Understanding lipogenesis by dynamically profiling transcriptional activity of lipogenic promoters in *Yarrowia lipolytica*

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Abstract

Lipogenesis is a complicated process involving global transcriptional reprogramming of lipogenic pathways. It is commonly believed that nitrogen starvation triggers a metabolic shift that reroutes carbon flux from Krebs cycles to lipogenesis. In this study, we systematically surveyed and dynamically profiled the transcriptional activity of 22 lipogenic promoters aiming to delineate a picture how nitrogen starvation regulates lipogenesis in *Y. lipolytica*. These lipogenic promoters drive the expression of critical pathways that are responsible for the generation of reducing equivalents (NADPH), carbon backbones (acetyl-CoA, malonyl-CoA and DHAP *et al*), synthesis and degradation of fatty acids. Specifically, our investigated promoters span across an array of metabolic pathways, including glycolysis, Krebs cycle, pentose phosphate pathway, mannitol cycle, glutamine – GABA cycle, fatty acid and lipid synthesis, glyoxylate, β-oxidation and POM (pyruvate-oxaloacetate-malate) cycle. Our work provides evidences that mannitol cycle, glutamine – GABA cycle and amino acid degradation, pyruvate oxidation and acetate assimilation pathways are lipogenesis-related steps involved in generating cytosolic NADPH and acetyl-CoA precursors. This systematic investigation and dynamic profiling of lipogenic promoters may help us better understand lipogenesis, facilitate the formulation of structure-based kinetic models as well as develop efficient cell factories for fuels and chemical production in oleaginous species.

Keywords: Oleaginous yeast, lipogenesis, nitrogen starvations, reducing equivalents, carbon backbones

Introduction

The transition to a sustainable bioeconomy represents a major challenge for our society. The past three decades' progress in metabolic engineering and biocatalysis has proven the metabolic capacity of microbes can be harnessed to upgrade chemical manufacturing and provide fuels, pharmaceuticals and fine chemicals for our everyday use. In particular, various biosynthetic pathways have been exploited to produce fuels and green chemicals with the aim to reduce our dependence on fossil-fuels and mitigate climate change concerns. Among the promising fuel targets, fatty acid-based fuels are receiving growing attention due to the higher energy density, lower hygroscopicity, miscibility with diesel fuels, reduced purification costs and compatibility with existing infrastructure (Knothe 2010). As such, extensive efforts have been made to engineer various microbes to produce free fatty acids (Xu et al. 2013; Xu et al. 2014), fatty alcohols (Runguphan and Keasling 2014), FAEEs (de Jong et al. 2014; Shi et al. 2014b; Tai et al. 2015) and alkanes (Blazeck et al. 2013; Runguphan and Keasling 2014).

Recently, oleaginous yeast or lipoyeast, has attracted wide attentions as they naturally can accumulate large quantity of neutral lipids. These lipids could be easily upgraded to green diesels, cosmetic additives, and oleochemicals via chemical or biological conversion (Dourou et al. 2017; Xu et al. 2017b). The high precursor acetyl-CoA flux along with the versatile carbon-utilization capability makes this yeast a superior host to upgrade low-value carbons into high-value fuels and oleochemicals (Pfleger et al. 2015; Xu et al. 2016).

Y. lipolytica is oleaginous yeast that internalizes substantial portion of carbon feedstock as fatty acids. It has been recognized as a generally regarded as safe (GRAS) organism for the production of organic acids and natural products (Groenewald et al. 2014) in the food and nutraceutical industry. Coupled with its ability to degrade a wide range of substrates, including

hexose/pentose, glycerol, hydrocarbons, volatile fatty acids (VFAs), agricultural waste and even urea or urine as nitrogen source, its low pH tolerance and strictly aerobic nature (Abghari and Chen 2014; Brabender et al. 2018; Ledesma-Amaro et al. 2016b), making this yeast an attractive candidate for industrial applications. In particular, engineering the carboxyl termination and functionalization module offers the opportunity to achieve a variety of biorefinery options, including reduction, hydroxylation, hydrolysis, β-oxidation, ω-oxidation, decarbonylation and condensation *et al* (Fig. 1). The lipogenic pathway in oleaginous yeast has allowed the production of an arrange of commodity chemicals including 3-hydroxyl propionic acid, fatty alcohol, hydroxyl fatty acids and dicarboxylic acid and fatty acids with varied chain-length (Xu et al. 2016), pointing out the huge potential to build a sustainable biorefinery platform from the oleaginous yeast.

Eukaryotes have evolved to partition specialized metabolic functions into distinct cellular compartments. The biogenesis of NADPH, acyl-CoAs and acyl-ACPs occurs in discrete subcellular compartments and often involves spatially separated enzymatic reactions (Li-Beisson et al. 2010; Mlícková et al. 2004). For example, free fatty acids and acyl-CoA intermediates are degraded into acetyl-CoAs via β-oxidation pathways in peroxisome, and lipids are synthesized in ER but stored in oleosome (Bates and Browse 2012; Hiltunen et al. 2003). In the past few years, systems biology approaches including ^{13}C-metabolic flux data (Kerkhoven et al. 2016; Liu et al. 2016; Wasylenko et al. 2015) and transcriptomics data (Liu et al. 2015a; Morin et al. 2011; Wang et al. 2018) or genome-scale metabolic models (Kavšček et al. 2015; Loira et al. 2012) have been used to elucidate lipogenesis mechanisms. These findings have generated fundamental insights on how to efficiently engineer oleaginous yeast for industrial applications. Nevertheless, it is still unclear how the transcriptional activity of lipogenesis-associated promoters would change under

nitrogen starvation conditions. Centering around the supply of acetyl-CoA, malonyl-CoA and NADPH, transcriptional activity of critical pathways in glycolysis, TCA, pentose-phosphate pathway, glyoxylate, fatty acid synthesis/degradation and amino acid metabolism was quantified to derive hypothesis how nitrogen starvation controls lipid accumulation in *Y. lipolytica*. This study may help us better understand the dynamic trend of lipogenic promoters and draw a detailed picture of the metabolic activity of lipogenic pathways, and the insights obtained here will guide us to engineer more efficient cell factories for fuel and oleochemical production with improved pathway yield and process economics.

Materials and methods

Strains and culture conditions

Escherichia coli NEB5α high efficiency competent cells were obtained from NEB and used for plasmid construction, preparation, propagation and storage. The *Y. lipolytica* Po1f strain was purchased from ATCC (MYA-2613). The auxotrophic Po1g (Leu−) was obtained from Yeastern Biotech Company (Taipei, Taiwan).

LB broth or agar plate with 100 μg/mL ampicillin was used to cultivate *E. coli* strains. Yeast rich medium (YPD) was prepared with 20 g/L Bacto peptone (Difco), 10 g/L yeast extract (Difco), and 20 g/L glucose (Sigma-Aldrich), and supplemented with 20 g/L Bacto agar (Difco) for solid plates. YNB medium for seed culture was made with 1.7 g/L yeast nitrogen base (without amino acids and ammonium sulfate) (Difco), 5 g/L ammonium sulfate (Sigma-Aldrich), 0.69 g/L CSM-Leu (Sunrise Science Products, Inc.), and 20 g/L glucose. Selective YNB plates were made with YNB seed culture media supplemented with 20 g/L Bacto agar (Difco).

For promoter activity characterization, the YNB medium with carbon/nitrogen (C/N) ratio 10 used for the shake flask fermentations contained 1.7 g/L yeast nitrogen base (without amino acids and

ammonium sulfate), 11 g/L ammonium sulfate, 0.69 g/L CSM-Leu and 50 g/L glucose. The components in YNB media with C/N ratio 50 and C/N ratio 100 were as same as them in C/N ratio 10 except the content of ammonium sulfate changed to 2.2 g/L and 1.1 g/L, respectively. Single *Y. lipolytica* colonies were picked from YNB selective plates and inoculated into YNB seed culture medium, which were grown at 30 °C 250 rpm for 48 h. The seed culture was then inoculated into 30 mL of YNB media in 250 mL shake flasks.

Plasmids construction

The NanoLuc luciferase was PCR-amplified and cloned downstream of the *Y. lipolytica* TEF promoter in the vector pYaliA1 backbone containing TEF promotor at the SnaBI and KpnI site via Gibson assembly as shown in previous work (Gibson et al. 2009; Wong et al. 2017b).The 22 endogenous promoters were PCR-amplified using *Y. lipolytica* genomic DNA as the template (Supplementary Table S2). About 1000 bp of genome DNA sequence upstream of the coding sequence was amplified to generate the respective promoters. Then the TEF promoter in pYaliA1-NLuc(Wong et al. 2017a) was replaced with the chosen promoter. Specifically, pYaliA1-NLuc was linearized with AvrII and XbaI and gel purified, then the linearized plasmid backbone containing the Nanoluc luciferase reporter gene was Gibson assembled with PCR-amplified 22 endogenous promoters, yielding a total of 22 plasmids with different promotors (Supplementary Table S1).

Luciferase assay for promoter characterization

All plasmids constructed were transformed into the *Y. lipolytica* host strain Po1g ΔLeu using the lithium acetate/single-strand carrier DNA/PEG method (Chen et al. 1997). Single colonies were picked for culturing and subsequently harvested the supernatant from flask culture at various timepoint to perform the luciferase assay using the Nano-Glo Luciferase Assay System kit from

Promega (Catalog number: N1120). To simplify promoter activity assay, all luciferase detected represent the secreted luciferase from the media under different C/N ratios. Luminescence was measured with a Biotek H1 Synergy microplate reader. All measurements were performed with triplicates and the results are reported as mean with standard deviations.

Results

Reducing equivalents and source of lipogenic NADPH

Biosynthesis process involves carbon condensation reaction and electron reduction reaction (Fig. 2). The degradation of carbon feedstock provides the basic building block (acetyl-CoA or malonyl-CoA, IPP, DMAPP *et al*) and the reducing equivalents in the form of NADH or NADPH. The rearrangement of carbon building-blocks requires electrons to reduce the carbonyl group and extend the carbon backbone (Fig. 2), a process very similar to a chemical plant. Similarly, reducing equivalents in the form of NADPH, provide the electrons to reduce and extend the fatty acid carbon backbone. Because NADPH directly affects pathway yield and process economics (Dugar and Stephanopoulos 2011; Qiao et al. 2017; Ratledge 2014), it is necessary to investigate whether there are alternative cytosolic NADPH pathways that could be used for lipid biosynthesis under nitrogen starvation conditions.

Y. lipolytica carries multiples functional NADP+-specific dehydrogenases that complement the oxidative branch of the pentose phosphate pathway. To test alternate pathways that may generate reducing equivalents for lipogenesis, we have mapped the potential NADPH-source pathway in *Y. lipolytica* (Fig. 2). This pathway includes the glucose-6-phosphate dehydrogenase (ZWF1, YALI0E22649g), 6-phosphogluconate dehydrogenase (GND2, YALI0B15598g), sorbitol dehydrogenase (MnDH1 and MnDH2, YALI0B16192g, YALI0D18964g), malic enzyme (MAE1, YALI0E18634g), cytosolic NADP-specific isocitrate

dehydrogenase (IDP2, YALI0F04095g), succinate semialdehyde dehydrogenase (UGA2, YALI0F26191g) and NADP- specific glutamate dehydrogenase (GDH3, YALI0F17820g). These putative NADP-specific dehydrogenases are involved in pentose phosphate pathway, pyruvate-oxaloacetate-malate (POM) cycle, mannitol cycle and keto acids/amino acid salvage (GABA) pathway. Most of the NADPH pathway were chosen on the basis of literature search and sequence alignment in phylogenetically closely-related fungal species. We have chosen these targets because these enzymes have been found to prefer NADPH as cofactor in other fungi, and sequence alignment gives us high scores about conserved domains with *Y. lipolytica*.

To test which enzymatic steps may contribute to generating lipogenic NADPH, we amplified the ~1,000 bp promoter and 5'UTR (5'-untranslated region) sequence of the target genes. Using highly sensitive ATP-independent luciferase (NanoLuc) as reporter gene (Wong et al. 2017a), we systematically characterized the transcriptional activity of NADPH-source pathways (Fig. 3) under different nitrogen starvation conditions. Our results indicate that, transcriptional activity of pentose phosphate pathway promoters ZWF1 (Fig. 3 A) and GND2 (Fig. 3 B) were positively correlated with carbon-nitrogen (C/N) ratios. The transcriptional activity of ZWF1 in C/N ratio 100 increased about two times higher than it in C/N ratio 10 after 96 h, while the activity of GND2 increased more than five times in the same cases (Supplementary Fig. S1). In addition, as the cell enters lipogenic phase (after 40 hr), promoter strength of both ZWF1 and GND2 pathways were rapidly increased, indicating their essential role to generate NADPH for lipid synthesis. Interestingly, our results also identified that mannitol dehydrogenase (encoded by MnDH1 and MnDH2) played an essential role in modulating cytosolic NADPHs, as their transcriptional activity was significantly increased as we tune up the C/N ratios in the lipogenic phase, and especially MnDH2 promoter showed a similar transcriptional trend with TEF promoter (Fig. 3 C, Fig. 3 D and Supplementary

Fig. S1). This is not counterintuitive, as mannitol has been found as the major byproduct accompanying lipid accumulation (Dulermo et al. 2015; Xu et al. 2017b).

Malic enzyme has been extensively studied as the primary source for lipogenic NADPH in both fungi and plants (Wise and Ball 1964; Wynn et al. 1999; Zhu et al. 2018). However, recent studies indicate that *Y. lipolytica* malic enzyme was localized in the mitochondria inner membrane and was not involved in lipid accumulation (Zhang et al. 2013). This led us to investigate whether the transcriptional activity of malic enzyme would change as we limit the nitrogen level in the media. Interestingly, promoter strength of malic enzyme was increased 1.5 times as we increase the C/N ratios from 10 to 100 (Fig. 3 E), but displayed overall decreasing activity as the cell enters lipogenic phase (after 40 hr). These results indicate that malic enzyme in *Y. lipolytica* may be subject to complex regulations that has not been fully understood.

We next investigated the glutamine/GABA-related pathway and tested whether they would contribute to the generation of cytosolic NADPH. Transcriptional activity of putative $NADP^+$-specific isocitrate dehydrogenase (IDP2), succinate semialdehyde dehydrogenase (UGA2) and glutamine dehydrogenase was investigated (Fig. 3). Interestingly, IDP2 promoter appears to be less dependent on the nitrogen starvation conditions (Fig. 3 F), but displayed increasing transcriptional activity as the cell enters lipogenic phase. Most strikingly, UGA2 promoter was strongly activated by nitrogen starvation (Fig. 3 G) at lipogenic phase, indicating its important role to maintain cytosolic NADPHs. Transcriptional activity of GDH3 promoter is relatively unchanged during the lipogenesis phase (Fig. 3 H).

Carbon backbones and source of lipogenic acetyl-CoA

Acetyl-CoA is an essential metabolic intermediate that links both anabolism and catabolism. It is the basic building block for *de novo* fatty acids and sterols biosynthesis. It is also

the end product of lipid β-oxidation, and it is used in the glyoxylate shunt pathway to synthesize C4 carboxylic acids for cell proliferation when C2 feedstock is used as sole carbon source. Due to its amphiphilic nature and the relatively large CoA moiety, acetyl-CoA cannot freely traverse cellular membranes (Chen et al. 2012). Similarly, Krebs cycle intermediates dicarboxylic acids including oxaloacetic acids (OAA), malate and succinate are found in cytoplasm, mitochondria and peroxisome. The activation of glyoxylate shunt pathway is almost exclusively used to replenish the C4 carboxylic acids for TCA cycle and gluconeogenesis when there is an overflow of fatty acids. Therefore, it is important to understand metabolism of acetyl-CoA, malonyl-CoA and C4 carboxylic acids. Overcoming the carbon backbone limitations may unleash the potential of lipogenesis for the manufacturing of various acetyl-CoA derived compounds in this yeast.

It is generally believed that nitrogen starvation induces the expression of AMP deaminase (AMD) which converts AMP to IMP (inosine monophosphate) (Ratledge and Holdsworth 1985; Ratledge and Wynn 2002). Since AMP is an activator for TCA metabolic enzyme isocitrate dehydrogenase (IDH1), TCA metabolic activity will be repressed in response to decreasing level of AMP. As a result, most of the carbon flux will be drained away from Krebs cycle to lipogenesis under nitrogen starvation conditions. To test this hypothesis, we characterized the transcriptional activity of the primary lipogenic precursor pathways (Fig. 4). Our investigated promoters are derived from pathways including ATP citrate lyase (ACL2, YALI0D24431), pyruvate decarboxylase (PDC1, YALI0D10131), acetyl-CoA carboxylase (ACC1, YALI0C11407), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, YALI0C06369), hexokinase (HXK1, YALI0B22308) and pyruvate carboxylase (PYC1, YALI0C24101).

The promoters of ACL2 and ACC1 both displayed decreasing transcriptional activity as the cell enters lipogenic phase (Fig. 4 A and Fig. 4 C). This result is consistent with previous findings

that ATP-citrate lyase and acetyl-CoA carboxylase are subject to tight post-translational phosphorylation modifications (Hynes and Murray 2010; Shirra et al. 2001; Xing et al. 2014). These results indicate that both cytosolic acetyl-CoA and malonyl-CoA might be rate-liming precursors for efficient lipid synthesis in *Y. lipolytica*. Most notably, SNF1-mediated phosphorylation of acetyl-CoA carboxylase has been recently identified as the major suppressing factor for the full function of acetyl-CoA carboxylase (Shi et al. 2014a). Recent studies demonstrate that ATP citrate lyase (encoded by ACL2) is responsible for chromosomal histone acetylation and regulating gene expression in eukaryotic cells, its transcriptional activity was also observed under non-oleaginous conditions (Bellou et al. 2016; Wellen et al. 2009) in *Y. lipolytica*, indicating its important role as a global regulator. Understanding the details of the phosphorylation regulation may require mapping the function of the carbon-source related Snf1 kinase, which is out of the scope of current investigations.

Interestingly, both PDC1 and HXK promoters displayed increasing transcriptional activity as the cell enters lipogenic phase (Fig. 4 B and Fig. 4 E). HXK1 catalyzes the entry point for glucose utilization, and lipid biosynthesis in general has a high demand of glycolytic precursors (i.e. DHAP). The high HXK1 transcriptional activity probably reflects increased glycolytic carbon flux leading to lipid synthesis. Unexpectedly, PDC1 transcriptional activity was also significantly increased about 2 times after 48 h when the C/N ratio increased from 10 to 100. (Fig. 4 B). Scrutiny of *Y. lipolytica* genome would reveal multiple copies of aldehyde dehydrogenase and one copy of acetyl-CoA synthetase (YALI0F05962g, ACS2). The highly upregulated PDC1 promoters at lipogenic phase point the important role of pyruvate decarboxylation, aldehyde dehydrogenase and acetate assimilation as potential carbon backbone pathways to generate cytosolic acetyl-CoAs.

Indeed, engineering PDC-ALDH bypass has led to the increased production of acetyl-CoA-derived compounds in this yeast (Markham et al. 2018; Xu et al. 2016).

Transcriptional activities of GAPDH and PYC1 promoters are generally less dependent on carbon-nitrogen ratios (Fig. 4 D and Fig. 4 F). However, increase in the PYC1 promoter activity at lipogenic phase may indicate that the cell has a need to replenish oxaloacetate (OAA) precursors or other TCA intermediates during lipogenesis. Since POM (pyruvate-oxaloacetate-malate) cycle involves the reductive carboxylation of pyruvate by PYC1 and the subsequent oxidative decarboxylation of malate to generate cytosolic NADPH (Fig. 2), the increase in PYC1 transcriptional activity may provide the evidence that malic enzyme (encoded by MAE1) indeed contributes to supplying cytosolic NADPH during lipogenesis. GAPDH is the glycolytic pathway that is associated with NADH and ATP generation, seems least affected by nitrogen starvation during lipogenesis.

Fatty acids synthesis and degradation

Lipogenesis in oleaginous yeast involves the dynamic interaction of fatty acid biosynthetic pathway and the degradation pathway. Major lipogenesis players involve the multifunctional fatty acids synthase (FAS1 and FAS2), diacylglycerol acyltransferase (DGA1 and DGA2) and the peroxisomal acyl-CoA oxidase (POX1 to POX4) *et al*. To systematically understand how nitrogen starvation regulates the transcriptional activity of fatty acid synthesis and degradation pathway, we surveyed a number of promoters including the YALI0B15059g (Fatty acid synthase β subunit, FAS1), YALI0B19382g (Fatty acid synthase α subunit, FAS2), YALI0E32769g (Acyl-CoA: diacylglycerol acyltransferase, DGA1) and the YALI0E27654g (Fatty acyl-CoA oxidase, POX4).

Surprisingly, the transcriptional activity of FAS1 promoter displays decreasing trend as the cell enters lipogenic phase (Fig. 5 A), and transcriptional activity at C/N 50 demonstrates overall

stronger strength than that of C/N 100. In contrast, the transcriptional activity of FAS2 promoter displays increasing trend, and the promoter strength correlates well with the C/N ratio in the media, almost 50% improvement from C/N 10 to C/N 100 (Fig. 5 B). This inconsistent transcriptional activity of FAS1/FAS2 promoter responding to nitrogen starvation indicates the complexity of transcriptional regulation in fatty acids synthesis. It may also suggest that only FAS1 limits the efficiency of the fatty acid synthase system in *Y. lipolytica*. A few studies have demonstrated that a downstream regulatory element within the coding sequence of FAS1 could activate the transcription of FAS2 (Wenz et al. 2001), which may involve multiple transcriptional factors including Ino2, Ino4 and Fbf1 *et al*. It is interesting to find that only the first 66 nt of the FAS2 coding region was necessary and sufficient for FAS1-dependent gene expression (Wenz et al. 2001). Since our FAS2 promoter didn't contain any of the coding regions, it could possibly explain that FAS1 promoter didn't follow the transcriptional trend with the FAS2 promoter. To explore how the cell coordinates the expression of FAS1 and FAS2 and why there is a discrepancy of the transcriptional activity between FAS1 and FAS2 promoter, molecular details of fatty acids synthesis regarding regulatory roles of major transcriptional factors should be discovered.

DGA1 involves the last step of triacylglycerol synthesis by transferring the acyl group from acyl-CoA to diacylglycerol. Previous studies with overexpression of DGA1 from a strong TEF promoter has led to the construction of high lipid strains (Qiao et al. 2015; Tai and Stephanopoulos 2013), indicating its important role to sink acyl-CoA flux to lipid bodies. Our studies indicate that DGA1 promoter exhibits relatively low transcriptional activity, and nitrogen starvation conditions induced gene expression from DGA1 promoter (Fig. 5 D). DGA2, an isozyme of DGA1, was also discovered as an important contributor in lipid synthesis in both oleaginous yeast (Beopoulos et al. 2012; Silverman et al. 2016) and microalgae species (Xin et al. 2017). To unravel the

underpinnings how DGA1 and DGA2 control lipid synthesis, it is worth to study the dynamic interplay between DGA1 and DGA2 and understand the time-events of metabolic activity of these two enzymes.

POX4 promoter, which drives the expression of peroxisomal acyl-CoA oxidase (Aox) and initiates fatty acid β-oxidation, was found to be highly induced after the cell entered lipogenic phase (Fig. 5 C). Extensive studies on the function and chain-length specificity of the six Aox enzymes reveal a 'leaky - hose pipe model' of the β-oxidation cycle in *Y. lipolytica*, where various intermediates of acyl-CoAs could be released from the oxidation cycle (Haddouche et al. 2010). Targeting enzymes into peroxisome and activating the β-oxidation cycle have been proven as effective strategies to produce or secrete various oleochemicals with different chain-length (Ledesma-Amaro et al. 2016a; Xu et al. 2016). Morphological studies indicate that Aox proteins regulate the size of cellular triacylglycerol pools and the size and number of lipid bodies in this yeast (Mlícková et al. 2004). Aox enzymes were also found to degrade a broad range of chemicals, including alkanes, hydroxy fatty acids, dicarboxylic acids (Fickers et al. 2005) and aromatic toluene (Jain et al. 2004) *et al*. The highly induced nature of POX promoter highlights the potential applications of these Aox enzymes for biotechnological applications.

Krebs cycle, glyoxylate and GABA-associated amino acid degradation

Lipogenesis is a complicated process involving reallocation of central carbon flux toward the fatty acid pathway. Apart from the reducing equivalents, carbon backbone and fatty acids synthesis/degradation pathways, we also investigated the transcriptional activity of the promoters participating in TCA cycle, glyoxylate shunt, and amino acid degradation pathway. We specifically chose the promoters for the mitochondrial isocitrate dehydrogenase (IDH2, YALI0D06303), peroxisomal isocitrate lyase (ICL1, YALI0C16885), the cytoplasmic γ-

aminobutyrate (GABA) transaminase (UGA1, YALI0E18238) and the glutamate decarboxylase (GAD, YALI0C16753).

A central hypothesis on lipogenesis in oleaginous yeast is the repression of TCA cycle due to depletion of AMP by AMP deaminase (Ratledge and Wynn 2002; Wynn et al. 1999), and the overflown citrate flux is redirected to cytosolic acetyl-CoA by ATP citrate lyase. Indeed, our promoter test data indicates that the mitochondrial IDH2 transcriptional activity is reduced as the cell enters lipogenic phase (Fig. 6 A). Interestingly, nitrogen starvation conditions appear to induce the transcriptional activity of IDH2 promoter (Fig. 6 A). It is not clear whether the mRNA transcripts encoding the isocitrate dehydrogenase will be increased or not. Systematic studies of the transcriptomic level gene expression change should be performed to further validate the relationship between TCA metabolic activity and lipogenesis.

Peroxisomal glyoxylate shunt pathway converts two acetyl-CoA units to C4 dicarboxylates (malate, succinate) to replenish TCA intermediates and generate most of the biomass precursors (Zhu et al. 2012). We also surveyed the isocitrate lyase promoters (ICL1) to understand how glyoxylate shunt connects with lipogenesis. ICL1 promoter was significantly induced at the lipid accumulation phase (Fig. 6 B). The transcriptional activity of ICL1 promoter was also positively correlated with nitrogen starvation and it could be induced at same transcriptional level with other strong promoters after 96 h cultivation (Supplementary Fig. S1). This data suggests that the functional operation of TCA cycle may require the replenish of C4 intermediates (malate or succinate) from glyoxylate shunt, equivalently to say that α-ketoglutarate dehydrogenase or succinyl-CoA synthetase steps were possibly impaired under nitrogen starvation conditions.

A few recent studies have indicated that amino acid metabolism might be related with lipogenesis (Blazeck et al. 2011; Kerkhoven et al. 2017; Kerkhoven et al. 2016; Rodríguez-

Frómeta et al. 2013). Under nitrogen starvation conditions, amino acid should have a higher turnover rate to meet essential cell functions. Since glutamate/aspartate-ketoacids transaminases are at the central hub of amino acid degradation, we specifically investigated the promoter activity of glutamate decarboxylase (GAD) and γ-aminobutyrate (GABA) transaminase (UGA1). Our results indicate that transcriptional activity of GAD promoter was decreased (Fig. 6 C), presumably pointing out that the pool of glutamate or glutamine is critical to maintain basic cell functions. Instead, promoter activity of UGA1 (encoding γ-aminobutyrate transaminase) was strongly induced by nitrogen starvation, about 20% (Fig. 6 D). Coupling with the strong induction of UGA2 (Fig. 3 G) and GDH3 (Fig. 3 H) promoters, it clearly indicates that glutamate-GABA transaminase cycle and succinate semialdehyde dehydrogenase may be involved in generating lipogenic precursor (i.e. NADPHs) in oleaginous yeast (Fig. 2).

Discussion

Core promoter sequence in fungi is approximately 200 bp. 1000 bp coding sequence in front of the ATG start codon was chosen as the promoter in the reported work. It should be noted that this is only a proximal promoter without distantly regulatory sequence. But this 1000 bp should provide enough binding sites for endogenous transcriptional factors that may better mimic the native transcriptional regulation. Thorough investigation of endogenous transcriptional network is required to fully understand lipogenic mechanism in this yeast.

The source of cytosolic NADPH may dependent on both the genetic background and physiological conditions. There have been extensive studies on alternative source of NADPH in Baker's yeast. For example, a well-performed study demonstrates that disruption of glucose-6-phosphaste dehydrogenase (Zwf1) makes Baker's yeast more sensitive to oxidative stress. Then "overexpression of the ALD6 gene coding for cytosolic acetaldehyde dehydrogenase, which

utilizes NADP+ as its cofactor" could mitigate oxidative stress and restore cell viability (Grabowska and Chelstowska 2003). These results indicate the reaction catalyzed by Ald6p (aldehyde dehydrogenase) as an important source of reducing equivalents (NADPH) in yeast cells. In a similar study, other group proved that cytosolic NADP+-specific isocitrate dehydrogenase (Idp2p) were alternative source of NADPH (Minard and McAlister-Henn 2005) when non-fermentative carbon source (lactate or other short chain fatty acids) were used. In this study, we investigated the possible NADPH source pathways in *Y. lipolytica*. Apart from pentose phosphate pathway, our results indicate that mannitol cycle, malic enzyme, aldehyde dehydrogenase (succinate semialdehyde dehydrogenase encoded by UGA2) and glutamate dehydrogenase (GDH3) all involved in generating cytosolic NADPHs for lipid biosynthesis in *Y. lipolytica*.

When glucose is used as carbon source, metabolic flux analysis indicates that NADPH is primarily generated at the expense of glucose oxidation in the pentose phosphate pathway (Liu et al. 2016; Ratledge 2014; Wasylenko et al. 2015). Consequently, the overall pathway yield will be decreased due to the supply of NADPH by oxidative glucose decarboxylation (Qiao et al. 2017). Centering around the supply of NADPH, engineering alternative NADPH pathways to bypass pentose phosphate pathway or recycling NADPH from central carbon metabolism have proven as the most efficient strategy to increase pathway yield and improve process economics in oleaginous yeast species (Qiao et al. 2017). Since the previously published metabolic flux map (Liu et al. 2016; Wasylenko et al. 2015) only incorporates a limited number of NADPH pathways, it will be challenging to conclude whether there are alternative NADPH pathways contributing to lipid synthesis. The major function of pentose phosphate pathway is to generate ribose sugars for DNA/RNA synthesis. However, lipogenic cells were find non-dividing with constant non-lipid biomass (Xu et al. 2017b), which is different from prolific cancer cells. The increase in biomass

was primarily a result of the increase of carbon storage as lipid bodies in oleaginous yeast (Xu et al. 2017b). Our findings indicate that we could harness these alternative NADPH pathways to further improve the cost-efficiency of oleaginous yeast cell factories for various applications.

Lipogenesis is triggered by nitrogen starvation, and previous omics-studies have demonstrated that amino acid metabolism plays important roles in regulating lipogenesis (Kerkhoven et al. 2017; Kerkhoven et al. 2016). Under nitrogen starvation conditions, nitrogen is precious resource for replenishing and synthesizing important amino acids and nucleic acids. Our promoter assay data indicates that nitrogen salvage pathways provide alternative NADPH that could be used for lipid biosynthesis. Genome alignment indicate *Y. lipolytica* possesses at least four aldehyde dehydrogenases (Ald2, Ald4, Ald5 and Ald6) prefer $NADP^+$ as cofactors. A putative $NADP^+$-specific isocitrate dehydrogenase (IDP2) have been recently characterized in *Y. lipolytica* (Li et al. 2013). Since GABA (gamma-aminobutyrate) pathway is at the central hub of amino acid degradation (Marzluf 1997), with all these findings, we mapped the potential cytosolic NADPH source pathways in *Y. lipolytica* (Fig. 2). Interestingly, Ald6 in *Y. lipolytica* is annotated as UGA2 that encodes succinate semialdehyde dehydrogenase, which connects amino acid degradation with replenish of C4 intermediates (succinate). Likewise, an evolutionary process has revealed a novel lipid production enhancer (uga2) and provided the evidence that GABA-associated glutamate degradation may contribute to lipogenesis (Liu et al. 2015b).

In terms of lipogenic carbon backbones, our studies revealed the complicated regulation of ACL2 (ATP citrate lyase) and ACC1 (acetyl-CoA carboxylase) promoters. The low transcriptional activity of ACL2 and ACC1 promoters also indicate their possible rate-limiting roles that determine or control lipogenic efficiency (Fig. 3). The remarkable induction of PDC1 promoter by nitrogen starvation suggests that lipogenic acetyl-CoA may be primarily generated

from the pyruvate bypass steps instead of ATP citrate lyase. Pyruvate bypass involves pyruvate decarboxylase (PDC), aldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS), which accounts for majority of cytosolic acetyl-CoAs in Bakers' yeast (Chen et al. 2012; Kozak et al. 2014; Shiba et al. 2000; van Rossum et al. 2016). Although there is no detectable acetate in glucose fermentation of *Y. lipolytica* culture (Xu et al. 2017a), the high acetate utilization efficiency indicates that ACS was active enough to convert acetate to acetyl-CoA in this yeast. It is worth to engineer the pyruvate bypass to further improve the lipid production of this yeast.

Our systematic investigation of 22 lipogenic promoters provides a special angle how nitrogen starvation controls lipid synthesis in *Y. lipolytica*. Promoter transcriptional activity data may not be able to accurately correlate with mRNA abundance of pathway genes, the protein expression level of essential enzymes, and the metabolites pool of critical intermediates. A more holistic approach that incorporates transcriptomics, proteomics, phospho-proteomics or metabolomics data should be put forward for understanding the exact metabolic events that are associated with lipogenesis and nitrogen starvation. Systematic reconstruction of transcriptional regulation by studying the interactions of hundreds of various transcriptional factors will also provide fundamental insight on lipid synthesis. Nevertheless, our mapping of the transcriptional activity of 22 lipogenic promoters helps us draw a clear picture how the carbons and reducing equivalents flow into lipid pathway. This systematic investigation and dynamic profiling of major lipogenic promoters may help us better understand lipogenesis, facilitate the formulation of structure-based kinetic models as well as the engineering of more efficient cell factories that upgrade lipoyeast biomanufacturing platform.

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Author contributions

PX and HL designed the study. HL performed this study with the promoter cloning work helped by MM and Ms. Lynn Wong. PX and HL wrote the manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no competing interests.

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Table 1. Twenty-two lipogenic promoters investigated in this study.

Genomic loci	Annotation	Enzyme Reaction	Promoter name	Vector
YALI0E32769g	Acyl-CoA: diacylglycerol acyltransferase	DAG + LCAcCoA → TAG	ylDGA1	pYaliB1
YALI0C11407g	Acetyl-CoA-carboxylase 1	Ac-CoA + CO_2 + ATP → Mal-CoA + ADP	ylACC	pYaliC1
YALI0D24431g	ATP citrate lyase 2	Cit + ATP + CoA → OAA + Ac-CoA + ADP + Pi	ylACL2	pYaliD1
YALI0D06303g	Isocitrate dehydrogenase mitochondrial	Iso-Cit + NAD^+ ⇌ α-KG + CO_2 + NADH + H^+	ylIDH2	pYaliE1
YALI0B15059g	Fatty acid synthase beta	Ac-CoA + 8 Mal-CoA + 16 NADPH → stearic acid + 8 CO_2 + $NADP^+$	ylFAS2	pYaliF1
YALI0B19382g	Fatty acid synthase alpha		ylFAS1	pYaliG1
YALI0C16885g	Isocitrate lyase 1	Iso-Cit → Suc + Glx	ylICL1	pYaliH1
YALI0E27654g	POX4 Fatty-acyl-CoA oxidase	Acyl-CoA → QH_2 + 2-Enoyl-CoA	ylPOX4	pYaliJ1
YALI0E22649g	G-6-P dehydrogenase	G-6-P + $NADP^+$ ⇌ 6-PGL + NADPH	YlZWF1	pYaliK1
YALI0F04095g	Cytosolic NADP-specific isocitrate dehydrogenase	Iso-Cit + $NADP^+$ → α-KG + NADPH + CO_2	ylIDP2	pYaliL1
YALI0C06369g	Glyceraldehyde 3-phosphate dehydrogenase	GAP + NAD^+ →1,3-P-G + NAD	ylGAPDH	pYaliM1
YALI0E18238g	γ-aminobutyrate (GABA) aminotransferase	GABA + α-KG ⇌ SSA + Glu	ylUGA1	pYaliN1
YALI0F26191g	succinate semialdehyde dehydrogenase	SSA + $NADP^+$ ⇌ SUC + NADPH	ylUGA2	pYaliO1
YALI0F17820g	NADP- glutamate dehydrogenase	Glu + $NADP^+$ ⇌α-KG + NH_3 + NADPH + H^+	ylGDH3	pYaliP1
YALI0C16753g	glutamate decarboxylase	Glu → GABA + CO_2	ylGAD	pYaliQ1
YALI0E18634g	Malic enzyme	Mal + $NADP^+$ ⇌ PYR + CO_2 + NADPH	ylMAE1	pYaliR1
YALI0B15598g	6- phosphogluconate dehydrogenase	6-PGL+$NADP^+$ → 5-PRL + CO_2 + NADPH	ylGND2	pYaliS1
YALI0B22308g	HXK1 Hexokinase	Hexose + ATP → Hexose-6-P+ ADP	ylHXK1	pYaliT1
YALI0B16192g	mannitol dehydrogenase	MNT + $NADP^+$ → FRU + NADPH	ylMnDH1	pYaliU1
YALI0D18964g			ylMnDH2	pYaliV1
YALI0C24101g	pyruvate carboxylase	PYR + CO_2+ ATP → OAA + ADP + P	ylPYC1	pYaliW1
YALI0D10131g	pyruvate decarboxylase	PYR + TPP → HETPP + ALD+ CO_2	ylPDC1	pYaliX1

Figure captions

Fig. 1. Opportunity for engineering lipogenic chemistry to synthesize a broad range of fuels, oleochemicals and green chemicals in oleaginous yeast. All these oleochemicals are derived from acyl-CoAs and acyl-ACPs, which are initially derived from acetyl-CoA. Oleaginous yeast provides the platform for synthesizing these chemicals in a more efficient way.

Fig. 2. Lipogenesis mechanism and involved metabolic pathway in Y. lipolytica. Red-colored gene are potential NADPH source pathway. Blue-colored gene are potential carbon precursor pathways. Cellular compartmentalization of major catabolic and anabolic pathways is colored with green (mitochondria), brown (peroxisome), light blue (ER). Detailed list of genes and the encoded enzymes are compiled at Table 1. The genes upregulated to respond to increasing CN ratio were underlined.

Fig. 3. Time-profile of transcriptional activity of NADPH-source pathways under nitrogen starvation conditions. Nanoluc luciferase genes was used a reporter gene to quantify the promoter strength. Detailed annotation of each gene could be found in Table 1.

Fig. 4. Time-profile of transcriptional activity of carbon backbone pathways under nitrogen starvation conditions. Nanoluc luciferase genes was used a reporter gene to quantify the promoter strength. Detailed annotation of each gene could be found in Table 1.

Fig. 5. Time-profile of transcriptional activity of fatty acid synthesis and degradation pathways under nitrogen starvation conditions. Nanoluc luciferase genes was used a reporter gene to quantify the promoter strength. Detailed annotation of each gene could be found in Table 1.

Fig. 6. Time-profile of transcriptional activity of Krebs cycle, glyoxylate and GABA-associated amino acid degradation pathways under nitrogen starvation conditions. Nanoluc luciferase genes

was used a reporter gene to quantify the promoter strength. Detailed annotation of each gene could be found in Table 1.