



Benchmarking two *Saccharomyces cerevisiae* laboratory strains for growth and transcriptional response to methanol



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ABSTRACT

One-carbon compounds, such as methanol, are becoming potential alternatives to sugars as feedstocks for the biological production of chemicals, fuels, foods, and pharmaceuticals. Efficient biological production often requires extensive genetic manipulation of a microbial host strain, making well-characterised and genetically tractable model organisms like the yeast *Saccharomyces cerevisiae* attractive targets for the engineering of methylotrophic metabolism. *S. cerevisiae* strains S288C and CEN.PK are the two best-characterised and most widely used hosts for yeast synthetic biology and metabolic engineering, yet they have unpredictable metabolic phenotypes related to their many genomic differences. We therefore sought to benchmark these two strains as potential hosts for engineered methylotrophic metabolism by comparing their growth and transcriptomic responses to methanol. CEN.PK had improved growth in the presence of methanol relative to the S288C derivative BY4741. The CEN.PK transcriptome also had a specific and relevant response to methanol that was either absent or less pronounced in the BY4741 strain. This response included up-regulation of genes associated with mitochondrial and peroxisomal metabolism, alcohol and formate dehydrogenation, glutathione metabolism, and the global transcriptional regulator of metabolism *MIG3*. Over-expression of *MIG3* enabled improved growth in the presence of methanol, suggesting that *MIG3* is a mediator of the superior CEN.PK strain growth. CEN.PK was therefore identified as a superior strain for the future development of synthetic methylotrophy in *S. cerevisiae*.

1. Introduction

The yeast, *Saccharomyces cerevisiae*, is one of the most intensely studied model eukaryotic microorganisms. This single-celled fungus has a well-characterised genetic system amenable to a large variety of advanced genetic manipulation tools, and robust industrial growth. Thanks to its long history in the food, beverage and bioethanol industries, its safety record, and ability to grow robustly at an industrial scale, *S. cerevisiae* has been widely engineered and deployed as a “cell factory” for the production of chemicals, fuels, foods, materials, and pharmaceuticals [1]. With the eventual decline of global oil reserves and mounting environmental concerns over fossil-resource use, renewable methods of biological chemical production are becoming increasingly important. One limitation to the use of cell factories, such as yeast, to achieve this is the fact that they rely on sugars as a carbon source for growth and production. The sugarcane and maize that is

commonly used as a bioprocess feedstock relies on arable land, water, and fertiliser that competes with the human food supply. Moreover, the complete transfer of petrochemical production processes to sugar-fed biological production would have a significant impact on food supplies.

An emerging alternative to sugar as a bioprocess feedstock is to use substrates such as carbon dioxide, methane, and methanol as carbon sources for microbial production processes. Compared to the gases carbon dioxide and methane, methanol is a relatively safe and stable liquid at room temperature and therefore does not require alternative infrastructure for transportation, storage and fermentation. Methanol can be derived from the gasification of biomass to synthesis gas with subsequent reduction to methane, followed by oxidation to methanol. This can be achieved chemically or biologically via a variety of emerging technologies [2,3]. These processes would enable the use of biomass, municipal waste, or natural gas as feedstocks for bio-production via methanol, enabling independence from arable land and sugar

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production. For these reasons, methanol has become an attractive carbon source for the metabolic engineering of sustainable chemical production [4].

There are many microorganisms that naturally grow using methanol, yet they usually do not have the depth of characterisation and genetic tools of model organisms such as *Escherichia coli* and *S. cerevisiae*. Native methylotrophic metabolism is also optimised for growth, making metabolite production from methanol a challenge. Synthetic methylotrophy in model organisms has therefore become a focus in the fields of synthetic biology and metabolic engineering with recent demonstrations in *E. coli* [5–8]. Engineering methylotrophy in yeast is also an attractive option since it would enable production of compounds that require the functional expression of eukaryotic proteins, and could be coupled to a multitude of optimised metabolite production pathways [9].

Laboratory strains of *S. cerevisiae*, such as the S288C derivatives and semi-industrial CEN.PK series, are commonly used for synthetic biology and metabolic engineering projects due to their depth of characterisation and genetic tools. However, significant variation exists in their physiology and genetics with roughly 22,000 point mutations and 83 genes absent in the CEN.PK strains relative to S288C [10,11]. There is an enrichment of single nucleotide polymorphisms (SNPs) in genes and regulatory regions that encode enzymes involved in metabolism, and these two popular laboratory strains of *S. cerevisiae* have inherently different capacities for different engineered functions. For example, S288C was shown to produce 10-fold more vanillin from an engineered pathway than a CEN.PK strain in continuous culture [11]. In contrast, a CEN.PK strain engineered for *p*-coumaric acid production made between 20 and 50% more than its S288C counterpart [12]. Given these fundamental and unpredictable metabolic differences in *S. cerevisiae* strain backgrounds, we sought to benchmark and compare the S288C derivative BY4741 and the CEN.PK derivative CEN.PK113-5D strains for their potential as hosts for synthetic methylotrophy, and understand any differences using RNA-seq mediated transcriptomics.

2. Materials and methods

2.1. Growth media

S. cerevisiae strains were grown in synthetic dropout (SD) media containing Yeast Nitrogen Base Without Amino Acids mix (Sigma-Aldrich Y0626) supplemented with carbon source and/or 0.1% Yeast Extract (Merck 103753) as indicated.

2.2. Growth conditions

For spot assays, swabs from streaked agar plates were pre-cultured twice in 10 mL of 1x YNB, 1% glucose in sterile 50 mL Falcon tubes. During the log phase of the final pre-culture, cells were washed twice in 10 mL of sterile MilliQ water and serially diluted 10-fold prior to spotting 6 μ L of each dilution onto the indicated agar plates. The resulting plates/colonies were photographed using a Singer Instruments Phenobooth after 5 days of growth. Spot assay photos are representative of many repeated experiments.

Liquid cultures were pre-cultured and washed as described for the spot assays. Separate pre-cultures were used to inoculate 50 mL of either 1x YNB plus 0.1% Yeast Extract or 1x YNB, 0.1% Yeast Extract and 2% methanol in triplicate 250 mL baffled shake-flasks. Flasks were shaken at 200 rpm in a 20 mm orbital Infors incubator set to 30° Celsius. Optical density readings at 600 nm (OD_{600nm}) were used to track growth over 48 h. This experiment was repeated with the harvesting of all cultures for RNA extraction after 24 h. OD_{600nm} values were not significantly different between these two experiments. At time of harvesting OD_{600nm} values for BY4741 were 0.11, 0.10, 0.12 in yeast extract medium and 0.10, 0.10, 0.11 in yeast extract methanol medium; for CEN.PK 0.13, 0.14, 0.13 in yeast extract medium and 0.16, 0.16,

Table 1
Plasmids used in this study.

Name	Details	Origin
pRS415	Yeast centromeric plasmid, LEU2 marker	Euroscarf [18]
pRS416	Yeast centromeric plasmid, URA3 marker	Euroscarf [18]
pFDH1-ADR1-pRS416	<i>FDH1</i> promoter mediated <i>ADR1</i> expression from the pRS416 plasmid	This study
pFDH1-MIG3-pRS416	<i>FDH1</i> promoter mediated <i>MIG3</i> expression from the pRS416 plasmid	This study

0.14 in yeast extract medium. Significance testing of optical density differences was conducted using a two-sided student's t-test with equal variance.

2.3. Strain construction

Cells were transformed using the lithium acetate/polyethylene glycol/ssDNA transformation method [13]. A prototrophic BY4741 strain was generated by transforming functional versions of the *MET17* and *HIS3* genes amplified from S288C genomic DNA, and the pRS415 and pRS416 plasmids (Table 1) and selecting on minimal medium without amino acids. This engineered strain is referred to as BY4741 throughout the manuscript. A growth comparison was also performed between this prototrophic BY4741 and the parental strain S288C (Supplementary Fig. 1). A prototrophic CEN.PK113-5D strain was generated by transforming pRS416 (Table 2). The *ADR1* and *MIG3* expression strains were made by Gibson Assembling [14] the CEN.PK113-5D genomic DNA PCR amplified *FDH1* promoter and respective open reading frames into *Sma*I linearized pRS416 vector. Genbank files of these two vectors can be found in Supplementary file 1. The CEN.PK113-5D *mig3 Δ* strain was generated by replacing the entire *MIG3* open reading frame with a kanamycin marker that was PCR-amplified from the BY4741 knockout collection. Colonies resulting from *KanMX* transformation were selected on YPD agar plates with Geneticin (200 μ g/mL; Gibco™ 10131035) and screened via PCR.

2.4. RNA extraction

50 mL samples of YE or YEM cultures were pelleted after 24 h of growth by spinning at 4000 \times g for 2 min and removing the supernatant. Pellets were resuspended in 1 mL of sterile MilliQ water and pelleted again. Total RNA was extracted by digesting culture pellets with 5 units of zymolyase in the digestion buffer of the YeaStar RNA extraction kit (Zymo Research catalog number R1002) for 1 h at 37 °C, followed by column purification using the RNeasy Plus Mini Kit (QIAGEN catalog number 74136), which excludes genomic DNA.

2.5. RNA-sequencing and analysis

Library preparation and sequencing was carried out by the Ramaciotti Centre for Genomics using a TruSeq Stranded mRNA-seq kit and NextSeq 500 1x75bp high-output sequencing. 2.25 Gbp were sequenced per sample, with > 96% of reads having Q30 quality. Untrimmed reads were aligned to the S288C reference genome using the Geneious RNA algorithm with default settings in Geneious Prime v11 software [15]. RPKM, FPKM, and TPM read counts were calculated using Geneious prior to differential expression analysis using the DeSeq2 Geneious plugin [16] with a false discovery rate of 0.1. Genes having adjusted p values less than 0.01 were considered significantly different. Supplementary Files 2–5 contain gene lists, adjusted p-values, and log2 fold changes for transcriptome comparisons i, ii, iii (Fig. 3A), and for CEN.PK and BY4741 on yeast extract medium, respectively.

All significantly up-regulated or down-regulated genes were analysed for Gene Ontology Process and Pathway enrichment using the Saccharomyces Genome Database (<https://www.yeastgenome.org>)

Table 2
Yeast strains used in this study.

Name	Genotype, plasmids	Notes	Origin
S288C	<i>MATa SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6</i>	Haploid prototrophic laboratory strain, mating type α	Euroscarf [18]
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Haploid auxotrophic laboratory strain, mating type 'a'	Euroscarf [18]
BY4741–HIS3/MET17	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; HIS3; MET17, pRS415, pRS416</i>	Haploid auxotrophic laboratory strain with mating type 'a'	This study
CEN.PK113-5D	<i>MATa; ura3-52</i>	Haploid auxotrophic laboratory strain with mating type 'a'	Euroscarf [18]
CEN.PK113-5D-pRS416	<i>MATa; ura3-52, pRS416</i>	Haploid auxotrophic laboratory strain with mating type 'a' and pRS416	This study
CEN.PK113-5D-ADR1	<i>MATa; ura3-52, pFDH1-ADR1-pRS416</i>	<i>FDH1</i> promoter mediated <i>ADR1</i> expression	This study
CEN.PK113-5D-MIG3	<i>MATa; ura3-52, pFDH1-MIG3-pRS416</i>	<i>FDH1</i> promoter mediated <i>MIG3</i> expression	This study
BY4741–HIS3/MET17-ADR1	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; HIS3; MET17, pFDH1-ADR1-pRS416</i>	<i>FDH1</i> promoter mediated <i>ADR1</i> expression	This study
BY4741–HIS3/MET17-MIG3	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; HIS3; MET17, pFDH1-MIG3-pRS416</i>	<i>FDH1</i> promoter mediated <i>MIG3</i> expression	This study
CEN.PK113-5D-mig3 Δ	<i>MATa; ura3-52, mig3Δ</i>	<i>MIG3</i> deletion	This study
CEN.PK113-5D-mig3 Δ -pRS416	<i>MATa; ura3-52, mig3Δ, pRS416</i>	<i>MIG3</i> deletion and pRS416	This study

with Holm-Bonferroni correction and maximum p-values of 0.05. The Saccharomyces Genome Database GO Slim mapper was used to assign differentially expressed genes to GO terms. The STRING database was used to identify and visualise interactions between genes [17]. No extra shell proteins were used to visualise the interaction network in Fig. 4 B, which displays interactions based on text mining, experiments, databases, and co-expression for the CEN.PK methanol up-regulated genes with greater than 2-fold expression changes. Line thickness indicates confidence in the interaction.

3. Results

3.1. Impact of methanol on the growth of BY4741 and CEN.PK113-5D

To test if the presence of methanol in the medium had any effect on strain growth, serial 10-fold dilutions of BY4741 and CEN.PK113-5D were spotted onto solid minimal (Yeast Nitrogen Base) media containing either 2% glucose; 0.1% yeast extract; 2% methanol plus 0.1% yeast extract; 1% methanol; 2% methanol; 3% methanol; or 4% methanol (Fig. 1). The same cell density was observed between the yeast strains in media containing 2% glucose, only Yeast Nitrogen Base (YNB), or 0.1% yeast extract, however, growth on YNB-methanol resulted in distinct growth phenotypes. Despite BY4741 and CEN.PK113-5D displaying equal growth on minimal medium with 2% methanol and 0.1% yeast extract, BY4741 was unable to grow on methanol plates in the absence of yeast extract, even at the lowest methanol concentration

(1%), no growth was observed past the 10^{-1} dilution. CEN.PK113-5D had much higher growth on methanol at 1% and 2% compared to BY4741, with the presence of methanol becoming detrimental at 3%.

Having established that methanol has a distinct and strain-specific influence on agar-plate growth, we tested whether this trend could be re-created in liquid medium. Culturing of either strain in minimal medium with methanol as the sole carbon source did not result in any measurable growth. Previous studies have suggested yeast extract is needed to initiate growth in liquid methanol medium [19], thus 1 g/L was added to the liquid methanol medium. When 0.1% yeast extract was included in the media alongside 2% methanol (YEM medium), CEN.PK113-5D had a 30% ($p = 0.026$) increase in its final optical density (48 h) as measured at 600 nm (OD_{600nm}) compared to the yeast extract only medium (YE medium; Fig. 2B). This methanol-specific difference was also evident at the 36 h time point (Fig. 2B, 29% increase, $p = 0.01$). In contrast, BY4741 showed no methanol-dependent growth effect at 48 h ($p = 0.49$) or 36 h ($p = 0.10$) (Fig. 2A). Both strains had a marginally significant methanol-specific growth effect at 24 h with an increase of 22% for BY4741 ($p = 0.04$) and an increase of 23% for CEN.PK ($p = 0.05$) (Fig. 2A and 2B). These strain and methanol-specific growth differences were consistent with the results obtained from the spot assays, which demonstrated that CEN.PK113-5D has a greater capacity for growth in the presence of methanol than BY4741 (Fig. 1). Growth of the two strains was not different in the yeast extract only medium (Fig. 2C). Although the CEN.PK113-5D strain grew to a higher final OD_{600nm} than the BY4741 strain in YEM medium after

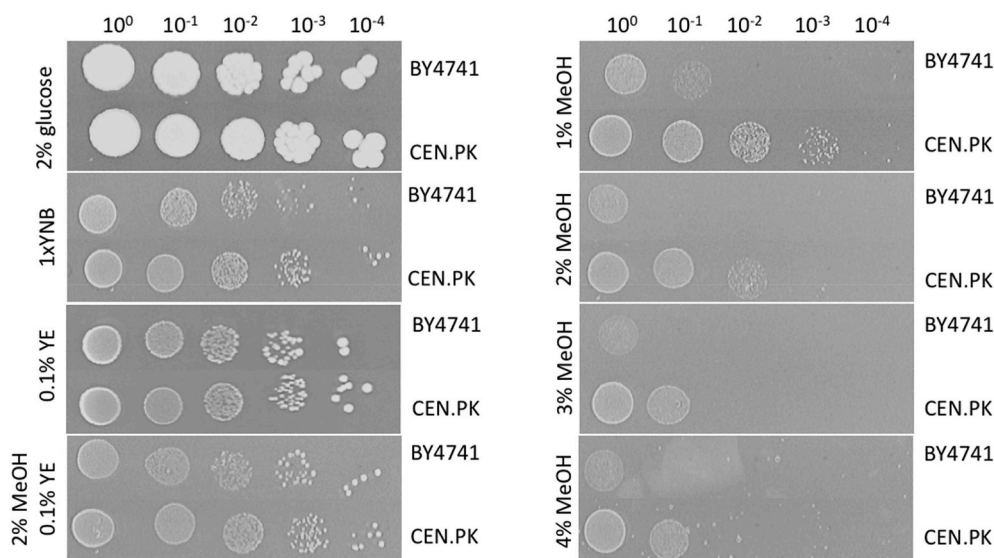


Fig. 1. Spot assays of BY4741 and CEN.PK113-5D on different carbon sources. Growth of serially 10-fold diluted BY4741 and CEN.PK113-5D strains on solid 1x Yeast Nitrogen Base medium with different carbon sources as indicated. Yeast Nitrogen Base (YNB), Yeast Extract (YE), Methanol (MeOH). Images were taken after incubating at 30 °C for 5 days.

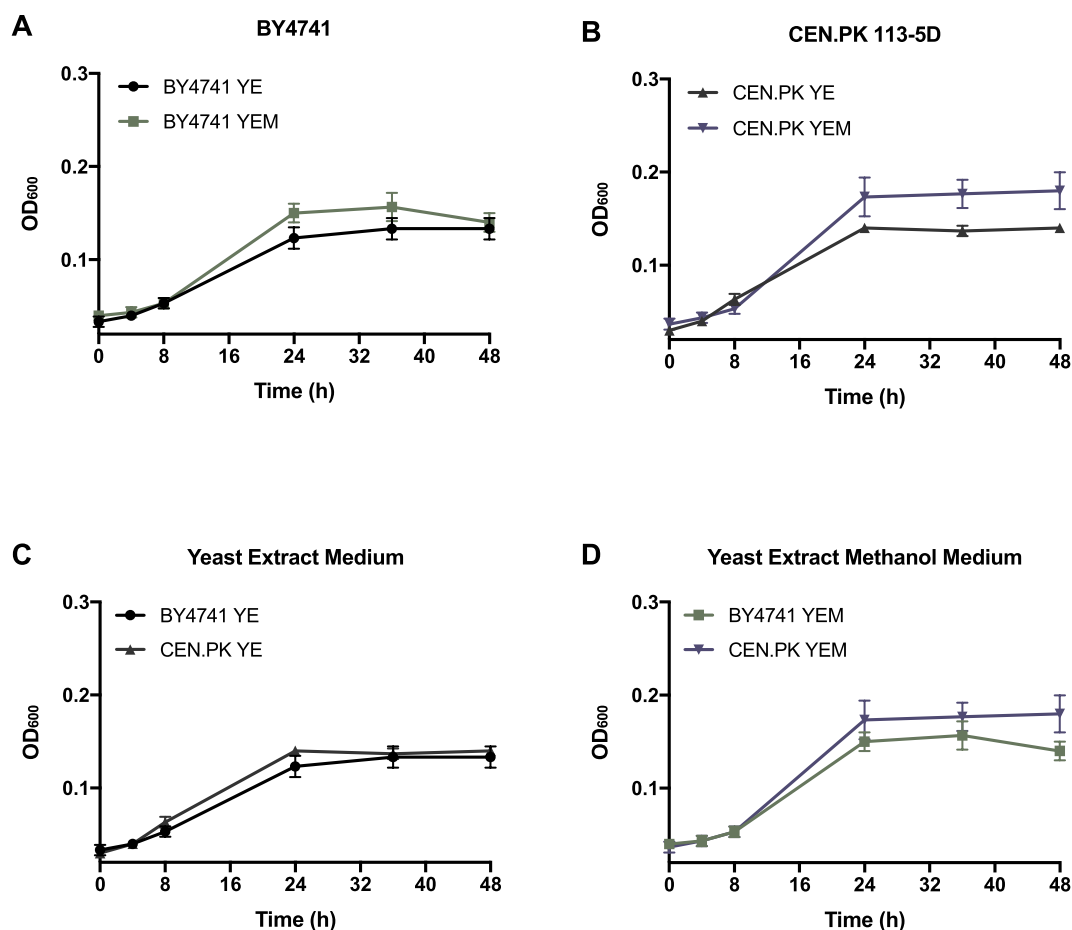


Fig. 2. Growth of BY4741 and CEN.PK113-5D in liquid yeast extract containing medium with or without 2% methanol. Growth of prototrophic BY4741 and CEN.PK113-5D strains was tracked for 48 h in 0.1% yeast extract, 1x Yeast Nitrogen Base medium with (YEM) or without 2% methanol (YE). Data points and error bars represent the mean and ± 1 standard deviation from triplicate cultures.

48 h (Fig. 2D), this was mainly due to a decrease in OD_{600nm} in the BY4741 strain. Given that there were only marginal (statistically insignificant) differences between the two strains in YEM medium after 24 or 36 h (Fig. 2D), the transcriptomics analyses were subsequently focused on comparing differences between YEM and YE media for each strain (Fig. 3A).

3.2. Global transcriptional changes in response to methanol

To understand differences in global transcription patterns during exposure to methanol, the transcriptomes of BY4741 and CEN.PK113-5D grown in YEM medium were compared to those from YE medium. RNA was extracted from each culture at the 24-h time point of the liquid growth experiment (Fig. 2). mRNA sequencing and differential expression analysis was used to elucidate changes in global expression profiles between the YEM and YE medium conditions for each strain separately, and between the two strains grown on YEM (Fig. 3A). Principle component analysis demonstrated that the strain and growth medium differences explained the sample variance, with methanol treated cultures and each strain clustering together (Fig. 3B).

Global changes were visualised using volcano plots (Fig. 3C–E), with Gene Ontology (GO) process mapping used to ascertain higher-level trends (Supplementary Files 6–9). The only significant GO-process or pathway enrichment that was found for the up- or down-regulated genes from the medium comparison for each strain was ‘glycolysis’ which had 11 up-regulated genes when BY4741 strain was exposed to methanol (comparison i, Fig. 3A). For methanol-treated BY4741, 1133 genes were significantly up-regulated ($p < 0.01$), with top GO-process

categories being ‘cytoplasmic translation’, ‘response to chemical’, and ‘transmembrane transport’ (Supplementary File 6). A total of 1224 genes were significantly down-regulated, with top GO categories including ‘transcription by RNA Polymerase II’, ‘response to chemical’, and ‘transmembrane transport’ (Supplementary File 7). The CEN.PK113-5D medium comparison revealed 1298 up-regulated genes, with top GO-processes being ‘response to chemical’, ‘transmembrane transport’, and ‘carbohydrate metabolic process’ (Supplementary File 8). From 1449 significantly down-regulated genes, the top down-regulated GO-processes for CEN.PK methanol grown cultures were ‘rRNA processing’, ‘transcription by RNA Polymerase II’, and ‘cytoplasmic translation’ (Supplementary File 9). While there was limited GO-term enrichment for these intra-strain medium comparisons (i, ii, Fig. 3A), comparing the two strains directly (comparison iii, Fig. 3A) showed significant enrichment ($p = 0.03$) of the peroxisome-associated fatty acid oxidation pathway in CEN.PK. This enrichment was obtained using a list that comprised of genes with fold changes above 2 and p -values less than 0.01. However, enrichment of this pathway was also found when both yeast extract only medium conditions were compared between the two strains, and is therefore strain- rather than methanol-specific.

3.3. Highly up-regulated CEN.PK113-5D genes exhibit a specific and metabolically relevant response to methanol

Compared to the methanol specific transcriptional response in the BY4741 strain, the CEN.PK113-5D strain had some striking differences (comparison iv, Fig. 3A) when only highly up-regulated genes were

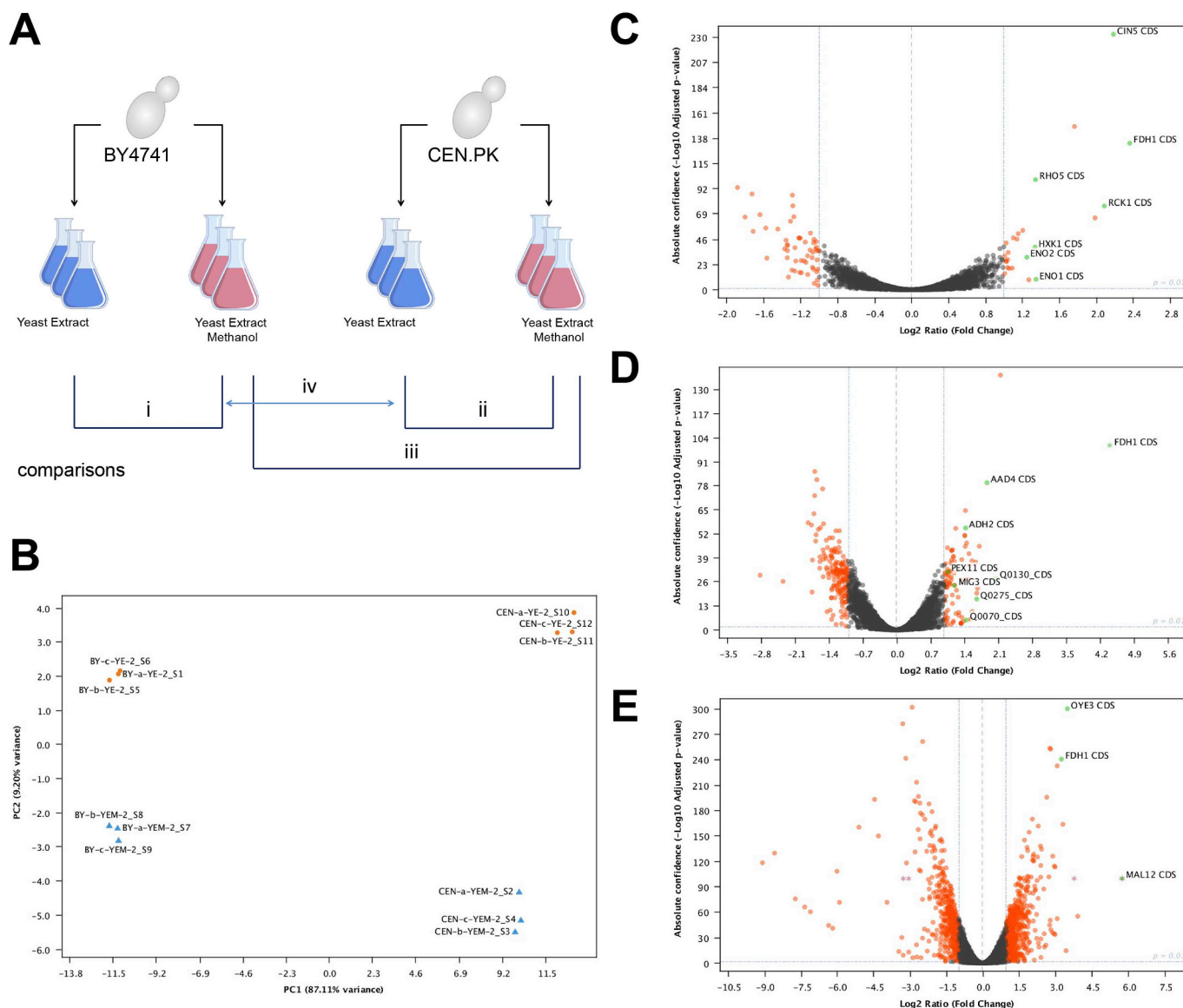


Fig. 3. Transcriptomic experimental design and overview. (A) BY4741 and CEN.PK113-5D were grown in triplicate in media containing 0.1% yeast extract with (YEM) and without 2% methanol (YE). RNA was extracted after 24 h of growth and sequenced. Transcriptomic comparisons were made for each strain on the different media (i and ii), and between the two strains when grown with methanol (iii). Finally, the differences between the strain and medium specific transcriptomes were compared (iv). (B) Principle component analysis was used to determine the relative contributors to variance in the expression profiles from each sample, with volcano plots used to visualise the distribution of fold changes specific to (C) BY4741 methanol treatment, (D) CEN.PK113-5D methanol treatment, and (E) CEN.PK113-5D methanol treatment relative to BY4741 methanol treatment. Genes with fold-changes greater than 2 and adjusted p-values less than 0.01 are coloured orange. PCA and volcano plots were generated using Geneious Prime version 11 [15].

considered (fold-changes above 2 with adjusted p-values < 0.01). Firstly, several mitochondrial genes were up-regulated in the presence of methanol in the CEN.PK113-5D strain (*TRR2*, *CIT3*, *OM45*, *Q0130*, *Q0045*, *Q0070*, *Q0065*, *Q0060*, *Q0055*, *Q0275*) suggesting an active respiratory metabolism when methanol is present in the medium (Fig. 4). Mining of the literature for interactions between genes in this list using the STRING database [20] revealed a greater than expected level of interaction, with a strong cluster of mitochondrial genes evident (Fig. 4, Supplementary File 10).

The second major difference between the two strains when they were grown on YEM relative to YE medium (Fig. 3A, iv) was the up-regulation of several genes that resemble the methanol-specific response of the methylotrophic yeast, *Pichia pastoris* (Fig. 5A). For

example, peroxisome biogenesis and metabolism (*PEX11*, *RTN2*, *EC11*), glutathione metabolism (*GTT2*, *GSH2*, *ECM4*), D-xylulose formation (*XYL2*), and alcohol oxidation (*ADH2*) were all highly up-regulated CEN.PK genes that were absent from the equivalent BY4741 transcriptome (Fig. 5B). The final difference was the up-regulation of the *MIG3* transcription factor, which is thought to be a master regulator of central carbon metabolism that is inactive in some laboratory yeast strains [21]. *MIG3* was up-regulated by 1.6-fold in CEN.PK113-5D relative to BY4741 when methanol was present (but not in yeast extract medium) and by 2.3-fold in CEN.PK113-5D relative to the yeast extract medium. Expression of *FDH1*, which encodes a formate dehydrogenase involved in formaldehyde and formate detoxification, was the most highly up-regulated gene in both strains in the presence of methanol.

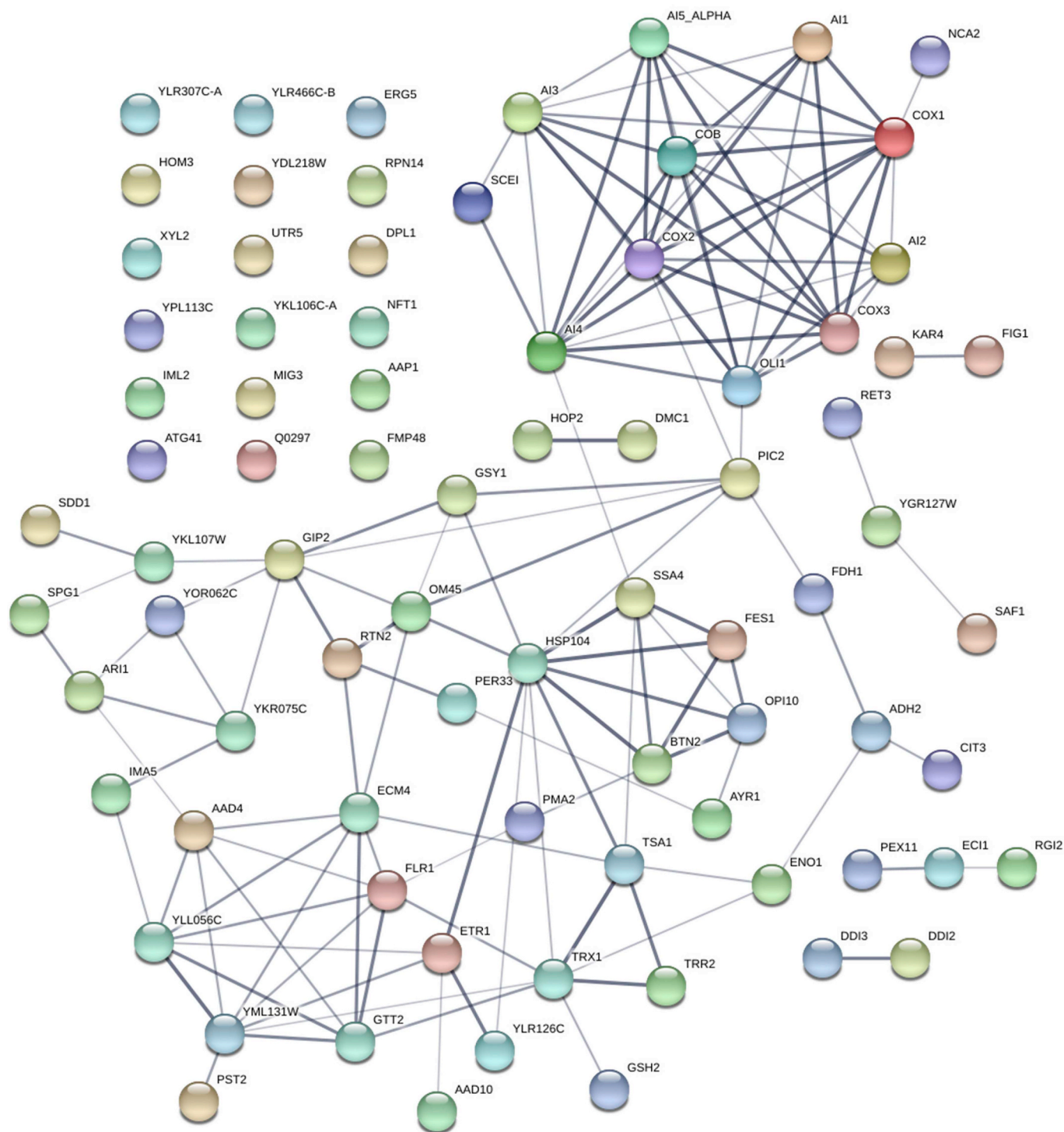


Fig. 4. Interaction network of highly up-regulated methanol specific genes in CEN.PK113-5D. Eighty highly up-regulated methanol-specific CEN.PK113-5D genes (adjusted p-value < 0.01, > 2-fold change) were used to map protein interactions using the STRING database [20]. Interactions (edges) were assigned using text mining, experiments, databases, and co-expression, with increasing edge thickness indicating increasing confidence in node interactions.

Interestingly, in the methanol-medium inter-strain comparison, *FDH1* was 9.6-fold higher in CEN.PK113-5D relative to BY4741.

3.4. Reverse engineering of the CEN.PK methanol response

Based on the up-regulated genes that were specific to the methanol-treated CEN.PK transcriptome, we sought to introduce genes into BY4741 to improve growth in the presence of methanol and ‘reverse engineer’ the superior growth phenotype of CEN.PK. To achieve this,

the *ADR1* and *MIG3* transcriptional regulators from the highly expressed CEN.PK gene set (Fig. 4) were expressed using the *FDH1* promoter on a low-copy pRS416 vector in each strain background. We chose to express these two genes because targets of Adr1p such as *ADH2* and *PEX11* were found in the highly up-regulated methanol-specific CEN.PK transcriptome, while the *MIG3* transcript was itself present in this gene set (Fig. 4). Adr1p is a transcriptional regulator of ethanol and peroxisomal metabolism [22], while Mig3p is a transcriptional regulator of carbon catabolite repression and possibly ethanol metabolism

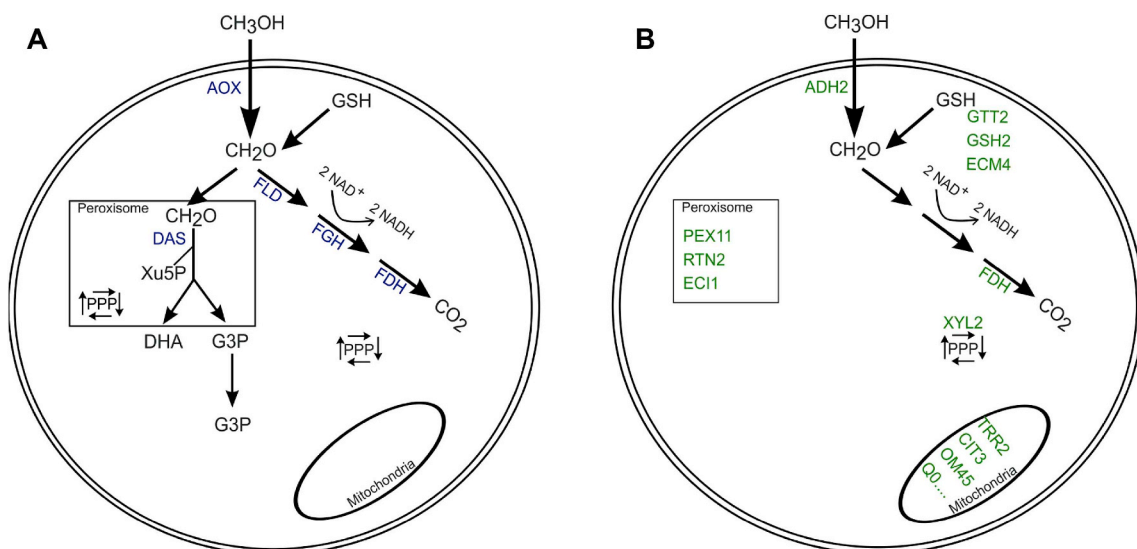


Fig. 5. CEN.PK113-5D exhibits a specific metabolic response to methanol. (A) Schematic representation of methanol assimilation in the methylotrophic yeast *P. pastoris*. Methanol is oxidised to formaldehyde, which is assimilated to glyceraldehyde-3-phosphate for biomass production via the xylulose-5-phosphate (Xu5P) dependent dihydroxyacetone synthase (DAS) or dissimilated to CO₂ for energy using formaldehyde dehydrogenase (FLD), S-formylglutathione hydrolase (FGH), and formate dehydrogenase (FDH). (B) Highly up-regulated *S. cerevisiae* CEN.PK113-5D YEM-specific genes (adjusted p-value < 0.01 >, 2-fold change) were intuitively mapped to potential methanol-associated metabolic functions. Alcohol dehydrogenase (ADH2), glutathione S-transferase (GTT2), glutathione synthetase (GSH2), S-glutathionyl-(chloro)hydroquinone reductase (ECM4), PEX11, RTN2 (involved in peroxisome biogenesis), peroxisomal delta3, delta2-enoyl-CoA isomerase (ECI1), xylitol dehydrogenase (XYL2), mitochondrial thioredoxin reductase (TRR2), mitochondrial citrate and methylcitrate synthase (CIT3), mitochondrial outer membrane protein of unknown function (OM45).

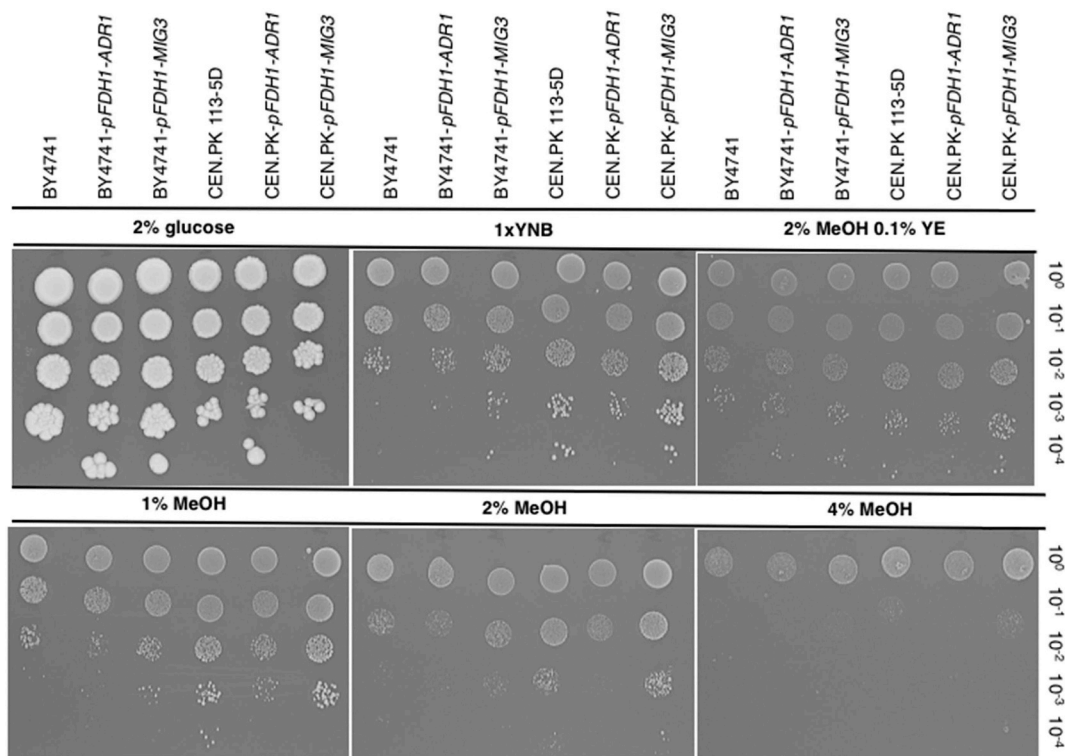


Fig. 6. ADR1 and MIG3 expression spot-assays on different carbon sources. Growth on solid 1x Yeast Nitrogen Base medium with different carbon sources was tested using serial 10-fold dilutions of strains with empty vectors or pFDH1-mediated expression of the ADR1 or MIG3 transcriptional regulators. Yeast Nitrogen Base (YNB), Yeast Extract (YE), Methanol (MeOH). Images were taken after incubating at 30 °C for 5 days.

[21,23]. The FDH1 promoter was chosen because FDH1 was the most highly up-regulated gene in both strains when exposed to methanol (Fig. 3C and D). FDH1-promoter mediated expression of ADR1 did not improve growth when either BY4741 or CEN.PK strains were exposed to methanol (Fig. 6), in fact it appeared to be detrimental to growth.

However, FDH1-promoter mediated MIG3 expression improved growth in both strains when they were exposed to methanol (Fig. 6). Interestingly, this improved growth was also evident in both strains when no carbon-source was added to YNB medium (1x YNB, Fig. 6).

To further investigate the improved growth in the presence of

methanol after *MIG3* over-expression, all strains were grown in liquid yeast extract methanol medium (Supplementary Fig. 2). However, no significant differences between the strains were observed, which is consistent with the results from solid minimal medium with 2% methanol and 0.1% yeast extract (Fig. 6, 3rd panel). The CEN.PK-*pFDH1-MIG3* strain displayed a slight increase in growth compared to all the others but this increase is almost negligible (Supplementary Fig. 2). Given the apparent importance of *MIG3*, we sought to investigate if growth would be hindered by the absence of *MIG3*. *MIG3* was deleted from the genome to create the strain CEN.PK *mig3Δ*, which was grown and spotted onto solid methanol minimum medium alongside CEN.PK (Supplementary Fig. 3). Interestingly, CEN.PK *mig3Δ* had no growth effect on solid minimal media with different methanol concentrations and had the same growth profile as the parent strain in 2% glucose, YEM, and YNB-only media (Supplementary Fig. 3).

4. Discussion

We have compared the two commonly used laboratory strains of *S. cerevisiae*, BY4741 and CEN.PK113-5D, for growth and transcriptional response to methanol. We found that the CEN.PK strain grows better in the presence of methanol than the BY4741 strain on both solid and liquid medium, but that yeast extract is required to support growth in liquid medium. These differences are important for determining which strain could best serve as a host for the engineering of methylotrophic metabolism. Synthetic methylotrophy has become an active area of research in the field of synthetic biology due to the potential attractiveness and sustainability of methanol as a bioprocess feedstock, and the genetic plasticity of model organisms, such as *S. cerevisiae* and *E. coli*. We therefore used transcriptomics to gain insight into differences in genes expression that might explain the methanol-specific growth differences between these two laboratory yeast strains.

In contrast to a previous transcriptome study on methanol toxicity in *S. cerevisiae* S288C where growth medium with yeast extract, peptone, dextrose, and 5% methanol was used [24], we used a lower concentration of methanol (2%), included the semi-industrial CEN.PK113-5D strain, and focused on both strain- and medium-specific differences in transcription. Despite these differences, we found some similar trends to this previous study. For example, we also observed up-regulated aryl alcohol dehydrogenases, alcohol dehydrogenases, aldehyde dehydrogenase, and enzymes involved in glutathione metabolism. These genes are likely involved in the detoxification of methanol and formaldehyde and their up-regulation suggests that *S. cerevisiae* has a native metabolic response to methanol, potentially via promiscuous alcohol dehydrogenase activity followed by formaldehyde detoxification (Fig. 5B). For example, a study by Grey et al. (1996) found that over-expression of *ADH1* lead to hyper-resistance to formaldehyde via possible reduction to methanol, this reaction could also be occurring in the reverse direction oxidising methanol to formaldehyde [25]. We also found that the CEN.PK strain has a specific response to methanol that differs to that of BY4741, and has some similarities to methylotrophic yeasts such as *Pichia pastoris* (Fig. 5A). This response included up-regulation of genes that are specific to the ethanol responsive *ADR1* transcription factor [22], such as peroxisome biogenesis genes, alcohol dehydrogenase (*ADH2*), and glutathione enzymes. The CEN.PK strain also had significant up-regulation of genes involved in mitochondrial respiration, suggesting a more active metabolism involved in ATP synthesis. In theory, NADH would be derived from formaldehyde and formate detoxification by the CO₂ forming formate dehydrogenase (*FDH1*), which was the most highly up-regulated gene in both strains. The NADH generated from Fdh1p-mediated formate oxidation could then be oxidised via the mitochondrial electron transport chain, as long as the tricarboxylic acid cycle (TCA) was active. Despite *FDH1* being up-regulated in both strains under methanol exposure, *FDH1* was still significantly up-regulated by 9.6-fold in CEN.PK grown on YEM relative to BY4741 grown on YEM.

Another interesting finding was the CEN.PK-specific up-regulation of the *MIG3* transcription factor. *MIG1* and *MIG2* are well-characterised glucose-responsive regulators of metabolism [26–28], yet the role of *MIG3* is less well-characterised. Recent work has suggested that *MIG3* is involved in responding to ethanol, and is non-functional in S288C strains [21]. Given the observed up-regulation of *MIG3* in methanol-treated CEN.PK cultures, which was absent in the equivalent BY4741 cultures, and the fact that we were able to improve the growth of both strains by increasing the expression of *MIG3*, it is possible that this transcription factor mediates the observed CEN.PK-specific transcriptional differences in the presence of methanol. *MIG3* represses *SIR2* expression [29], and decreased *SIR2* expression has been shown to improve growth on non-fermentable carbon sources such as ethanol [21]. It is therefore possible that *MIG3* improves growth on non-fermentable carbon sources in general, rather than methanol specifically, which is supported by our observation that the *MIG3* over-expressing CEN.PK strain also has improved growth on YNB-only medium, without methanol present (Fig. 6). However, this does not rule out the possibility that *MIG3* mediates the differences in growth and global transcription patterns that we observed between methanol-treated BY4741 and CEN.PK (Figs. 1–2). This is supported by the fact that *MIG3* expression in BY4741 enabled an increase in growth under methanol exposure on solid minimal media (Fig. 6).

Both synthetic and native methylotrophy are becoming attractive targets for metabolic engineering due to the potential of methanol as an industrial bioprocess feedstock. Our results, alongside some recent studies, suggest that *S. cerevisiae* is a promising host for the engineering of either native or synthetic methylotrophy. For example, a synthetic methylotrophic pathway involving four genes from *P. pastoris*, including an alcohol oxidase and dihydroxyacetone synthase, was recently expressed in *S. cerevisiae* with a subsequent increase in growth in the presence of methanol [30]. However, this study did not demonstrate methanol assimilation using ¹³C-methanol labelling. The potential for formate assimilation to glycine by redirecting fluxes in the native glycine cleavage complex in *S. cerevisiae* was also recently demonstrated [31], and it is not inconceivable that this pathway contributes to the small boost in growth that we observed in the CEN.PK strain grown in liquid culture supplemented with methanol. Alternatively, the NADH generated from the detoxification of methanol and formaldehyde to CO₂ by the Fdh1p enzyme could also boost growth via subsequent ATP generation. Previous work has shown that over-expression of the native formaldehyde dehydrogenase (*SFA1*) can enable the use of formaldehyde as an auxiliary substrate in *S. cerevisiae* [32], demonstrating the potential for C1 metabolism in yeast.

5. Conclusions

Due to inherent and unpredictable differences in the S288C and CEN.PK laboratory yeast strains and the need to identify a host strain for the development of synthetic methylotrophy, we compared the methanol-specific growth and transcriptomic responses of the commonly used yeast strains BY4741 and CEN.PK113-5D. We found that the CEN.PK strain had dramatically improved growth on solid media supplemented with methanol relative to the S288C strain (BY4741), and slightly improved growth in liquid yeast extract medium containing methanol compared to yeast extract only medium. In contrast, the S288C derivative had no growth response to methanol in liquid medium. Consistent with this improved methanol-specific growth, the CEN.PK strain had a distinct transcriptomic response to methanol that included up-regulation of mitochondrial genes, peroxisome biogenesis, glutathione metabolism, the alcohol dehydrogenase *ADH2*, the formate dehydrogenase *FDH1*, and the global carbon-source specific transcriptional regulator *MIG3*. Reverse engineering of the CEN.PK phenotype by expressing *MIG3* in the BY4741 strain to improve growth in the presence of methanol also suggested that *MIG3* is a mediator of the improved growth phenotype and distinct transcriptome in CEN.PK. We

conclude that CEN.PK is a superior host strain for future studies on methylotrophic processes in *S. cerevisiae*, and for the development of synthetic methylotrophy.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.synbio.2019.10.001>.

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