Adaptive laboratory evolution of native methanol assimilation in *Saccharomyces cerevisiae*

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Utilising one-carbon substrates such as carbon dioxide, methane, and methanol is vital to address the current climate crisis. Methylotrophic metabolism enables growth and energy generation from methanol, providing an alternative to sugar fermentation. *Saccharomyces cerevisiae* is an important industrial microorganism for which growth on one-carbon substrates would be relevant. However, its ability to metabolize methanol has been poorly characterised. Here, using adaptive laboratory evolution and ¹³C-tracer analysis, we discover that *S. cerevisiae* has a native capacity for methylotrophy. A systems biology approach reveals that global rearrangements in central carbon metabolism fluxes, gene expression changes, and a truncation of the uncharacterized transcriptional regulator Ygr067cp supports improved methylotrophy in laboratory evolved *S. cerevisiae*. This research paves the way for further biotechnological development and fundamental understanding of methylotrophy in the preeminent eukaryotic model organism and industrial workhorse, *S. cerevisiae*. 
Methylophilic organisms are able to grow on one-carbon substrates such as carbon dioxide, carbon monoxide, methane, or methanol and are important primary producers for Earth’s ecosystems as they fix inorganic carbon into biologically available organic carbon. These organisms and their underlying metabolic networks are becoming increasingly important in global efforts to mitigate climate change and reduce our reliance on non-renewable resources such as fossil fuels. Our current dependence on fossil fuels is not sustainable owing to finite reserves and negative environmental impacts from extraction and use. By-products from fossil fuel combustion include a myriad of toxic air pollutants and CO₂, which is the main anthropogenic contributor to climate change. These complex environmental problems call for a global effort to move towards a bio-economy in which microbial metabolism is used for the conversion of renewable materials into useful products.

Typically, sugars derived from sugarcane or corn are used as feedstocks for the production of fuels and chemicals from microbial metabolism. However, sugar production can be prohibitively expensive and requires arable land.

As an alternative to sugars, one-carbon (C1) substrates are abundant and can be obtained from natural gas or waste resources such as agricultural, municipal or industrial waste. Methanol is a particularly attractive one-carbon substrate owing to its abundance and liquid state, which makes it more compatible with existing fermentation, storage and transportation infrastructure. Methanol can be obtained from methane and carbon dioxide stemming from industrial waste streams. Engineering microorganisms to convert C1 compounds such as methanol into food, fuels and chemicals has therefore become a major goal in the field of synthetic biology.

Methylophilic metabolism enables energy and biomass generation from methanol and is present in bacterial, archaeal and yeast species. Recent attempts have been made to produce valuable metabolites using methylophilic organisms. For example, *Methylo bacterium extorquens* AM1 has been engineered to produce 3-hydroxypropionic acid from methanol. The methylophilic yeast *Pichia pastoris* (renamed *Komagataella phaffii*) is currently used industrially for production of recombinant proteins. Recently, *P. pastoris* has also been used for production of metabolites including astaxanthin and isobutanol from sugar. Methanol is particularly attractive as a one-carbon substrate owing to its abundance and liquid state, which makes it more compatible with existing fermentation, storage and transportation infrastructure. Methanol can be obtained from methane and carbon dioxide stemming from industrial waste streams. Engineering microorganisms to convert C1 compounds such as methanol into food, fuels and chemicals has therefore become a major goal in the field of synthetic biology.

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Incorporation of 13C-methanol into central carbon metabolites and specialty products has been demonstrated in both species, with growth on methanol as the sole carbon source recently engineered in *E. coli*. Methanol utilisation research on model eukaryotes such as the yeast *Saccharomyces cerevisiae* has been limited, although *S. cerevisiae* has distinct advantages over organisms such as *E. coli* for use in industrial fermentation. For example, *S. cerevisiae* can correctly express, fold and post-translationally modify eukaryotic proteins, is not susceptible to phage contamination, has high tolerance to low pH concentrations and solvents like methanol, and has organelles that can be co-opted for localisation of specialised metabolism.

Previously, our work identified a methanol-specific transcriptional response in the laboratory strain *CEN.PK* as well as methanol-specific growth. This response was stronger than that of the laboratory strain S288C. Thus, we sought to further characterise methanol metabolism in *CEN.PK* using adaptive laboratory evolution (ALE). Here, we identify the native capacity of *S. cerevisiae* to assimilate methanol into central carbon metabolism. Using methanol-dependent growth assays and 13C-methanol tracer studies, we characterise and improve native methanol assimilation in *S. cerevisiae*.

**Results**

**Methanol-specific growth and 13C-methanol tracer analysis reveals native methanol assimilation in *S. cerevisiae*.** Our previous study compared two commonly used *S. cerevisiae* strains, S288C and *CEN.PK* 113-5D, for their transcriptional response and growth profile in the presence of methanol. *CEN.PK* was identified as the superior candidate to engineer methylophilotrophy as it had better methanol tolerance and a distinct methanol-specific transcriptional response that included upregulation of genes involved in peroxisomal biogenesis (*PEX*), alcohol oxidation (*ADH2*) and formate oxidation (*FDH1*). Having identified the potential for methanol-dependent growth and metabolism in *S. cerevisiae*, methanol-specific growth of *CEN.PK* 113-5D was further assessed using controlled bioreactor fermentations. Previous work with *E. coli* has highlighted the importance of yeast extract for growth on liquid methanol media. Liquid yeast nitrogen base (YNB) medium with and without 2% methanol and 0.1% yeast extract was therefore used to assess the effect of methanol on growth (Fig. 1a). The presence of methanol in the medium resulted in a final OD600 increase of 39% relative to yeast extract-only medium, confirming a methanol-specific growth increase in liquid medium containing yeast extract.

To further investigate the methanol response in *S. cerevisiae* and determine the extent to which methanol enters central carbon metabolism in the presence of the co-substrate yeast extract, a 13C-methanol tracer analysis was performed. Bioreactor off-gases such as 13C-methanol, 13C-CO₂, 13C-ethanol and ethanol were measured in real-time using a mass spectrometer connected to the bioreactors. Intracellular metabolites were analysed using Liquid Chromatography-Mass Spectrometry (LC-MS). 13C-ethanol (Fig. 1c, d) and fully 13C-labelled intracellular metabolites involved in the pentose phosphate pathway, glycolysis, and the TCA cycle were also detected (Fig. 1b). 13C-fructose-1,6-biphosphate and 13C-pyruvate could only arise through the conversion of 13C-methanol through central carbon metabolism (Fig. 1b), confirming *S. cerevisiae* has a native capacity for methanol assimilation. In particular, the identification of a high proportion of fully 13C-labelled metabolite in the case of fructose-1,6-biphosphate (33% of the metabolite pool) and of the structurally complex acetyl-CoA (60% of the metabolite pool) demonstrates methanol assimilation can occur in *S. cerevisiae*.

**ALE of native methanol assimilation in *S. cerevisiae*.** ALE was applied to characterise and improve native methanol metabolism in *S. cerevisiae*. The ALE strategy consisted of three independent lineages grown in 1× YNB medium with either yeast extract or yeast extract plus 2% methanol. Yeast extract-only medium was included to track any improvements in fitness specific to yeast extract. Cultures were grown in baffled shake flasks with alternating passages on 1% glucose or yeast extract with or without methanol (Fig. 2a). ALE relies on naturally occurring DNA replication errors to generate genetic diversity within a population. Owing to the relatively poor growth of *S. cerevisiae* on yeast extract methanol medium, pulsing between two different media was performed in order to increase the number of generations.
replicate.

and mutations over time, while still exposing the population to methanol alongside a poor co-substrate (yeast extract) as a selection pressure. All six independent lineages were grown for 230 generations until a biomass (OD_{600}) increase in yeast extract only medium or minimal medium with glucose. The three evolved lineages, the parental CEN.PK 113-5D, and reconstructed EC strains were grown on yeast extract methanol medium or minimal medium with glucose.

To test if truncation of the YGR067C transcription factor was responsible for improved growth on methanol, CRISPR-Cas9-mediated homologous recombination was used to introduce a stop codon in YGR067C of the wild-type strain as observed in the evolved lineage C (Fig. 2b). The mutation from evolved lineage C was chosen to reconstruct the phenotype as the truncation in Ygr067cp occurred midway between the evolved lineages A and B (Fig. 2b). This reconstructed CEN.PK 113-5D strain was referred to as reconstructed EC. The three evolved lineages, the parental CEN.PK 113-5D, and reconstructed EC strains were grown on yeast extract methanol medium to analyse growth differences (Fig. 2c). The evolved lineage A strain had a final biomass increase of 37% in the presence of methanol compared with the parental CEN.PK 113-5D strain, whereas the evolved lineage B and C strains showed a 22% and 44% increase, respectively. Importantly, the reconstructed EC strain has the same growth profile and final biomass increase (44%) as the evolved lineage C strain compared with the parental strain, indicating that the truncated transcription factor is responsible for increased growth in the presence of methanol. No growth improvement was observed between the parental and reconstructed EC strain on yeast extract only medium or minimal medium with glucose.

Reconstructed EC strain characterisation and $^{13}$C-methanol tracer analysis. To characterise the effect that the reconstructed EC strain had on native methanol metabolism in *S. cerevisiae*, CEN.PK 113-5D and the reconstructed EC strain were grown in bioreactors with 2% $^{13}$C-methanol and 0.1% yeast extract. The reconstructed EC strain was chosen for $^{13}$C-methanol tracer analysis as it provides a clearer genotype–phenotype relationship as the only genotypic change between this strain and the parental
CEN.PK 113-5D is the introduced truncation of Ygr067cp. Thus, any observed changes could most likely be attributed to the regulatory function of YGR067C. As previously noted (Fig. 2c), a growth advantage was observed in the reconstructed EC strain (Fig. 3a), which reached a higher final biomass compared with CEN.PK 113-5D. Both strains produced 80% of total CO₂ as 13C-CO₂ (Fig. 3b). However, the reconstructed EC strain produced less 13C-ethanol, particularly at 11 h (Fig. 3c, d). We hypothesised that the reconstructed EC strain could be redirecting methanol into biomass constituents and thus reducing ethanol production. 

13C-methanol tracer analysis revealed that the reconstructed EC strain had a higher percentage of fully 13C-labelled intracellular metabolites compared with CEN.PK 113-5D (Fig. 3e). An approximate threefold increase in 13C-labelled glyceraldehyde-3-phosphate and dihydroxyacetone phosphate was observed as well as increased 13C-labelling of metabolites involved in lower glycolysis, including 3-phosphoglyceric acid, phosphoenolpyruvate, and pyruvate. 13C-labelled pyruvate increased from 2.7% in CEN. PK 113-5D to 15.4% in the reconstructed EC strain. Higher 13C-labelling was also observed for metabolites in the pentose phosphate pathway but the proportion of 13C-labelled metabolites was still low in both strains (<5%). An interesting observation was that CEN.PK 113-5D had a higher proportion of 13C-labelled acetyl-CoA compared with the reconstructed EC strain, 67.6% compared with 46.6% (Fig. 3e). Finally, no un-labelled or 13C-labelled ketoglutarate, fumarate, malate, oxaloacetate or glyoxylate were detected in the TCA cycle. The only metabolites that were detected in the TCA cycle were citrate and succinate, 13C-labelled citrate increased from 2.4% to 11.4% in the reconstructed EC strain while the same levels (0.5%) were observed in both strains for 13C-labelled succinate.

To further characterise the changes in the reconstructed EC strain, global transcript and protein levels were compared with the parental strain (Fig. 4). During growth on 2% 13C-methanol and 0.1% yeast extract, 243 transcripts were found to be significantly differentially expressed in the reconstructed EC strain (adjusted p < 0.01) with 111 genes downregulated and 132 genes upregulated (Supplementary File 1). Gene-list analysis of the downregulated genes showed Gene Ontology (GO) pathway enrichment of the TCA cycle, respiration and the glyoxylate cycle. No pathway enrichment was found for the upregulated genes in the reconstructed EC strain but GO process enrichment was found for ‘carbohydrate transmembrane transport’, ‘carbohydrate metabolic process’, ‘glycolytic process’ and ‘pyruvate metabolic process’, among others. Proteomics analysis showed 103 proteins were significantly altered in abundance in the reconstructed EC strain relative to the parent (adjusted p < 0.05; Supplementary File 2) with 84 proteins increased and 19 proteins decreased. Gene-list analysis of proteins with increased expression showed pathway enrichment of the ‘superpathway of glucose fermentation’, whereas the proteins with decreased abundance showed GO process enrichment only for ‘trehalose metabolic process’.

Both transcriptomics and proteomics indicated there were significant changes in central carbon metabolic pathway expression between the evolved reconstructed and parental strain. Five genes involved in glycolysis had higher transcript abundance in the reconstructed EC strain compared with CEN.PK 113-5D...
(HXK1, GLK1, DAK2, TDH1 and ENO2), whereas FBP1, coding for fructose-1,6-bisphosphatase, a key enzyme involved in gluconeogenesis\textsuperscript{29,30}, had lower transcript abundance. Hxk1p, Glk1p, Tdh1p and Eno2p also had increased protein abundance, as well as Pfk1p, suggesting glycolysis rather than gluconeogenesis is predominantly occurring during growth on methanol in the reconstructed EC strain. The increased transcript abundance of the 6-phosphofructo-2-kinase glycolysis regulator PFK2\textsuperscript{31} in the reconstructed EC strain also supports this concept. Concordant with the metabolite profile of the reconstructed EC strain, lower transcript abundance was observed for nine genes involved in the TCA cycle (\textit{CIT1, CIT2, ACO1, IDH1, IDH2, KGD2, SDH1, SDH3 and FUM3}; Fig. 4), suggesting the TCA cycle is downregulated during growth on methanol and yeast extract. Sfa1p, which is involved in native formaldehyde detoxification and alcohol oxidation also had increased protein abundance in the reconstructed EC strain. Finally, the pentose phosphate pathway showed interesting results, with \textit{RKI1} having lower transcript abundance and Tkl2p lower protein abundance, whereas Tal1p and Fba1p had higher protein abundance.

Fig. 3 \textsuperscript{13}C-methanol fermentations to characterise the reconstructed evolved strain. a Growth profile of CEN.PK 113-5D and the reconstructed evolved strain grown in liquid YNB medium with 2\% \textsuperscript{13}C-methanol supplemented with 0.1\% yeast extract cultures in bioreactors. Data are from two independent biological replicates with lines representing the mean. Source data are provided as a Source Data file. b Percentage of \textsuperscript{13}C-CO\textsubscript{2}/CO\textsubscript{2} production in yeast extract \textsuperscript{13}C-methanol (2\%) medium from two biological replicates with bars representing the mean. c \textsuperscript{13}C-ethanol was produced by CEN.PK 113-5D and by the reconstructed evolved strain. The signal intensity was normalised to the inert gas nitrogen, and then to biomass for each strain. Data show \textsuperscript{13}C-ethanol intensity at 47 amu for two independent biological replicates, with bars representing the mean. d Comparison of \textsuperscript{12}C-ethanol and \textsuperscript{13}C-ethanol intensities during growth in \textsuperscript{13}C-methanol. The signal intensity was normalised to the inert gas nitrogen, and then to biomass for each strain. Data show the \textsuperscript{12}C-ethanol and \textsuperscript{13}C-ethanol intensity at 31 and 33 amu, respectively, for two independent biological replicates, with bars representing means. SEM secondary electron multiplier, \textit{amu} atomic mass unit. e Percentage of fully \textsuperscript{13}C-labelled intracellular metabolite pool relative to total metabolite pool of CEN.PK 113-5D and reconstructed evolved strains. \textit{F1,6BPP} fructose-1,6-bisphosphate, \textit{G3P} glyceraldehyde-3-phosphate, \textit{RL5P} ribulose-5-phosphate, \textit{DHAP} dihydroxyacetone phosphate, \textit{CIT} citrate, \textit{3PG} 3-phosphoglyceric acid, \textit{PEP} phosphoenolpyruvate, \textit{ACCoA} acetyl-coenzyme A. Data are from two independent biological replicates, with bars representing means, except for CEN.PK 113-5D \textit{F1,6BPP}, for which no \textsuperscript{12}C metabolite was detected in one replicate. Purple squares represent the Reconstructed EC strain, and black circles represent the CEN.PK 113-5D strain. Source data are provided as a Source Data file.
**ADH2 and ACS1 deletion reduces methanol-specific growth.**

Four genes were selected to analyse their potential role in native methanol metabolism in the reconstructed EC strain. First, the gene coding for an alcohol dehydrogenase 2 (ADH2) was selected as in a separate study it was significantly upregulated in response to methanol 25 and owing to the promiscuity of alcohol dehydrogenases in *S. cerevisiae*, it could be oxidising methanol to formaldehyde. CAT8, coding for a transcription factor involved in de-repressing genes during growth on non-fermentable carbon sources and thought to regulate the mutated transcription factor Ygr067cp32 (Fig. 2b) was chosen to analyse its effect on methanol growth. The serine hydroxymethyltransferase gene (SHM1) was

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chosen for deletion as another possibility for C1 carbon assimilation in *S. cerevisiae* involves formaldehyde detoxification to formate and then assimilation through the glycerol cleavage complex. Shm1p is responsible for converting serine to glycine and 5,10-methylenetetrahydrofolate and would be required if biomass formation was due to formaldehyde detoxification and subsequent formate assimilation. Finally, ACSI coding for an Acetyl-CoA synthetase was selected as a study by Oshawa et al. analysed the role of ACSI in *P. pastoris* and found that it is involved in ethanol repression, which is needed for the expression of methanol-induced genes.

Single deletion strains of the aforementioned genes were constructed in the reconstructed EC strain and tested by spotting the strains onto solid minimal YNB medium with 2% glucose, no additional carbon source, or increasing methanol concentrations (Fig. 5). The reconstructed EC strain Δadh2 grew similarly to the reconstructed EC strain in minimal medium with 2% glucose or no additional carbon source (1× YNB) but growth was dramatically reduced on media containing methanol (1–3%), suggesting it is needed for methanol oxidation to formaldehyde. When Δcat8 was deleted from the reconstructed EC strain, growth was only observed with 2% glucose as the carbon source, and its deletion dramatically decreased growth on methanol and 1× YNB, as previously reported. Any potential role of CAT8 in methanol assimilation could therefore not be analysed using this method. Deletion of SHM1 had no effect on growth compared with the reconstructed EC strain on media with either glucose or methanol, suggesting it is not involved in the native methanol assimilation pathway. Interestingly, ACSI deletion showed no growth effect with 1% methanol, but had almost no growth when methanol was present at higher concentrations (2 and 3%).

**Discussion**

Methanol is emerging as an important C1 feedstock for industrial biotechnology, making the characterisation and improvement of native methanol metabolism in model organisms critical to utilising this feedstock. Here, we analysed native methanol utilisation in *S. cerevisiae* CEN.PK 113-5D. First, a methanol-specific growth increase was seen in medium containing low amounts of yeast extract as a co-substrate (Fig. 1a). 13C-methanol trace analysis was then used to analyse the methanol metabolism of CEN.PK 113-5D. 13C-ethanol and 13C-labelled intracellular metabolites were produced (Fig. 1b, c), demonstrating methanol was not only being utilised by CEN.PK but also assimilated through central carbon metabolism. Importantly, *S. cerevisiae* has never been classified as a methylo troph, and no previous growth on- or assimilation of methanol has been recorded in the literature. The identification of fully 13C-labelled metabolites from 13C-methanol clearly showed that methanol can be assimilated through central carbon metabolism in *S. cerevisiae*.

After identifying native methanol assimilation, we sought to characterise and optimise this pathway using ALE (Fig. 2a).
Laboratory evolution experiments have been successful in optimising consumption of C1 carbon sources such as methanol and CO2 in E. coli, and CO2 in P. pastoris 14,36,37. ALE strategies are also helpful for elucidating non-obvious biological engineering solutions and understanding partially characterised systems. In this study, final biomass was increased in liquid yeast extract methanol medium by 44% (Fig. 2a) after 230 generations. Whole-genome sequencing revealed mutations in an uncharacterised putative transcription factor YGR067C that led to truncations of the protein (Fig. 2b). Ygr067cp truncation was reverse engineered into the parental CEN.PK 113-5D strain, and the phenotype was restored (Fig. 2c), confirming that mutations in YGR067C are responsible for the improved growth on methanol. Ygr067cp shares an identical DNA-binding domain with Adr1p and is regulated by Cat8p 28,32. Both Adr1p and Cat8p are involved in the upregulation of genes (Fig. 4). Together with the upregulated genes, ADH2 is an important regulator of methanol metabolism.

The reconstructed EC strain was grown on 13C-methanol and compared with the parental strain CEN.PK 113-5D (Fig. 3). The reconstructed EC strain had an improved capacity for methanol assimilation, with higher proportions of 13C-labelled intracellular metabolites observed (Fig. 3e). Moreover, integrated metabolomics, transcriptomics and proteomics analyses showed that the reconstructed EC strain has a different metabolic profile compared to the parent, with the TCA cycle and gluconeogenesis enzymes being downregulated (Fig. 4). Together with the upregulated genes (DAK2, TDH1 and ENO2) and higher protein abundance of Pfklp, Fablp, Tdhp1 and Enolp, it is likely that a net glycolytic flux increase occurs during methanol assimilation in the reconstructed EC strain relative to the parental control strain.

The downregulated transcripts and proteins that we observed in the TCA and glyoxylate cycles fully overlap with those that are normally de-repressed during growth on non-fermentable carbon sources in a CAT8- and SNF1-dependent manner 28,32. Given that CAT8 is known to regulate YGR067C 32, it is likely that truncation of the YGR067C protein facilitates a decoupling of methanol assimilation from the traditional non-fermentable carbon source utilisation phenotype in yeast. At present, it is unclear how these metabolic rearrangements favour methylotrophy in S. cerevisiae. One possibility is that glycolytic rather than gluconeogenic fluxes favour the pentose phosphate pathway fluxes necessary for methanol assimilation in the presence of yeast extract. Another point worth noting is that many obligate methylotrophs operate an incomplete, downregulated TCA cycle through the absence of α-ketoglutarate dehydrogenase activity, which is thought to preclude heterotrophic growth 38.

It is unclear how formaldehyde, the oxidation product of methanol, is assimilated in S. cerevisiae. However, the high levels of 13C-labelling we saw in dihydroxyacetone phosphate, fructose-1,6-bisphosphate, and glyceraldehyde-3-phosphate suggest assimilation occurs via a mechanism similar to the P. pastoris XuMP pathway where formaldehyde and xylulose-5-phosphate are converted into dihydroxyacetone and glyceraldehyde-3-phosphate. Based on the higher transcript abundance of Tal1p and Fablp, it is possible that the non-oxidative branch of the pentose phosphate pathway is going through different rearrangement reactions than those when S. cerevisiae is grown on glucose to recycle the co-substrates needed for formaldehyde assimilation. Transaldolase could be catalysing the rearrangements of fructose-6-phosphate and sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate and erythrose-4-phosphate as it was recently postulated in P. pastoris grown on methanol 39,40. To identify specific genes involved in native methanol assimilation, ADH2 and three other genes were deleted from the reconstructed EC strain. Another study from our group found that ADH2 was upregulated when S. cerevisiae was grown in the presence of methanol 41 and when the reconstructed EC strain with an ADH2 deletion was grown on solid minimal media with increasing concentrations of methanol, growth was severely hindered in all cases, suggesting its role in oxidising methanol to formaldehyde (Fig. 5). We also observed that deletion of acetyl-CoA synthetase (ACS1) reduced growth on solid methanol media (Fig. 5). ACS1p is important for growth on non-fermentable carbon sources 42, and its homologue is an important regulator of methylotrophic metabolism in P. pastoris 43,44. ACS1p is therefore likely to be an important source of acetyl-CoA in S. cerevisiae methylotrophic metabolism.

Native methanol assimilation was identified in S. cerevisiae and improved through ALE, providing the possibility of exploring native methanol metabolism. Higher biomass formation, a complete understanding of native methylotrophic metabolism, and the elimination of the requirement for yeast extract in liquid methanol medium are the most immediate challenges remaining for the implementation of growth with methanol as the sole carbon source in S. cerevisiae. After robust methylotrophy is established in S. cerevisiae, existing metabolite production pathways could potentially be coupled to a methanol converting ‘platform strain’ using the state-of-the-art genetic tools and the deep physiological characterisation available in this model organism. Together, the results from this work represent an exciting step towards using sustainable feedstocks during microbial fermentations for conversion to chemicals, fuels, materials, pharmaceuticals and foods, and provide a glimpse of an unexplored metabolic network in a model eukaryote.

**Methods**

**Strains and plasmids.** S. cerevisiae plasmids and strains used in this study are shown in Tables 1 and 2, respectively. All strains are available from the authors upon request. To delete ADH2, CAT8, SHM1 and ACS1 from the reconstructed EC strain, the orf:KanMX locus from the BY4741 knockout collection plus 500 bp up- and downstream was PCR amplified using primers 1–8. Amplified constructs were then transformed into the reconstructed EC strain and plated on yeast extract peptone dextrose (YPD) with Geneticin (200 μg/mL; Gibco 10131035). Independent colonies were screened via PCR using primers 9–16 that annealed 200 bp outside of the PCR-generated homology region. All primers and their sequences are listed in Supplementary Table 3.

**Media and growth conditions.** E. coli DH5α were used for plasmid propagation/storage and grown in lysogeny broth media (1% tryptone, 0.5% yeast extract, 1% NaCl) with ampicillin.

The strains were precultured on yeast nitrogen base (YNB) medium without amino acids and with 5 g/L ammonium sulphate (Sigma-Aldrich Y0626) and 10 g/L glucose. Growth experiments were performed on YNB medium with 1 or 2% methanol supplemented with 1 g/L yeast extract (Merck 103753). Optical density readings at 600 nm (OD600) were used to track growth. For spot assays, swabs from streaked agar plates were precultured twice in 10 mL of 1% YNB, 1% glucose in sterile 50 mL Falcon tubes. During the exponential phase of the final pre-culture, cells were washed twice in 10 mL of sterile MilliQ water and serially diluted 10-fold up to 10−4 prior to spotting 5 μL from each dilution onto the indicated agar plates. Plates were incubated at 30 °C for 6 days. Spot assay photos are representative of Table 1 Plasmids used in this study.

| Name | Details | Origin |
|------|---------|--------|
| pRS416 | Yeast centromeric plasmid, URA3 marker | Euroscarf |
Table 2 Saccharomyces cerevisiae strains used in this study.

| Name          | Genotype, plasmids | Notes | Origin |
|---------------|--------------------|-------|--------|
| CEN.PK 113-SD | MATa; ura3-52     |       | Euroscaf® |
| CEN.PK 113-SD | prS416             |       | This study |
| REC. EC       | CEN.PK 113-SD; prS416 |       | This study |
| REC. EC adh2Δ | CEN.PK 113-SD; prS416 |       | This study |
| REC. EC shm1Δ | CEN.PK 113-SD; prS416 |       | This study |
| REC. EC cat8Δ | CEN.PK 113-SD; prS416 |       | This study |
| REC. EC acs1Δ | CEN.PK 113-SD; prS416 |       | This study |

DNA extraction and whole-genome sequencing of ALE lineages. Glycerol stocks of the six independent evolutionary lineages were inoculated into 10 mL of YPD medium and grown overnight. Cells were pelleted and washed twice in 10 mL sterile MilliQ water by centrifuging at 4000 × g for 2 mins. Genomic DNA was extracted from pellets using the Yeast DNA Extraction Kit from ThermoFisher (catalogue number 78870) according to the instructions. Whole-genome sequencing was performed at the Ramaciotti Centre for Genomics using Nextera XT library preparation and NextSeq500 2 × 150 bp sequencing. A minimum of 11 million reads were generated per sample with >95% of reads having Q20 quality scores. For mutation reconstruction, evolutionary lineage B, which had 80% of reads mapped to at least Q20. An annotated CEN.PK 113-7D reference genome was generated by transferring annotated coding sequences with greater than 95% homology from the S288C genome to CEN.PK 113-7D FASTA files using Geneious Pro version 11.4. Untrimmed reads were mapped to the annotated CEN.PK 113-7D reference genome using Geneious Pro. Each sample had >99% of reads mapped to the reference with ~100-fold coverage. Non-synonymous single nucleotide variants (SNV) within coding sequences were identified at a minimum coverage of 10x, read-variant frequency of 0.9, maximum variant p value of 10^-5, and a minimum strand bias p value of 0.5 when excluding 65% bias. SNVs from each lineage were annotated onto the same CEN.PK reference sequence and inspected visually using Geneious Pro. SNVs present in the three methanol-exposed lineages but absent in the three yeast extract only lineages were identified as potentially causative mutations. Mutations present in all six lineages were assumed to be present in the parental CEN.PK 113-5D strain, and were not considered any further.

Reconstruction of evolved lineage C mutation in YGR067C. The methanol-exposed evolutionary lineages had convergent but non-identical mutations in YGR067C, which all encoded truncations in the protein through premature stop codons. These mutations were substitution of C to G at nucleotide position 788, absent in the three yeast extract only lineages were identified as potentially causative mutations. Mutations present in all six lineages were assumed to be present in the parental CEN.PK 113-5D strain, and were not considered any further.

RNA extraction, sequencing and transcriptome analysis. RNA samples were taken at 34 h during the 13C-methanol (2%) fermentations. In all, 30 mL of culture was centrifuged at 4 °C 17,000 × g for 10 min, with pellets resuspended in RNA later (Sigma-Aldrich R0901) and stored at −20 °C prior to RNA extraction. Total RNA was extracted by digesting cell pellets in five units of zymolase in the digestion buffer of the YeastStar RNA extraction kit (Zymo Research catalogue number R1002) for 1 h at 37 °C, followed by column purification using the RNeasy Plus Mini Kit (QiAGEN catalogue number 74136). Library preparation and sequencing was performed at the Ramaciotti Centre for Genomics using a TrueSeq Stranded mRNA-seq preparation kit and NextSeq 500 2 × 75 bp sequencing. Thirty million reads were generated per sample with Q30 > 95% and Q20 > 95%. Untrimmed reads were mapped to the S288C genome with read counting and differential expression analysis carried out using Geneious Pro version 11.4 by differential expression analysis using the DESeq2 within Geneious, and a false discovery rate (FDR) of 0.1. Approximately 72 million reads were generated per sample. Genes with adjusted p values < 0.01 were considered differentially expressed. Lists of up and downregulated genes were analysed for GO term and pathway enrichment using YeastMine at the Saccharomyces Genome Database (https://yeastmine.yeastgenome.org/yeastmine/bag.do). Significant enrichment against the background whole-genome gene list was identified with p-values less than 0.05 and Holm–Bonferroni correction for multiple comparisons. A list of all significant genes that were differentially expressed can be found in Supplementary File 1.

Off-gas data analysis. Real-time analysis of bioreactor culture off-gas was achieved using a Hiden HPR-20-QIC mass spectrometer (Hiden Analytical) that was connected to the bioreactors. The Faraday Cup detector was used to monitor the signal intensities of N2, Ar, CO2, 13C-CO2, ethanol, 13C-ethanol and 13C-methanol at 28, 40, 44, 45, 27, 40 atomic mass unit (amu), respectively. To increase sensitivity and detect the presence of 13C-ethanol from the off-gas data, the Secondary Electron Multiplier (SEM) detector was used to scan any intensities from 15 to 50 amu, with 47 and 48 amu corresponding to 13C-ethanol. The N2 intensity (constant during fermentation as nitrogen was an inert gas in our experiments) at 28 amu was used to normalise the intensity from 13C-ethanol. The SEM detector scanned the intensities during two to six independent cycles for each bioreactor and at two different time points.
Metabolomics. To measure intracellular metabolites, samples were taken at 11 h during the 13C-methanol (2%) fermentations, quenched in methanol and frozen at −80 °C for extraction, the pellet was resuspended in 2 mL of 50% acetonitrile and transferred to 2 mL microcentrifuge tubes with 0.1 mm diameter glass beads, every sample had two technical replicates. Samples were vortexed for 30 s using a Pre
cells 24 tissue homogeniser (Bertin Instruments) at 30 °C. Three rounds of vor
texting were performed allowing the samples to cool completely between rounds. Samples were centrifuged for 3 min at 9000 × g and the supernatant was transferred to a clean microcentrifuge tube and stored overnight at −80 °C. In all, 2 mL of samples were freeze-dried overnight, then resuspended in 100 μL of water with 10 μM of AZT as internal standard, then transferred to high-performance liquid chromatography (HPLC) mass insert for analysis.

Metabolites were analysed using liquid chromatography tandem mass spectrometry as adapted from ref. 30–32. In brief, analyses were performed using a Dionex Ultimate 3000 HPLC system coupled to an AB Sciex 4000 QTRAP mass spectrometer. Liquid chromatography was performed using a 50 min gradient, detailed in Supplementary Table 1, with 300 μL/min flowrate, on a Phenomenex Gemini-NX C18 column (150 × 2 mm, 3 μm, 110 A), with a guard column of (SecurityGuard Gemini-NX C18, 4 × 2 mm), and column temperature of 55 °C. The mobile phases used were: 7.5 mM aqueous tributylamine (Sigma-Aldrich) with pH adjusted to 4.95 (±0.05) using acetic acid (Labscan) for Solvent A, and acetonitrile (Merck) for Solvent B. Samples were loaded at 4 °C in a sample loop of 10 μL were controlled for injections, and supernatant was transferred to a clean microcentrifuge tube and stored at −20 °C. Samples were digested with trypsin following the S-trap mini method (Protifit Llc), protocol. The digested peptide mixtures were concentrated using Millipore® ZipTip C18 (Merck) eluting with 70% acetonitrile. Residual acetonitrile was removed by vacuum centrifugation and peptides resuspended in 5% acetonitrile, 0.1% formic acid (aqueous) before analysis. Pept
tides were analysed using a ThermoFisher Scientific UltiMate 3000 RSLCnano UHPLC system. Each sample was initially injected onto a ThermoFisher Acclaim PepMap C18 trap reversed-phase column (300 μm × 5 mm nano viper, 5 μm particle size) at a flow rate of 20 μL/min using 2% acetonitrile (aqueous) for 5 min with the solvent going to waste. The trap column was switched on-line with the separation column (ThermoFisher EasySpray PepMap RSLC C18, 150 μm × 150
mm, 2 μm) and the peptides were eluted using a flowrate of 1.0 μL/min using 0.1% formic acid in water (buffer A) and 80% acetonitrile in buffer A (buffer B) as mobile phases for gradient elution. Peptide elution employed a 4-30% acetonitrile gradient for 10 min followed by 30-50% acetonitrile for 10 min and 50-95% acetonitrile for 1 min at 40 °C. The total elution time was 60 min including 95% acetonitrile wash followed by re-equilibration. For each sample run, a volume of 2 μL equating to ~1 μg of peptide material from protein digestion was injected. The eluted peptides from the C18 column were introduced to the MS via a nanoESI and analysed using a Q-Exactive High-Resolution (ThermoFisher). The electrospray voltage was 1.8 kV in positive ion mode, and the ion transfer tube temperature was 275 °C. Employing a top-40ddaMS2 acquisition method, full MS-scans were acquired in the Orbitrap mass analyser over the range m/z 350–1400 with a mass resolution of 120,000 (at m/z 200). The AGC target value was set at 3.00E+06. The 40 most intense peaks with a charge state between 2 and 5 were fragmented in the high energy collision dissociation cell with a normalised collision energy of 28. MSMS spectra were acquired in the Orbitrap mass analyser with a mass resolution of 15,000 at m/z 200. The AGC target value for MSMS was set to 1.0E+05 while the ion selection threshold was set to 1E + 03 counts. The maximum accumulation times were 60 min for full MS-scans and MSMS. For all the experiments, the throughput experiments, the peptide FDR of 0.01 and a protein FDR of 0.01 were used as protein identification level cutoffs. Label-free quantification (LFQ)30 was performed using MaxQuant LFQ intensities. Protein quantitation analysis of the LFQ results was performed using Perseus26 version 1.6.10.0. The data were first filtered to remove identified proteins classified as ‘reverse’, ‘only identified by site’ and ‘potential contaminants’. The two corresponding biological replicates for CEN.PK 113-5D and the reconstructed EC strain were loaded separately and classified as replicates. Proteins that were not present in at least one of the two biological replicates were removed to further trim the data set. All LFQ values were log2 transformed, the median intensity was added to all intensities for all samples to normalise the distribution, and missing values were imputed to 0. Last, a two-sided t test was performed between CEN.PK 113-5D and the reconstructed EC strain, and proteins with FDR adjusted p values of <0.05 were designated as differentially expressed. The log2FC for the significant proteins was then calculated. A list of all significant proteins that were differentially expressed can be found in Supplementary File 2.

Statistics and reproducibility. The plotted data are from independent cultures and the spot assays in Fig. 5 were repeated twice with similar results.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data are included in the published article (and its supplementary information and Source Data files). Raw proteomics data can be downloaded using the following link http://data.mendeley.com/datasets/6ada27c1-2db7-46e9-83b4-fc34e809fcfb. Raw RNA-seq and genome sequencing reads have been deposited at the National Centre for Biotechnology Information under Bioproject number PRJNA162896. Lists of up and downregulated genes were analysed for GO term and pathway enrichment using YeastMine (https://yeastmine.yeastgenome.org/yeastmine/begin.do) at the Saccharomyces Genome Database (https://yeastmine.yeastgenome.org/yeastmine/index.jsp). The SGD Protein Sequence database (http://sgd-archive.yeastgenome.org/sequence?/sequence?5288c referenced/or_protein/) was used to assign proteomic mass spectra to yeast proteins. Prism 7 software was used to plot data. Any other relevant data are available from the authors upon reasonable request. Source data are provided with this paper.

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Author contributions

M.E. and T.C.W. conceived the study and designed the experiments. M.E. performed the experiments, with contributions from T.C.W. M.I.E. and T.C.W. analysed data. M.E., E.M., K.V., and R.A.G.G. designed and performed the 1C-labeling and bioassay experiments. M.P. designed the method to detect intracellular 13C-labelled metabolites. C.S., T.C.W., I.S.P. and I.T.P. participated in the design, support, and coordination of the project. All authors drafted and approved the final manuscript.

Competing interests

The authors declare no competing interests.
Additional information

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