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Global rewiring of cellular metabolism renders *Saccharomyces cerevisiae* Crabtree negative

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*Saccharomyces cerevisiae* is a Crabtree-positive eukaryal model organism. It is believed that the Crabtree effect has evolved as a competition mechanism by allowing for rapid growth and production of ethanol at aerobic glucose excess conditions. This inherent property of yeast metabolism and the multiple mechanisms underlying it require a global rewiring of the entire metabolic network to abolish the Crabtree effect. Through rational engineering of pyruvate metabolism combined with adaptive laboratory evolution (ALE), we demonstrate that it is possible to obtain such a global rewiring and hereby turn *S. cerevisiae* into a Crabtree-negative yeast. Using integrated systems biology analysis, we identify that the global rewiring of cellular metabolism is accomplished through a mutation in the RNA polymerase II mediator complex, which is also observed in cancer cells expressing the Warburg effect.
The yeast *Saccharomyces cerevisiae* is a widely used model organism for studying the biology of eukaryal cells as well as it is extensively used as a cell factory for the production of pharmaceuticals, chemicals, and biofuels. Its metabolism has evolved to have oxidative fermentation, meaning that even in the presence of oxygen, the yeast uses fermentative metabolism when glucose is in excess, a metabolic feature that is generally referred to as the Crabtree effect. This million-year-old evolution feature ensures the advantage in its ecological niche due to the antiseptic properties. However, it generally results in reduced yields when this yeast is used as a cell factory. There is therefore much interest in rewiring the central carbon metabolism to abolish the Crabtree effect.

Eliminating pyruvate decarboxylase activity in yeast completely abolishes the Crabtree effect, but the growth deficiency of pyruvate decarboxylase minus (Pdc−) strains in excess glucose conditions limits their application for biotechnology. Even though Pdc− strains have been studied for last 25 years, only one strategy has so far enabled successful restoration of the growth of Pdc− strains in a minimal medium with excess glucose. This strategy involves introducing *MTH1* mutations, which were originally identified from Pdc− strains evolved to grow in excess glucose. However, the specific growth rate of this strain was only 0.1 h−1, and acetyl-CoA generation in the cytosol only weakly supports lipid biosynthesis and hereby, for cell growth in a sugar-based media (Fig. 1a). This is the main reason for Pdc− strains being growth deficient in the glucose media.

To overcome this challenge, we create an alternative pyruvate dehydrogenase (PDH) bypass in *S. cerevisiae* with an ATP-independent acetyl-CoA synthetase pathway. With this, growth of a Pdc− strain is successfully restored in minimal media with excess glucose. Combining rational design, adaptive laboratory evolution (ALE), and reverse engineering, the specific growth rate of the best strain reaches 0.218 h−1, which is close to the maximum growth of *S. cerevisiae* with purely respiratory metabolism and the maximum specific growth rate of most Crabtree-negative yeasts. We find that, to unlock the millions of years of evolution that has determined metabolic features of *S. cerevisiae*, many different metabolic parts need to be engineered, and an important element is enabling global transcriptional alteration by having a mutation in the mediator complex that supports rewiring of cellular metabolism.

**Results**

**Establishing a functional ethanol overflow negative yeast.** In *S. cerevisiae*, deletion of *PDC1*, 5, and 6 completely abolishes ethanol production. However, pyruvate decarboxylase is also an indispensable enzyme of the PDH bypass, which provides cytosolic acetyl-CoA required for lipid biosynthesis and hereby, for cell growth in a sugar-based media (Fig. 1a). This is the main reason for Pdc− strains being growth deficient in the glucose media.

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**Fig. 1** Establishment of a cytosolic acetyl-CoA synthetic pathway in Pdc− *S. cerevisiae*. a The native and alternative cytosolic acetyl-CoA synthetic pathway in *S. cerevisiae*. The native metabolic network (yellow background) converts pyruvate to acetyl-CoA by pyruvate decarboxylase (Pdc1, 5, and 6), acetaldehyde dehydrogenase (Ald2, 3, 4, 5, and 6), and acetyl-CoA synthetase (Acs1 and 2). The metabolic network (green background) converts pyruvate to acetyl-CoA by pyruvate oxidase (PO) and phosphotransacetylase (PTA). b Growth curve of a Pdc− yeast strain carrying different PO/PTA plasmids in a synthetic medium containing 20 g l−1 glucose. sp: *S. pneumoniae*, lp: *L. plantarum*, av: *A. viridans*, se: *S. enterica*. c Growth and metabolite profiles of sZJD-11 (acs2Δ::POav acs1Δ::PTAse) in 20 g l−1 glucose minimal medium. d Acetyl-CoA-derived farnesene and 3-hydroxypropionate (3-HP) production in wild-type strain CEN.PK113-11C (orange bar) and sZJD-11 (blue bar) background strains. e Growth and metabolite profiles of sZJD-23 (acs2Δ::POav acs1Δ::PTAse gpp2Δ gpp2Δ) in 20 g l−1 glucose minimal medium. All data represent the mean ± s.d. of biological triplicates.
To restore growth of a Pdc− strain in excess glucose media, we established an ATP-independent cytosolic acetyl-CoA producing pathway (Fig. 1a) in a Pdc− S. cerevisiae strain, which enabled growth in an excess glucose medium (Fig. 1b and Supplementary Figure 1). In this pathway, pyruvate oxidase (PO, EC 1.2.3.3) from Aerococcus viridans (av) catalyzes the decarboxylation of pyruvate to acetate-phosphate, and subsequently, phosphotransacytelyase (PTA, EC 2.3.1.8) from Salmonella enterica (se) converts acetate-phosphate to acetyl-CoA (Fig. 1a), which was confirmed by the enzyme activity measurements (Supplementary Figure 2). To further verify the capacity of this pathway, the native acetyl-CoA synthetases, Acs1 and Acs2, were replaced by PTAse and PTA from Eremothecium sinecaudum (0.117–0.122 h⁻¹), which is similar to that of some natural Crabtree-negative yeasts, such as Kluyveromyces nonfermentans (0.101 h⁻¹) and Eremothecium sinecaudum (0.117–0.122 h⁻¹), it is still much lower than for most natural Crabtree-negative yeasts (0.249–0.429 h⁻¹) (Supplementary Table 2).

We therefore established three independent yeast populations based on exposing sZJD-24 (prototrophic strain based on sZJD-23) (Supplementary Table 1) to ALE for 40 days, which is a duration compromising the selection of clones with improved fitness and not accumulating too many mutations. All of the three evolved populations have a higher specific growth rate compared with starting strain sZJD-24. (Supplementary Figure 4). The maximum specific growth rate of clones picked from each of these three populations reached 0.217 h⁻¹, 0.221 h⁻¹, and 0.209 h⁻¹, respectively (Fig. 2a). Through genome sequencing of seven clones (two each from sZJD-24A and sZJD-24B, three from sZJD-24C), we found a total of 19 single nucleotide variations (SNVs) in 18 genes (Supplementary Table 3). Although there were no shared mutations among all seven clones (Fig. 2b), a nonstop mutation in MED3 was identified in five clones derived from lines sZJD-24A and sZJD-24C. The two clones of line sZJD-24B shared a mutation in MED2. Both Med2 and Med3 are components of the tail module of the RNA polymerase II mediator complex.10 These results indicated that the mediator complex may play a key role in regulating cell growth. Only three SNVs were identified in the clones from line sZJD-24C, which shared a nonsense mutation in GPD1 encoding a NADH-dependent glycerol-3-phosphate dehydrogenase.11 For all clones in line sZJD-24B, besides MED3, we found shared mutations in SIW14 and MHO1. Siw14 is tyrosine phosphatase involved in actin organization and endocytosis,12 Mho1 is a protein of unknown function.

Therefore, MED2, MED3, GPD1, HXK2, and SIW14 were chosen as reverse engineering targets to evaluate if mutations in these genes were causal. We successfully obtained the GPD1W71* and MED2A432Y single mutant strains sZJD-26 and sZJD-27, respectively, using the Cas9-expressing strain sZJD-25. The
specific growth rate of these two strains increased by 31.5 and 47.0% compared with the starting strain sZJD-25, reaching 0.139 h\(^{-1}\), and 0.156 h\(^{-1}\) respectively. A MED2\(^{432Y}\) and GPDI\(^{W71}\) double-mutant strain sZJD-28 reached an even higher specific growth rate of 0.205 h\(^{-1}\), which is 98% of the specific growth rate of the evolved line sZJD-24C (Fig. 2c), showing a clear causal effect of these two mutations. This is consistent with the finding that these two mutations were the only two SNVs found in the evolved strains sZJD-24C2 and sZJD-24C3 (Fig. 2b). sZJD-28 consumed glucose with faster rate and reached higher OD\(_{600}\) value compared with starting strain sZJD-23. The extracellular metabolites were also lower than those of sZJD-23 (Figs. 2d, 1e). Compared with wild-type strain CEN.PK113-11c, sZJD-28 had much higher biomass yield and lower RQ value, which is close to 1. The maximal specific growth rate of sZJD-28 reached 0.218 h\(^{-1}\) (Table 1). Thus, through identification of targets using ALE, we managed to engineer a better and faster growing Crabtree-negative S. cerevisiae with reduced carbon loss.

Transcriptional profiles of the Crabtree-negative strain. To understand the underlying mechanism of how the mutations results in faster growth rate of the reverse engineered strains, we used RNA-Seq to perform transcriptome analysis of the parental strain sZJD-25 and the three strains sZJD-26, sZJD-27, and sZJD-28.

Transcriptome analysis showed that in sZJD-28, totally, 2096 genes, about 33% of all genes of S. cerevisiae, were significantly (padj < 0.01) differentially expressed and 1562 genes in sZJD-27 compared with sZJD-25 (Fig. 3a). This indicated that the nonstop mutation of MED2 resulted in a global impact on the metabolic network. Med2 is one of the subunits of the tail module\(^{10}\), which is one of the four parts of the mediator complex, and it is required for the regulated transcription of nearly all RNA polymerase II-dependent genes in S. cerevisiae\(^{13-15}\). The tail module mediates mediator complex-associated transcriptional regulation on SAGA-regulated, TATA-containing genes which account for about 15% of all the genes in yeast\(^{16,17}\) (Supplementary Figure 5). Indeed, almost half of all TATA-containing genes had significantly altered expression in the MED2 mutant strains sZJD-28 (7.5%) and sZJD-27 (6.8%) (Fig. 3b). It indicated a clear causal effect of this mutation. Additionally, the expression level of MED2 containing genes in the MED2 mutant strain were predominantly downregulated, especially in the double-mutant strain sZJD-28 (Fig. 3c), which is clearly seen in the Volcano plot of sZJD-28 (Supplementary Figure 6). GO Slim Mapper analysis on the 114 shared TATA-containing genes showed that GO terms related to response to cellular amino acid metabolic processes, carbon metabolism, and chemical and oxidative stress were enriched (Supplementary Data 1). It indicated that altering the expression of genes with these GO terms may play an indispensable role in improving the cell growth rate.

The reporter GO term analysis showed that genes associated with GO terms related to translation are upregulated (upper part of heat map in Fig. 3d), whereas genes associated with GO terms related to carbon metabolism are downregulated (lower part of heat map in Fig. 3d). Protein synthesis is required for cell growth and needs ribosomes to polymerize amino acids into polypeptide chains. The cellular growth rate is linearly correlated with ribosome abundance, and the expression of ribosome-associated genes, including genes coding for ribosomal proteins and RNA biogenesis, therefore affects the growth rate\(^{18,19}\). Indeed, in sZJD-28 and sZJD-27, protein synthesis-associated genes were significantly upregulated (Fig. 3d and Supplementary Figure 7). In addition, reporter TFs analysis showed that genes controlled by chromatin remodeling-related TFs such as Snf2, Snf6, Sin3, Sas3, and Rsc1 were significantly changed in sZJD-27 and sZJD-28, in contrast to sZJD-25 (Supplementary Figure 8-9). It suggested that gene transcription may have become more active, supporting increased protein synthesis in these two strains. However, increasing the ribosomal protein fraction would reduce the fraction of metabolic proteins\(^{20}\). Indeed, GO terms related to carbon metabolism–contained genes were significantly downregulated in sZJD-27 and sZJD-28 (Fig. 3d and Supplementary Figure 7). Downregulating of these metabolic genes would save the resource for ribosomal proteins synthesis, as glycolytic enzymes account for a major fraction of the cellular proteome. These results suggested that the introduced mutations led to redistributed and active protein synthesis, which may support a faster cell growth rate.

**Discussion**
Pdc\(^{-}\) S. cerevisiae strains cannot grow in batch cultures on synthetic glucose medium. Two reasons are lacking of cytosolic acetyl-CoA supply and limited capacity of reoxidation of cytosolic NADH\(^{21}\). In sZJD-25, the PO/PTA pathway can produce acetyl-CoA in the cytosol, which supported the growth of Pdc\(^{-}\) S. cerevisiae strains in an excess glucose medium. However, the reoxidation of cytosolic NADH still mainly relies on the mitochondrial respiratory chain due to the absence of alcoholic fermentation. In sZJD-25, the unrestricted glucose uptake and high glycolytic activity would particularly cause problems with recycling of cytosolic NADH to NAD\(^{+}\). Compared with sZJD-25, the high-affinity glucose transporter genes HXT2, HXT4, HXT6, HXT7, HXT10, and HXT14 were upregulated and the low/medium-affinity glucose transporter genes HXT1, HXT3, HXT5, HXT9, and HXT11 were downregulated in sZJD-28. (Fig. 3e). The fold changes of high-affinity glucose transporter genes were higher than that of the low-affinity ones (Supplementary Table 1 Physiological parameters of the wild-type and engineered strains\(^{a}\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CEN.PK113-11c</th>
<th>sZJD-25</th>
<th>sZJD-28</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu) (h(^{-1}))</td>
<td>0.374 ± 0.013</td>
<td>0.140 ± 0.003</td>
<td>0.218 ± 0.006</td>
</tr>
<tr>
<td>(Y_{\text{mass}}) (g g(^{-1}))</td>
<td>0.126 ± 0.000</td>
<td>0.306 ± 0.003</td>
<td>0.368 ± 0.026</td>
</tr>
<tr>
<td>(q_{\text{eth}}) (mmol g(^{-1})h(^{-1}))</td>
<td>−16.405 ± 0.587</td>
<td>−2.284 ± 0.026</td>
<td>−3.255 ± 0.264</td>
</tr>
<tr>
<td>(q_{\text{gly}}) (mmol g(^{-1})h(^{-1}))</td>
<td>23.694 ± 0.485</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(q_{\text{ace}}) (mmol g(^{-1})h(^{-1}))</td>
<td>1.964 ± 0.061</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(q_{\text{co}}) (mmol g(^{-1})h(^{-1}))</td>
<td>0.361 ± 0.015</td>
<td>0.810 ± 0.017</td>
<td>1.397 ± 0.265</td>
</tr>
<tr>
<td>(q_{\text{pyr}}) (mmol g(^{-1})h(^{-1}))</td>
<td>0.152 ± 0.003</td>
<td>0.026 ± 0.001</td>
<td>0.047 ± 0.014</td>
</tr>
<tr>
<td>(q_{\text{CO}_2}) (mmol g(^{-1})h(^{-1}))</td>
<td>27.461 ± 0.877</td>
<td>5.461 ± 0.043</td>
<td>7.633 ± 0.869</td>
</tr>
<tr>
<td>OD(_{600})</td>
<td>3.575 ± 0.032</td>
<td>4.319 ± 0.021</td>
<td>6.400 ± 0.704</td>
</tr>
<tr>
<td>RQ</td>
<td>7.683 ± 0.314</td>
<td>1.264 ± 0.016</td>
<td>1.192 ± 0.010</td>
</tr>
</tbody>
</table>

\(^{a}\)Data are shown mean values ± standard deviations of triplicates

ND not detected.
Figure 3. Transcriptional analysis of reverse engineered strains and parental strain. **a** Venn diagram showing the numbers of significantly (padj < 0.01) regulated genes of reverse engineered strains compared with the parental strain. **b** Fraction of TATA-containing and TATA-less genes among differentially regulated genes in sZJD-28, sZJD-27, and sZJD-26. The bold line in the box represents the median and the yellow dot represents the mean. The lower and upper bounds of the box indicate the first and third quartiles, respectively, and whiskers represent ±1.5× the interquartile range (IQR). **c** Report GO term analysis of the transcription profiles of sZJD-28. The color key shows the rank of the GO terms, and the significance (p value) of the GO terms is included in each cell of the heatmap. For GO terms that have a consensus rank ≤10 in any of the groups shown in the heatmap, Dist (dn) is distinct-directional down, Mix (dn) is mixed-directional down, Nondir is non-directional change, Mix (up) is mix-directional up, and Dist (up) is distinct-directional up. **e** Transcriptional levels of genes related to glucose transportation, glycolysis pathway, and TCA cycle in sZJD-28, sZJD-27 and sZJD-26 compared with sZJD-25.

Expression of high-affinity glucose transporters were repressed by high levels of glucose and induced by low levels of glucose. Transcriptional changes in these glucose transporters indicated that glucose uptake was restricted in sZJD-28 compared with sZJD-25. Additionally, almost all of the genes in glycolysis were downregulated in sZJD-28 (Fig. 3e). Restricted glucose uptake and activity of glycolysis would release the burden on the respiratory chain, which is consistent with the expression profiles of oxidative phosphorylation (OXPHOS) genes, especially in sZJD-28 NADH dehydrogenase genes (ND31 and ND63) and ubiquinol-cytochrome c oxidoreductase genes (complex III), which were significantly downregulated compared with sZJD-25 (Supplementary Figure 11). These results indicate that the limited glucose consumption capacity of sZJD-25, which was enhanced by the introduced mutations led to higher glycolytic flux in sZJD-28, but now with balancing of NADH formation and oxidation back to NAD+. The faster glucose consumption rate, higher biomass yield and lower RQ of sZJD-28 compared with that of sZJD-25 (Table 1) further confirmed the higher glycolytic flux and respiration rate in the reverse engineered sZJD-28. The balance between fermentation and respiration may lead to efficient carbon and electron flux in the cell, which can support the faster growth rate. Taken together, we demonstrated that the Crabtree-positive S. cerevisiae can be turned into a Crabtree-negative yeast by systematic engineering, which included rational pathway design and
system biology analysis. The growth rate of this engineered \textit{S. cerevisiae} reached 0.218 h\(^{-1}\), which was two folds of previous \textit{MTH1} reverse engineered strains and almost reached the level of many natural Crabtree-negative yeasts. By systems biology analysis, the mediator complex was identified as a global regulator involved in rewiring the central carbon metabolism and allocating protein synthesis in a way that favors faster growth of this Crabtree-negative \textit{S. cerevisiae}. We believe that the derived yeast strain represents a possible platform strain for use in biotechnology as well as global rewiring of yeast metabolism through engineering; the mediator complex may be used as a strategy in metabolic engineering of yeast. Additionally, our finding on restricting glucose flux by modulation of the conservative mediator complex may give an insight into cancer metabolism due to the similarity between Crabtree effect and Warburg effect in cancer cells\(^{22}\). Thus, many cancer cells have altered pyruvate metabolism\(^{24}\) and mutations in the mediator complex\(^{3,5,22}\).

**Methods**

**Strains and plasmids.** The yeast \textit{S. cerevisiae} CEN.PK YMZ-E1, YM076, and CEN.PK 113-11C were used as host strain for strain engineering. The strains, plasmids, and primers used in this study are listed in the Supplementary Tables 1, 4 and Supplementary Data 2, respectively. POav, Pops, Polp, and PTase (Supplementary Data 2) were codon-optimized for yeast expression and synthesized by Genscript.

**Reagents.** Primers were synthesized by Sigma-Aldrich. DNA purification and plasmid extraction kits, Taq DNA polymerase, and restriction enzymes were the products of ThermoFisher Scientific. PrimeStar DNA polymerase was purchased from Takara Bio. For genomic DNA extraction, the Blood & Cell culture DNA Kit (Qiagen) was used. For RNA extraction, the RNeasy Mini Kit (Qiagen) was used. All chemicals used were purchased from Sigma-Aldrich, if not otherwise stated.

**Culture conditions and media.** All \textit{S. cerevisiae} strains were cultivated at 200 r.p.m., 30°C. To determine the growth rate, strains were inoculated into the medium with an initial OD\(_{600}\) of 0.05. YPD or YPE media (10 g l\(^{-1}\) yeast extract, 20 g l\(^{-1}\) peptone, 20 g l\(^{-1}\) glucose or ethanol) were used for preparing competent cells. Synthetic complete media without uracil (SCD-Ura or SC-E-Ura) were used to grow strains containing URA3-based plasmids. The media consisted of: 6.9 g l\(^{-1}\) yeast nitrogen base (YNB) without amino acids (Formedium), 770 mg l\(^{-1}\) MgSO\(_4\).7H\(_2\)O, 125 µl antifoam 204 (Sigma-Aldrich, USA), 40 mg l\(^{-1}\) histidine, if needed. For batch fermentation in bioreactors, the methods in previous work\(^{25}\) were followed. Initially, a sample was taken from the cultivation broth after 72 hours. An aliquot was taken from the dodecane phase and diluted five times in hexane for injection in GC-FID. The 2-µl samples were injected in splitless mode, with injection temperature at 200°C. ZB-50 column (30 m × 0.25 mm I.D., 0.25 µm film thickness; Phenomenex, Torrance, CA, USA), were used with helium as carrier gas at a flow rate of 1 ml min\(^{-1}\). Initial oven temperature was set at 50°C for 1.5 min, increased up to 170°C (30°C min\(^{-1}\)) and held for 1.5 min. The temperature was then increased to 300°C (15°C min\(^{-1}\)) and maintained for 3 min.

**Enzyme activity assay.** For cell extract preparation, yeast cells were harvested during the exponential phase and washed with cold enzyme activity detection buffer (1 M potassium phosphate, pH 6.7 and 100 mM Tris HCL, pH 7.4 for pyruvate oxidase and phosphotransacetylase, respectively). Then, the cell suspension was transferred into 2 ml lysis matrix tubes containing beads (MP Biomedicals). Cells were lysed by the MP FastPrep-24 instrument with four 20 sec cycles at the recommended maximum power (4.5 m). Cell debris was removed from the cell lysate by centrifugation at 18,000 × g for 5 min at 4°C, and the supernatant was used for enzyme activity assays. Protein concentration was determined using the Quick Start Bradford Protein Assay (Bio-rad) in an Ultimate 3000 HPLC system (ThermoFisher Scientific). To detect extracellular glucose, ethanol, glycerol, acetate, pyruvate, and succinate, the column was set at 45°C and eluted with 5 mM H\(_2\)SO\(_4\) at a flow rate of 0.6 ml min\(^{-1}\). For 3-HP analysis, the column was set at 60°C and eluted with 1 mM H\(_2\)SO\(_4\) at a flow rate of 0.6 ml min\(^{-1}\). For farnesene analysis, procedures and conditions in the previous work by Tippmann et al\(^{14}\) were followed. Spectrophotometric rate determination method was used. The assay condition was: 37°C, pH = 6.7, 565 nm, and light path = 1 cm. Reagent A: 1 M potassium phosphate buffer, pH 6.7 at 37°C, adjust to pH 6.7 with 1 M NaOH; Reagent B: 1 M NaCl; Reagent C: 0.2 M ammonium acetate, pH 7.0. Procedure: prepare a reaction cocktail by pipetting (in ml) the following reagents into a suitable container: deionized water (1.67 ml), Reagent A (0.2 ml), Reagent B (0.1 ml), and Reagent C (0.1 ml). Mix by swirling and equilibrating to 37°C. Pipette (in ml) the following reagents into suitable containers (test and blank): reactivation cocktail 0.60, Reagent H 0.30, and Reagent I 0.10. Mix and equilibrate to 37°C for 15 minutes; add to A between the A and A+556 nm until constant, then add: Reagent J 0.02 (blue); and enzyme 0.02 (test), immediately mix by inversion and incubate at 37°C for exactly 10 min. Then add Reagent K 0.2 ml in the both test and blank. Mix by inversion and incubate at 25°C for exactly 5 min. Transfer to suitable cuvettes and record at 565 nm. For phosphotransacetylase, continuous spectrophotometric rate determination method was used. The assay condition was: 25°C, pH = 7.4, 233 nm, and light path = 1 cm. Reagent A: 100 mM Tris HCl Buffer, pH 7.4 at 25°C, adjust to pH 7.4 at 25°C with
were cultured at 30°C, 200 r.p.m. in minimal medium with 20 g l⁻¹ ammonium sulfate solution; Reagent E: 25 mM Tris HCl Buffer with 500 mM ammonium sulfate, pH 8.0 at 25°C, adjust to pH 6.5 mM coenzyme A solution, prepare fresh; Reagent D: 220 mM acetyl phosphate 5 M HCl; Reagent B: 100 mM glutathione solution prepare fresh; Reagent C: 0.20 (blank), immediately mix by inversion and record the increase in A233 nm for approximately 5 min. Obtain the ΔA233 nm min⁻¹ using the maximum linear rate for both the test and blank.

Adaptive laboratory evolution. Adaptive laboratory evolution experiments with S. cerevisiae strains 1–24 were carried out by serial dilutions in shake flasks. The strains were cultured at 30°C, 200 r.p.m. in minimal medium to give an initial OD₆₀₀ of about 0.1. After 40 days, the three populations from three independent colonies were used for three independent evolution series. Serial transfer was performed every day or every second day. For every transfer, the cell culture was diluted by a factor ranging from 1:6 to 1:10 into minimal glucose medium to give an initial OD₆₀₀ of about 0.1. After 40 days, the three populations were spread on YPD plates, and three clones were randomly picked from each line. The strains were determined in shake 1 minimal glucose medium. C2 growth rates of these nine strains were determined in shake flasks with 20 g l⁻¹ minimal glucose medium.

Genome sequencing. The genomic DNA of the ALE strains was extracted by using the Blood & Cell culture DNA Kit. The quality of the genomic DNA was assessed by the Agilent 2100 Bioanalyzer according to the manufacturer’s instructions. The genomic DNA of SJD-24-1C and SJD-24-2C failed to meet the sequencing quality requirement and was discarded. DNA from strains SJD-24-1A, SJD-24-1B, SJD-24-3A, SJD-24-3B, and SJD-24-3C from the second line, and SJD-24-3A, SJD-24-3B, and SJD-24-3C from the third line. The specific growth rates of these nine strains were determined in shake flasks with 20 g l⁻¹ minimal glucose medium.

Transcriptome analysis. Cells were collected at OD₆₀₀=1 and stored at −80°C before processing for RNA extraction. Total RNA was extracted by using the RNeasy Mini Kit. The quality of RNA samples was assessed by using the Agilent 2100 Bioanalyzer according to the manufacturer’s instructions. The RNA samples were prepared by using the TrueSeq RNA Stranded HT Sample Prep Kit (Illumina) and sequenced by NextSeq Series Mid-Output Kit (2 × 150 bp paired-end method) according to manufacturer’s manual. S. cerevisiae CEN.PK.113-7D was used as the reference genome (cnpk.tudelft.nl) for mapping the reads. Bresq: 0.28.1 was used for detecting single nucleotide variants, insertions, and small deletions37.

Data availability. The RNA-Seq raw data of the reverse engineered strains and the control strain can be downloaded from the European Nucleotide Archive with the accession number PRJEB23677 (https://www.ebi.ac.uk/ena/data/search?query=+PRJEB23677). The data that support the findings of this study are available within the article and its Supplementary Information file or available from the corresponding author upon reasonable request.

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Author contributions
Z.J.D. and J.N. conceived the study; Z.J.D. designed and performed all the experiments and analyzed the data; M.T.H. assisted with transcriptional data analysis; Z.J.D., M.T.H., Y.C., V.S., and J.N. wrote the manuscript.

Additional information
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Competing interests: The authors declare no competing interests.

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