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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 ability to rapidly consume glucose and produce ethanol that has 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 relies on acetate supplementation and the native ATP-dependent acetyl-CoA synthetase.

To overcome this challenge, we create an alternative pyruvate dehydrogenase (PDH) bypass in *S. cerevisiae* with an ATP-independent acetyl-CoA synthesis 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 thereby, 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::PDAV acs1Δ::PTase) in 20 g l−1 glucose minimal medium. d Acetyl-CoA-derived farnesene and 3-hydroxypropanoate (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 gpp1Δ gpp2Δ) in 20 g l−1 glucose minimal medium. All data represent the mean ± s.d. of biological triplicates.
EBP2, identifi from line sZJD-24C. Kluyveromyces nonfermentans similar to that of some natural Crabtree-negative yeasts, such as hydroxypropionate (61.4 mg l\(^{-1}\)) and Farnesene (20.5 mg l\(^{-1}\)), derived from chemicals (Supplementary Table 2).

Increasing growth of the Crabtree-negative S. cerevisiae. Although the specific growth rate of this S. cerevisiae strain 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 MED2 was identified in five clones derived from lines sZJD-24A and sZJD-24C. The two clones of line sZJD-24B shared a mutation in MED3. 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 GPD1\(^{W71T}\) and MED2\(^{ΔG32Y}\) 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-wt, 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 OD₆₀₀ 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, the expression level of strain sZJD-28 (Fig.3c), which is clearly seen in the Volcano plot (Fig. 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.

Table 1 Physiological parameters of the wild-type and engineered strains

| Parameter          | CEN.PK113-11c     | sZJD-25          | sZJD-28          |
|--------------------|-------------------|------------------|------------------|
| μ (h⁻¹)            | 0.374 ± 0.013     | 0.140 ± 0.003    | 0.218 ± 0.006    |
| Y_biomass (g.g⁻¹)  | 0.126 ± 0.000     | 0.306 ± 0.003    | 0.368 ± 0.026    |
| q₆₅5 (mmol.BD₆⁻¹h⁻¹) | -16.405 ± 0.587  | -2.284 ± 0.026  | -3.255 ± 0.264  |
| q₆₁₈ (mmol.BD₆⁻¹h⁻¹) | 23.694 ± 0.485    | ND               | ND               |
| q₆₁₆ (mmol.BD₆⁻¹h⁻¹) | 1.964 ± 0.061     | ND               | ND               |
| q₆₄₄ (mmol.BD₆⁻¹h⁻¹) | 0.361 ± 0.015     | 0.810 ± 0.017    | 1.397 ± 0.265    |
| q₆₅5 (mmol.BD₆⁻¹h⁻¹) | 0.152 ± 0.003     | 0.026 ± 0.001    | 0.047 ± 0.014    |
| q₆₄₆ (mmol.BD₆⁻¹h⁻¹) | 27.461 ± 0.877    | 5.461 ± 0.043    | 7.633 ± 0.869    |
| q₆₅5 (mmol.BD₆⁻¹h⁻¹) | 3.575 ± 0.032     | 4.319 ± 0.021    | 6.400 ± 0.704    |
| RQ                 | 7.683 ± 0.314     | 1.264 ± 0.016    | 1.192 ± 0.010    |

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. 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. The fold changes of high-affinity glucose transporter genes were higher than that of the low-affinity ones (Supplementary Table 3). The expression of genes with these GO terms may play an indisputable role in improving the cell growth rate.
Glucose22. Transcriptional changes in these glucose transporters repressed by high levels of glucose and induced by low levels of glucose were signifi cantly downregulated compared with sZJD-25 (Table 1) further conﬁ rmed the higher glycolytic ﬂ ux and respiration rate in the reverse engineered sZJD-28. The balance between fermentation and respiration may lead to efﬁ cient carbon and electron ﬂ ux 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 systematic engineering, which included rational pathway design and
system biology analysis. The growth rate of this engineered S. cerevisiae reached 0.218 h⁻¹, which was two folds of previous 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 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. Thus, many cancer cells have altered pyruvate metabolism and mutations in the mediator complex.

Methods

Strains and plasmids. The yeast S. cerevisiae CEN.PK YMY-Z1, IM076, 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, P osp, P olp, and PTase (Supplementary Data 2) were codon-optimized for yeast expression and synthesized by Generspin.

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 S. cerevisiae strains were cultivated at 200 °C. To determine the growth rate, strains were inoculated into the medium with an initial OD₅0₅ 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 complete supplement mixture (CSM, w/o uracil) (Formedium), and 20 g l⁻¹ glucose or ethanol. SCΔ-5’-FOA or SCΔ-5’-FOA plates were used to select against the URA3 marker and which contained 6.9 g l⁻¹ YNB, 770 mg l⁻¹ CSM, 0.8 g l⁻¹ 5-fluorocytosine, 0.1 g l⁻¹ glucose or ethanol. URA3 plasmid cassette were selected on YPD plates containing 200 mg l⁻¹ G418. Shake flask batch fermentation was carried out in minimal medium, pH 6.3, consisting of 7.5 g l⁻¹ (NH₄)₂SO₄, 14.4 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ MgSO₄·7H₂O, 20 g l⁻¹ glucose, trace metal, and vitamin solution supplemented with 40 mg l⁻¹ uracil and/or 40 mg l⁻¹ histidine, if needed. For batch fermentation in bioreactors, the methods in previous work were followed. Specifically, strains were grown in defined minimal medium containing 20 g l⁻¹ glucose, 5 g l⁻¹ (NH₄)₂SO₄, 3 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ MgSO₄·7H₂O, 125 ± 50 antifoam 204 (Sigma-Aldrich, USA), 40 g l⁻¹ uracil, 40 g l⁻¹ histidine, trace metal solutions, and vitamins. Batch fermentations were performed at 30 °C in 1.21 bioreactors (DasaGip, Germany) with a working volume of 500 ml. Cultures were operated with 800 r.p.m. agitation and 1vvm gas flow of pure dried air. Culture pH was maintained at 5.0 by automated addition of 2 M KOH or 1 M NaOH or 1 M HCl; Reagent J: 10 mM potassium phosphate with 10 mM sodium chloride, adjust to pH 5.5 with either 1 M NaOH or 1 M HCl; Reagent K: 100 mM citric acid solution; Reagent L: McIlvain Buffer, 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 mM fluorine adenine dinucleotide solution, prepare fresh; Reagent C: 10 mM thionine pyrophosphate solution, prepare fresh; Reagent D: 150 mM sodium pyrophosphate solution; Reagent E: peroxidease enzyme solution containing 50 purpuragalins units ml⁻¹ in deionized water using peroxidase, immediately before use; Reagent F: 100 mM MgCl₂; Reagent G: 0.2% (v/v) N,N-diethylmethylamine solution; Reagent H: prepare by combining 1 ml of Reagent F and 2 ml of Reagent G, prepare fresh; Reagent I: 1 mM sodium pyruvate solution; Reagent J: 200 mM sodium phosphate solution; Reagent K: 100 mM citric acid solution; Reagent L: McIlvain Buffer, prepare 100 ml by adding 63.15 ml of Reagent J and 36.85 ml of Reagent K; Reagent M: 100 mM ethylenediaminetetraacetic acid solution, adjust to pH 5.5 with either 1 M NaOH or 1 M HCl; Reagent N: 10 mM potassium phosphate with 10 mM fluorine adenine dinucleotide solution, pH 7.0; Procedure: to prepare the reaction cocktail by pipetting (in ml) the following reagents into a suitable container: deionized water 1.70, Reagent A 2.00, Reagent B 0.10, Reagent C 0.20, Reagent D 1.00, and Reagent E 1.00. Mix by swirling and equilibrating to 37 °C. Pipette (in ml) the following reagents into suitable containers (test and blank): reagent cocktail 0.60, Reagent H 0.30, and Reagent J 1.00. Mix by swirling and equilibrating until the A₅₆₅ nm until constant, then add: Reagent N 0.02 (blank) and enzyme 0.02 (test), immediately mix by inversion and incubate at 37 °C for exactly 10 min. Then add Reagent M 2.00 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 activity analysis, 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 8.0 at 25°C with 1 M HCl. Procedure: pipette (in ml) the following reagents into UV cuvettes (test and blank): Reagent A 2.60, Reagent B 0.05, Reagent C 0.20, Reagent D 0.10, and Reagent E 0.03. Mix by inversion and equilibrate to 25°C. Monitor A233 nm until constant, then add Reagent G 0.02 (test) and emerin 0.02 (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 (strain fi 24) were carried out by serial dilutions in shake flask cultures at 30°C, 200 r.p.m. in minimal medium with 20 g l⁻¹ glucose. Cells from three independent colonies were used for three independent evolution series. Serial transfer was performed every one 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. 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. Cells from three independent colonies were used for three independent evolution series. Serial transfer was performed every one 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.

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 S. cerevisiae (strain fi 24) and S. cerevisiae (strain fi 24-1C) was sequenced by NextSeq Series Mid-Output Kit (2 × 75) (Illumina). Reads were aligned on the yeast genome by using Bowtie2 and then further processed by SAMTools and BEDTools to count the number of reads aligning to each gene.

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 TruSeq RNA Stranded HT Sample Prep Kit (Illumina) and sequenced by NextSeq Series Mid-Output Kit (2 × 75) (Illumina). Reads were aligned on the yeast genome by using Bowtie2 and then further processed by SAMTools and BEDTools to count the number of reads aligning to each gene. Differential gene expression was analyzed by using the DESeq package in the R programming language. Reporter analysis on GO terms and transcription factors was performed by using the Platform for Integrative Analysis of Omics (PIANO) R package. Differential expression levels and p-values were used as input in reporter analysis. GO slim mapper analysis was conducted with the online tool at the SGD website (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl).

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 access 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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