag D	166
Tag J	119
Tag K	399
Tag L	130
Tag M	225
Tag F	735

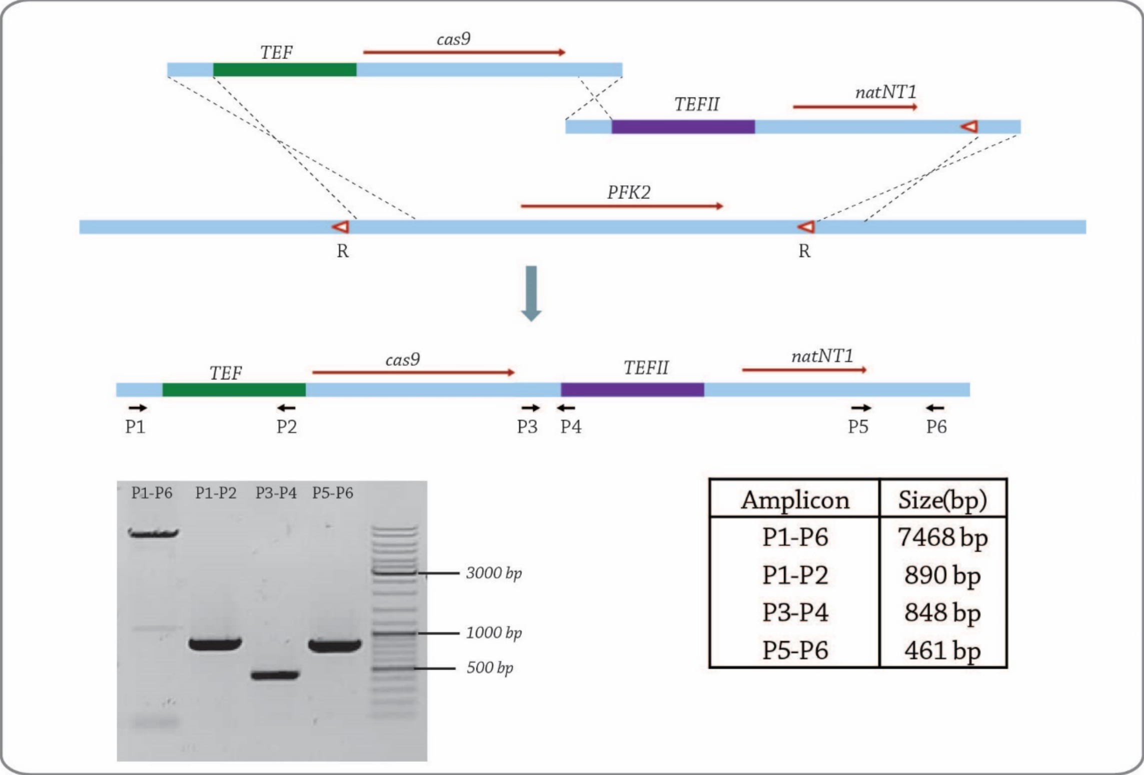
Supporting Figure S2



Supporting Figure S3

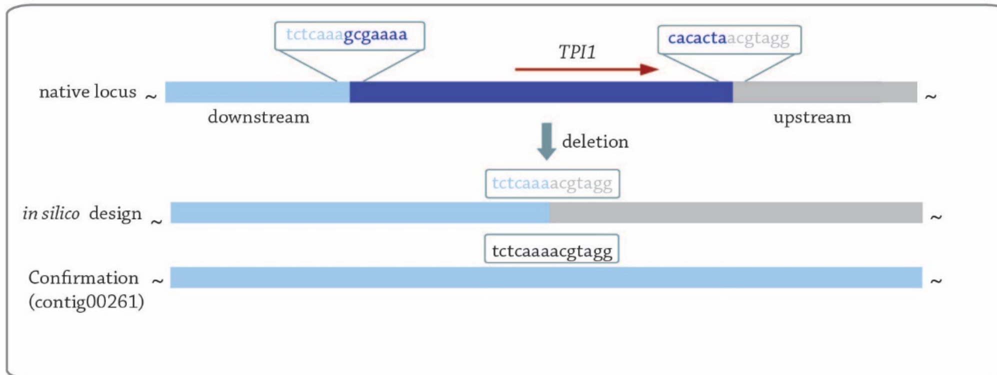


Supporting Figure S4



Amplicon	Size(bp)
P1-P6	7468 bp
P1-P2	890 bp
P3-P4	848 bp
P5-P6	461 bp

Supporting Figure S5



Gene		Sequence	
tpi	WT:	aataagaacatcttctaaa..acgtaggattattataactc	
	in silico design:	aataagaacatcttctaaaacgtaggattattataactc	
	Contig00261:	aataagaacatcttctaaaacgtaggattattataactc	
fba1	WT:	tgactttgaaatgactccgc..aactattacgtattacgata	
	in silico design:	tgactttgaaatgactccgcaactattacgtattacgata	
	Contig00222:	tgactttgaaatgactccgcaactattacgtattacgata	
gpm1	WT:	atcaaggttattgctataa..tatatatatatatatatata	
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	Contig00085:	atcaaggttattgctataatatatatatatatatatata	
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	Contig00361:	aacctcctcgcacattggtagcttaattttaaatttttttt	*insertion*
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	Contig00001:	caatacgactacaattaaggcatgtttttccatcgttttc	
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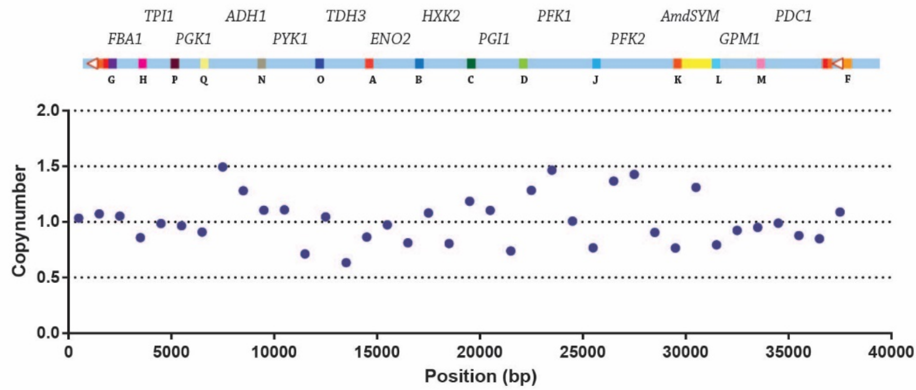
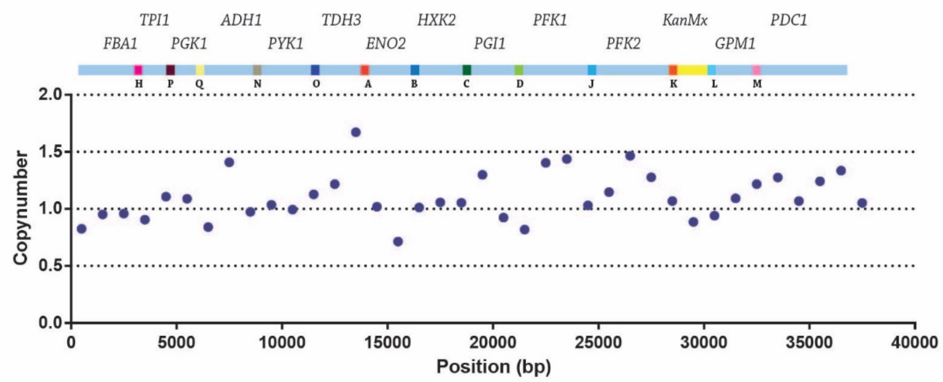
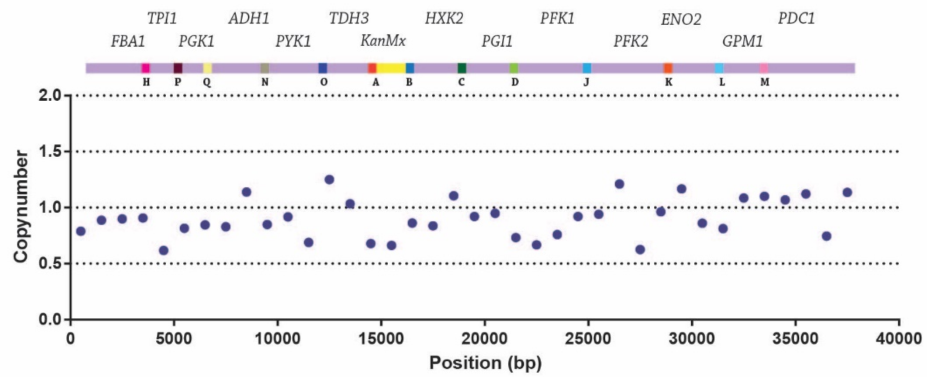
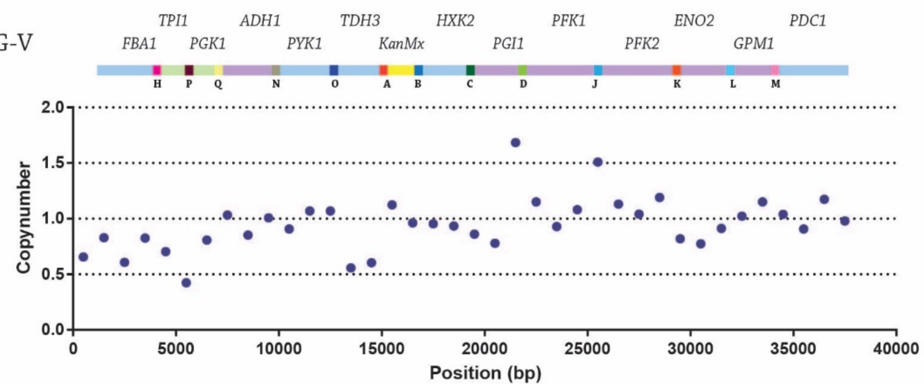
Supporting Figure S6

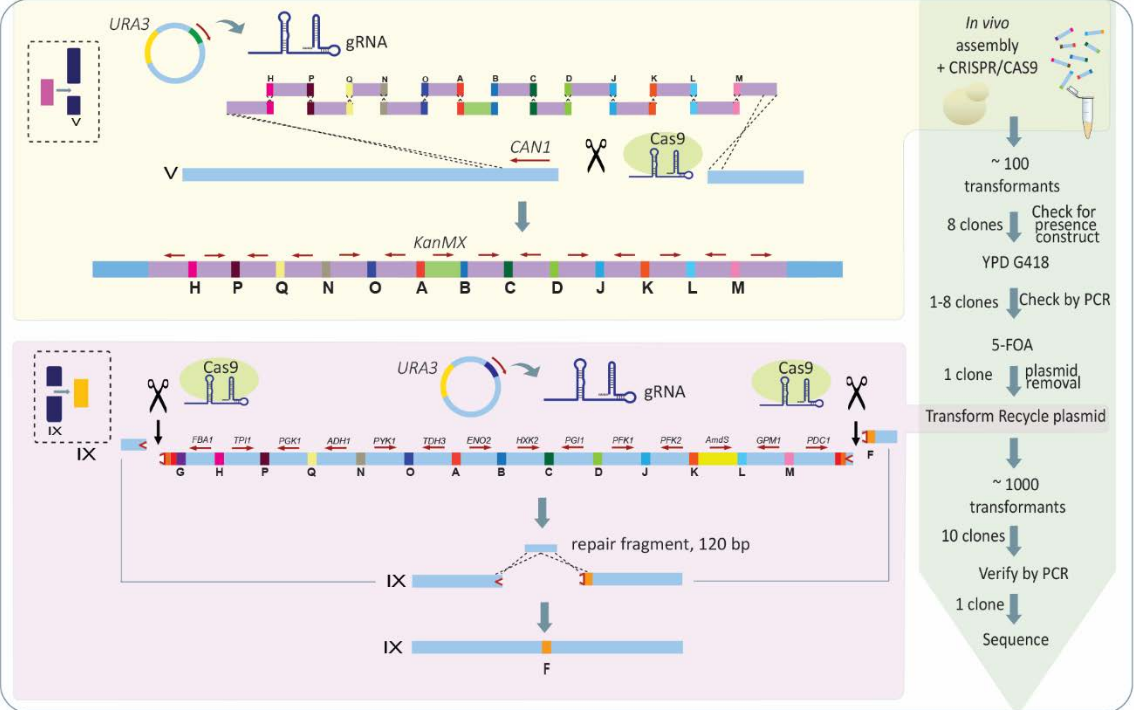




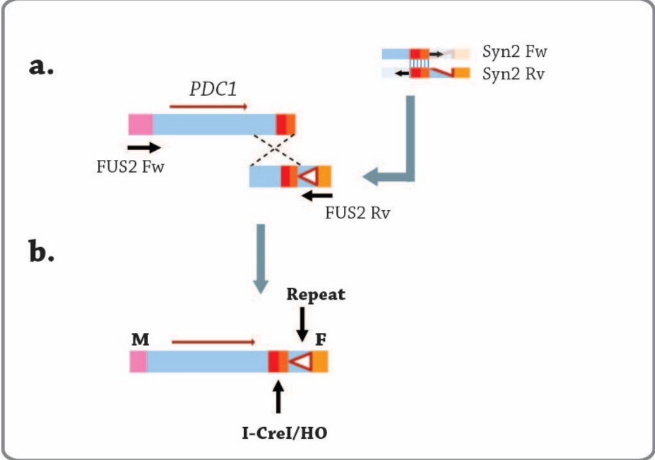




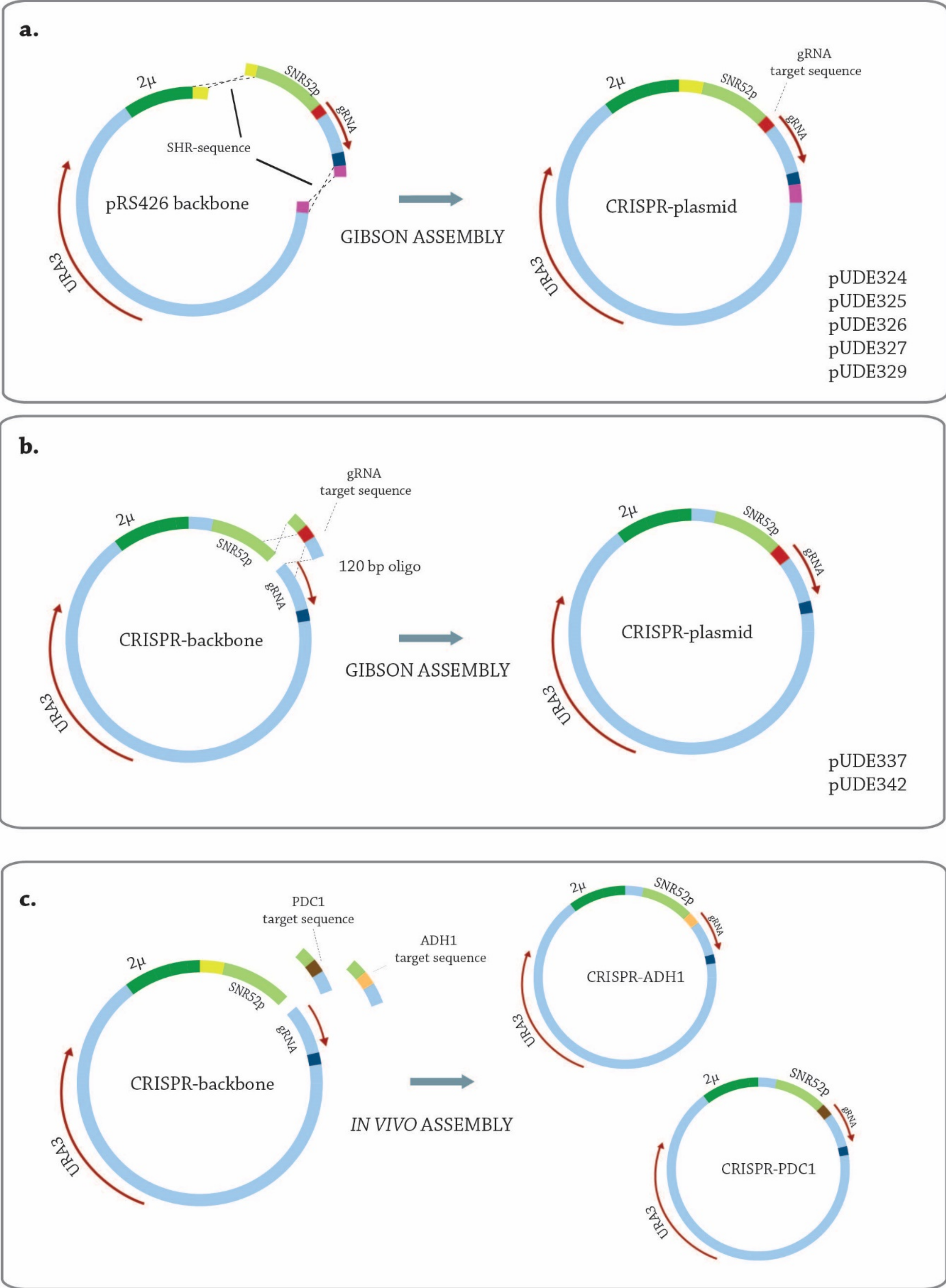
Supporting Figure S7



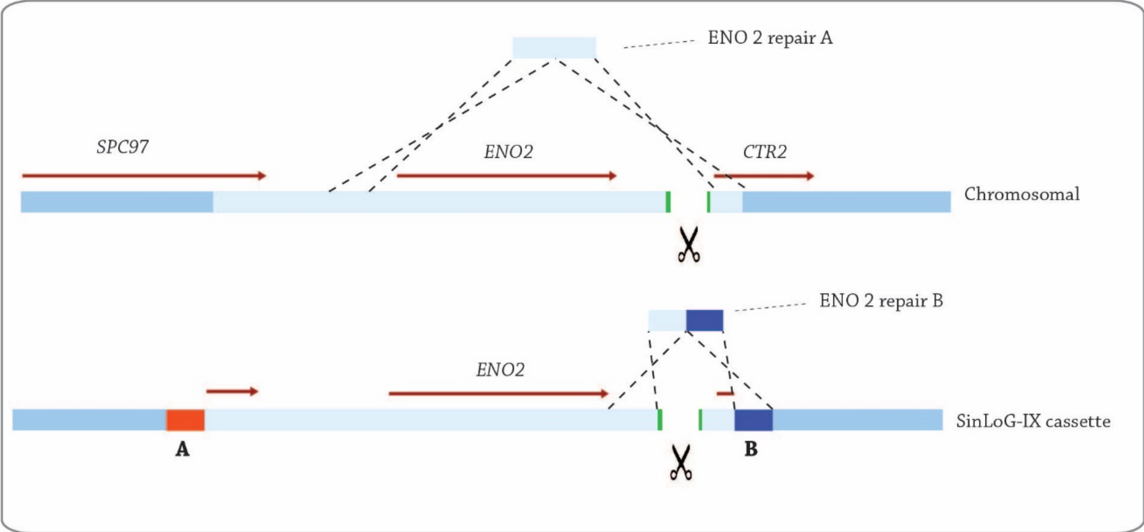
Supporting Figure S8



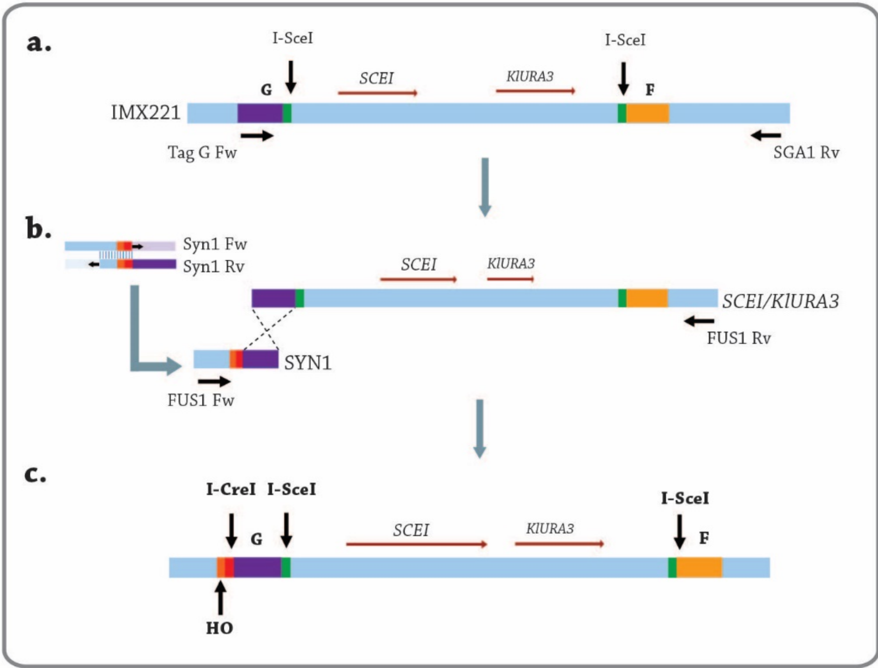
Supporting Figure S9



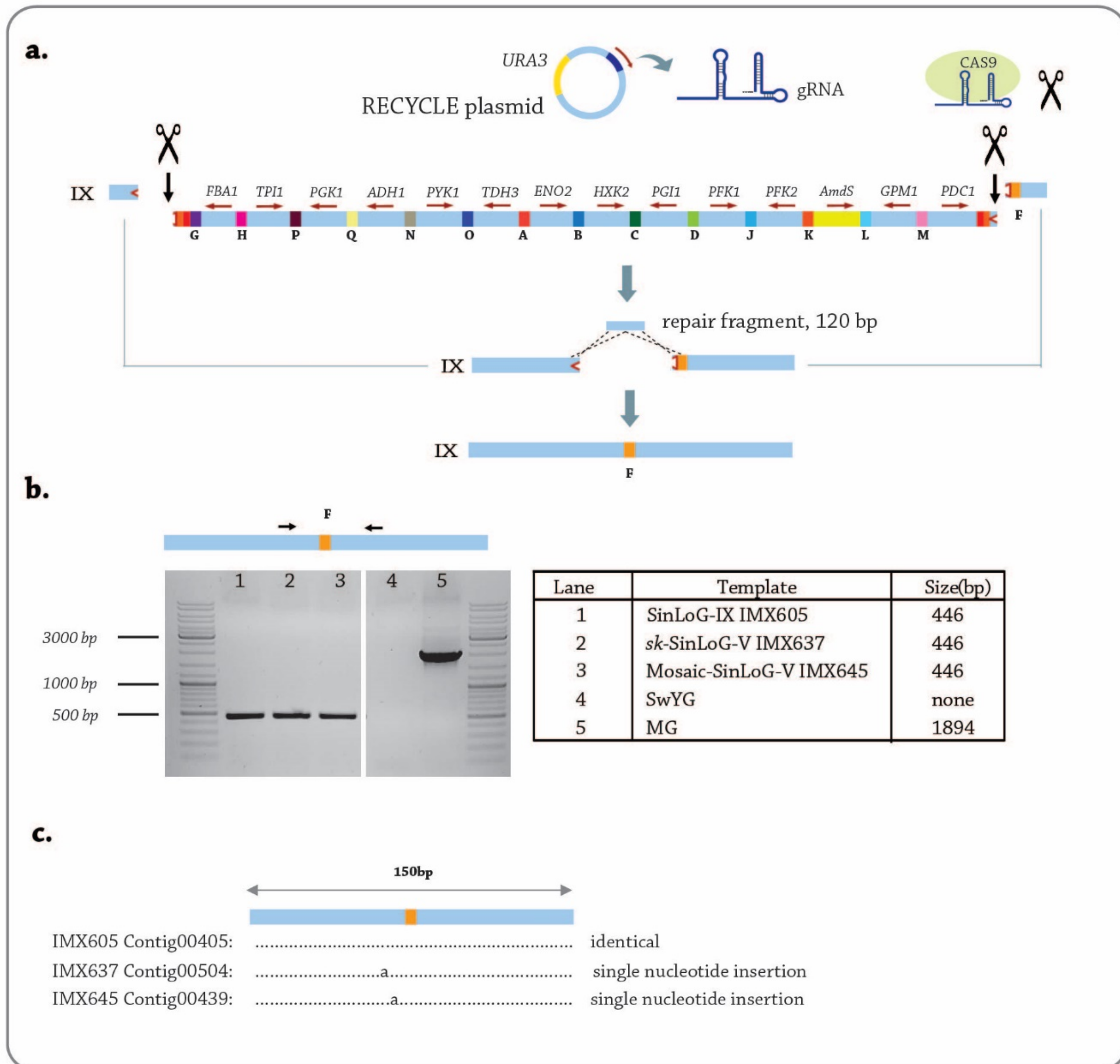
Supporting Figure S10



Supporting Figure S11



Supporting Figure S12



Lane	Template	Size(bp)
1	SinLoG-IX IMX605	446
2	*sk*-SinLoG-V IMX637	446
3	Mosaic-SinLoG-V IMX645	446
4	SwYG	none
5	MG	1894

Reference list for supporting information

1. Lopes CA, Barrio E, Querol A (2010) Natural hybrids of *S. cerevisiae* x *S. kudriavzevii* share alleles with European wild populations of *Saccharomyces kudriavzevii*. FEMS Yeast Res 10**:** 412-421.

2. Nijkamp JF, van den Broek M, Datema E, de KS, Bosman L, Luttik MA, Daran-Lapujade P, Vongsangnak W, Nielsen J, Heijne WH.et al. (2012) *De novo* sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. Microb Cell Fact 11**:** 36.

3. Entian KD, Kötter P (2007) Yeast genetic strain and plasmid collections. *Yeast Gene Analysis*, eds. Stansfield I, Stark MJR (Academic Press, Elsevier, Amsterdam), pp 629-666.

4. van Dijken JP, Bauer J, Brambilla L, Duboc P, Francois JM, Gancedo C, Giuseppin MLF, Heijnen JJ, Hoare M, Lange HC.et al. (2000) An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microb Technol 26**:** 706-714.

5. Kuijpers NG, Chroumpi S, Vos T, Solis-Escalante D, Bosman L, Pronk JT, Daran JM, Daran-Lapujade P (2013) One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in *Saccharomyces cerevisiae*. FEMS Yeast Res 13**:** 769-781.

6. Verduyn C, Postma E, Scheffers WA, van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8**:** 501-517.

7. Pronk JT (2002) Auxotrophic yeast strains in fundamental and applied research. Appl Environ Microbiol 68**:** 2095-2100.

8. Hensing MC, Rouwenhorst RJ, Heijnen JJ, van Dijken JP, Pronk JT (1995) Physiological and technological aspects of large-scale heterologous-protein production with yeasts. Antonie van Leeuwenhoek 67**:** 261-279.

9. Güldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 24**:** 2519-2524.

10. Goldstein AL, McCusker JH (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast 15**:** 1541-1553.

11. Gritz L, Davies J (1983) Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25**:** 179-188.

12. Bertani G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 62**:** 293-300.

13. Bertani G (2004) Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. J Bacteriol 186**:** 595-600.

14. Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol 350**:** 87-96.

15. Gritsenko A, Reinders MJ, de Ridder D (2013) *Pattern Recognition in Bioinformatics*, eds. Ngom A, Formenti E, Hao JK, Zhao XM, van Laarhoven T (Springer Berlin Heidelberg.

16. Solis-Escalante D, Kuijpers NG, Bongaerts N, Bolat I, Bosman L, Pronk JT, Daran JM, Daran-Lapujade P (2013) amdSYM, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. FEMS Yeast Res 13**:** 126-139.

17. Güldener U, Heinisch J, Koehler GJ, Voss D, Hegemann JH (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res 30**:** e23.

18. Solis-Escalante D, Kuijpers NG, van der Linden F, Pronk JT, Daran JM, Daran-Lapujade P (2014) Efficient simultaneous excision of multiple selectable marker cassettes using I-SceI-induced double strand breaks in *Saccharomyces cerevisiae*. FEMS Yeast Res 14**:** 741-754.

19. DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. Nucleic Acids Res 41**:** 4336-4343.

20. Mans R, van Rossum HM, Wijsman M, Backx A, Kuijpers NG, van den Broek M, Daran-Lapujade P, Pronk JT, van Maris AJ, Daran JM (2015) CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. FEMS Yeast Res 15.

21. Kuijpers NG, Chroumpi S, Vos T, Solis-Escalante D, Bosman L, Pronk JT, Daran JM, Daran-Lapujade P (2013) One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in *Saccharomyces cerevisiae*. FEMS Yeast Res 13**:** 769-781.

22. Yamashita I, Fukui S (1985) Transcriptional control of the sporulation-specific glucoamylase gene in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol 5**:** 3069-3073.

23. Kihara K, Nakamura M, Akada R, Yamashita I (1991) Positive and negative elements upstream of the meiosis-specific glucoamylase gene in *Saccharomyces cerevisiae*. Mol Gen Genet 226**:** 383-392.

24. Solis-Escalante D, Kuijpers NG, Pronk JT, Daran JM, Daran-Lapujade P (2015) A minimal set of glycolytic genes reveals strong redundancies in *Saccharomyces cerevisiae* central metabolism. Euk Cell 14**:** 804-816.

25. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, Cuomo CA, Zeng Q, Wortman J, Young SK.et al. (2014) Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One 9**:** e112963.

26. Nijkamp JF, van den Broek MA, Geertman JM, Reinders MJ, Daran JM, de RD (2012) *De novo* detection of copy number variation by co-assembly. Bioinformatics 28**:** 3195-3202.

27. Postma E, Verduyn C, Scheffers WA, van Dijken JP (1989) Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 55**:** 468-477.

28. Jansen MLA, Diderich JA, Mashego MR, Hassane A, de Winde JH, Daran-Lapujade P, Pronk JT (2005) Prolonged selection in aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* causes a partial loss of glycolytic capacity. Microbiology-SGM 151**:** 1657-1669.

29. Cruz LA, Hebly M, Duong GH, Wahl SA, Pronk JT, Heijnen JJ, Daran-Lapujade P, van Gulik WM (2012) Similar temperature dependencies of glycolytic enzymes: an evolutionary adaptation to temperature dynamics? BMC Syst Biol 6**:** 151.

30. Lowry OH, Rosebrough HJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193**:** 265-275.

31. Solis-Escalante D, Kuijpers NG, Barrajon-Simancas N, van den Broek M, Pronk JT, Daran JM, Daran-Lapujade P (2015) A minimal set of glycolytic genes reveals strong redundancies in *Saccharomyces cerevisiae* central metabolism. Eukaryot Cell 14**:** 804-816.

32. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215**:** 403-410.

33. Solis-Escalante D, Kuijpers NG, Pronk JT, Daran JM, Daran-Lapujade P (2015) A minimal set of glycolytic genes reveals strong redundancies in *Saccharomyces cerevisiae* central metabolism. Euk Cell 14**:** 804-816.

34. Lopes CA, Barrio E, Querol A (2010) Natural hybrids of *S. cerevisiae* x *S. kudriavzevii* share alleles with European wild populations of *Saccharomyces kudriavzevii*. FEMS Yeast Res 10**:** 412-421.

35. Solis-Escalante D, Kuijpers NG, Bongaerts N, Bolat I, Bosman L, Pronk JT, Daran JM, Daran-Lapujade P (2013) amdSYM, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. FEMS Yeast Res 13**:** 126-139.

36. Gueldener U, Heinisch J, Koehler GJ, Voss D, Hegemann JH (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res 30**:** e23.

37. Mumberg D, Muller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156**:** 119-122.

38. Solis-Escalante D, Kuijpers NG, van der Linden FH, Pronk JT, Daran JM, Daran-Lapujade P (2014) Efficient simultaneous excision of multiple selectable marker cassettes using I-SceI-induced double-strand DNA breaks in *Saccharomyces cerevisiae*. FEMS Yeast Res 14**:** 741-754.