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Lack of the NAD^+-dependent glycerol 3-phosphate dehydrogenase impairs the function of transcription factors Sip4 and Cat8 required for ethanol utilization in *Kluyveromyces lactis*

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ABSTRACT

The NAD^+-dependent glycerol 3-phosphate dehydrogenase (KlGpd1) is an important enzyme for maintenance of the cytosolic redox balance in the milk yeast *Kluyveromyces lactis*. The enzyme is localized in peroxisomes and in the cytosol, indicating its requirement for the oxidation of NADH in both compartments. *Klgpd1* mutants grow more slowly on glucose than wild-type cells and do not grow on ethanol as a sole carbon source. We studied the molecular basis of the latter phenotype and found that Gpd1 is required for high expression of *KlICL1* and *KlMLS1* which encode the key enzymes of the glyoxylate pathway isocitrate lyase and malate synthase, respectively. This regulation is mediated by CSRE elements in the promoters of these genes and the Snf1-regulated transcription factors KlCat8 and KlSip4. To study the transactivation function of these factors we developed a modified yeast one-hybrid system for *K. lactis,* using the endogenous ß-galactosidase gene *LAC4* as a reporter in a *lac9* deletion background. In combination with ChIP analyses we discovered that Gpd1 controls both the specific binding of Cat8 and Sip4 to the target promoters and the capacity of these factors to activate the reporter gene expression. We propose a model in which KlGpd1 activity is required for maintenance of the redox balance. In its absence, genes which function in generating redox balance instabilities are not expressed. A comparison of mutant phenotypes further indicates, that this system not only operates in *K. lactis*, but also in *Saccharomyces cerevisiae*.

1. Introduction

The yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase (Gpd) has received considerable attention in basic and applied research, due to its biotechnological interest in wine, beer and bioethanol production. Together with the glycerol 3-phosphate phosphatase, Gpd participates in glycerol synthesis, which is activated under osmotic stress and is also required to maintain the redox balance under anaerobic conditions (Bakker et al., 2001; Hohmann, 2009; Saito and Posas, 2012). In the presence of oxygen Gpd participates in the glycerol 3-phosphate shuttle, which is important for cells growing on glucose during the switch from fermentation to respiration and for growth on ethanol (Bakker et al., 2001; Larsson et al., 1998). This shuttle couples the reoxidation of cytosolic NADH to the electron transport chain *via* the mitochondrial FAD-dependent glycerol 3-phosphate dehydrogenase Gut2 (Fig. 1). In addition, Gpd provides glycerol 3-phosphate as a precursor for lipid biosynthesis and reduces the concentration of toxic methylglyoxal by metabolizing its precursor dihydroxyacetone phosphate (DHAP; Henry et al., 2012; Klug and Daum, 2014).

In *Saccharomyces cerevisiae* two Gpd isoenzymes exert both overlapping and different functions (Ansell et al., 1997; Nissen et al., 2000). Thus, Gpd1 is mainly involved in the adaptation to osmotic stress, a response mediated by the high osmolarity glycerol (HOG) pathway (Hohmann, 2009; Saito and Posas, 2012). Gpd1 resides in the cytosol and in peroxisomes, but rapidly enters the nucleus under high salt stress (Jung et al., 2010). On the other hand, Gpd2 is a mitochondrial enzyme induced under anaerobic conditions (Valadi et al., 2004).

Kluyveromyces lactis is another yeast of biotechnological interest (Rodicio and Heinisch, 2013). Like most other yeasts, and similar to mammalian cells, it relies primarily on a respiratory catabolism and is therefore fundamentally different from *S. cerevisiae*, which specializes in alcoholic fermentation (Breunig et al., 2000; Gonzalez-Siso et al., 2000; Rodicio and Heinisch, 2013). A substantial portion of glucose in *K. lactis* is metabolized through the pentose phosphate pathway, which

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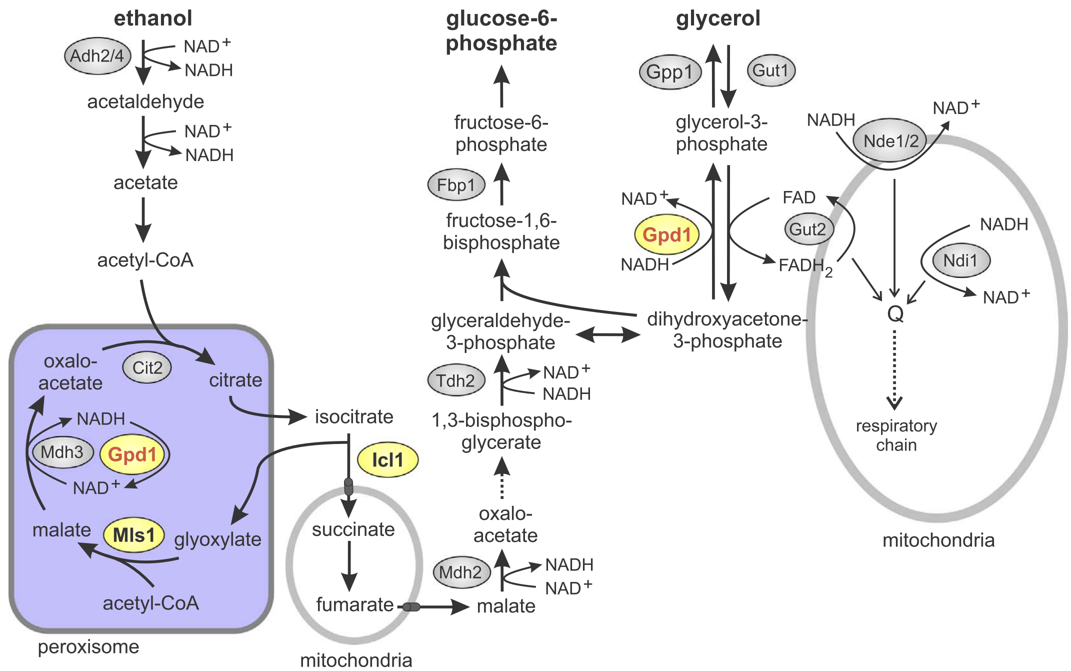

Fig. 1. Overview on ethanol metabolism and the role of Gpd1 in *K. lactis*. Carbon fluxes and cofactors in redox pairs are shown. For clarity and due to space limitations, only key enzymes mentioned in the text are depicted as filled ovals and the prefix "Kl" has been omitted from the enzyme names. Enzymes (or their encoding genes) employed in this work are highlighted on yellow background, with glycerol 3-phosphate dehydrogenase further emphasized in red letters. Barrels in the mitochondrial membrane at the lower left designate the succinate-fumarate-carrier protein Sfc1, which is required for shuttling the two metabolites between mitochondria and cytosol. Only the relevant isoforms of *K. lactis* enzymes are depicted, with abbreviations as follows: Adh2/4 = two isoforms of alcohol dehydrogenase, Mdh2/3 = two isoforms of malate dehydrogenase, Mls1 = malate synthase, Icl1 = isocitrate lyase, Tdh2 = glyceraldehyde 3-phosphate dehydrogenase, Fbp1 = fructose 1,6-bisphosphatase, Gpd1 = glycerol 3-phosphate dehydrogenase, Gpp1 = glycerol 3-phosphate phosphatase, Gut1 = glycerol kinase, Gut2 = mitochondrial glycerol 3-phosphate dehydrogenase, Nde1/2 = two isoforms of mitochondrial external NADH dehydrogenase, Ndi1 = NADH:ubiquinone oxidoreductase, Q = quinone as an electron acceptor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

requires reoxidation of the generated cytosolic NADPH (Tarrio et al., 2006a). For this purpose, *K. lactis* has two external dehydrogenases at its mitochondrial surface, KlNde1 and KlNde2, and two mitochondrial alcohol dehydrogenases, KlAdh3 and KlAdh4, which accept both NADH and NADPH (Tarrio et al., 2006b, 2005; Bozzi et al., 1997). Two cytosolic alcohol dehydrogenases required for fermentation, KlAdh1 and KlAdh2, are strictly NAD^+-dependent, with KlAdh2 possibly also playing a role in ethanol catabolism (Mazzoni et al., 2006). The mitochondrial dehydrogenase KlGut2, a component of the glycerol 3-phosphate shuttle, and the internal mitochondrial dehydrogenase KlNdi1 also participate in the maintenance of the redox balance (Fig. 1; Saliola et al., 2010, 2008).

Yeast carbohydrate metabolism is regulated by several signaling pathways which detect the availability of glucose (Broach, 2012; Conrad et al., 2014; Kim et al., 2013; Rodicio and Heinisch, 2017; Zaman et al., 2009). In this context, the SNF1 kinase complex is of central importance. SNF1 belongs to the family of AMP kinases (AMPK), which is highly conserved in evolution. AMPKs maintain the cells energy balance by activating catabolic pathways governing ATP production and down-regulating anabolic pathways that consume ATP (Hardie, 2015). Since they also mediate energy homeostasis in humans, AMPKs are an attractive drug target for the control of cancer and metabolic diseases such as diabetes type II and obesity (Carling, 2017).

In yeasts the SNF1 complex is required for the reprogramming of carbon metabolism from glycolysis to gluconeogenesis after glucose exhaustion and for growth on alternative carbon sources (Hedbacker and Carlson, 2008). In *S. cerevisae* it also regulates a variety of other processes, including biogenesis of peroxisomes (Simon et al., 1992), aging (Ashrafi et al., 2000; Lu et al., 2011), filamentous growth (Orlova et al., 2010), oxidative stress and sodium tolerance responses (Hong and Carlson, 2007; Ye et al., 2008). Moreover, the SNF1 complex was recently shown to affect the cell wall composition in *S. cerevisiae* and in *K. lactis*, thus having an influence on cell integrity (Backhaus et al., 2013; Rippert et al., 2017).

Similar to all members of the AMPK family the yeast SNF1 complex is a heterotrimer. In *S. cerevisiae* it is composed of a catalytic α-subunit (Snf1), which contains a catalytic and an autoinhibitory domain, a β-subunit providing a scaffold, which can be either Sip1, Sip2 or Gal83, and a regulatory γ-subunit (Snf4; Broach, 2012; Hedbacker and Carlson, 2008). The alternative β-subunits allow the formation of three different SNF1 complexes in *S. cerevisiae*, whilst *K. lactis* has only one α, β, and γ subunit (Goffrini et al., 1996; Rippert et al., 2017). The activity of the SNF1 complex of *S. cerevisiae* is regulated at different levels. Thus, Snf4 mediates inactivation of the inhibitory domain in the α-subunit in the absence of glucose (Hedbacker and Carlson, 2008). The kinase activity is positively regulated by phosphorylation of Thr210, which is located in the catalytic domain of the α-subunit, a residue conserved in its *K. lactis* homolog (Thr190). During growth on non-fermentable carbon sources such as glycerol and ethanol, the SNF1 complex of *S. cerevisiae* gets activated and phosphorylates the transcription factors Cat8 and Sip4, which recognize carbon source responsive elements (CSREs) in the promoters of target genes and trigger their expression (Schuller, 2003; Turcotte et al., 2010). In addition, the complex is required for the synthesis of Cat8 and Sip4. In fact, the promoter of the *SIP4* gene contains functional CSRE elements, which bind to and are activated by Cat8, but are not autoregulated by Sip4 itself (Vincent and Carlson, 1998). *CAT8* gene transcription is repressed by Mig1 on glucose medium and the Snf1 kinase phosphorylates and thereby inactivates the Mig1 repressor under derepressing conditions (Schuller, 2003; Zaragoza et al., 1999). Whereas Cat8 is essential for growth on non-fermentable carbon sources, Sip4 is apparently dispensable in *S. cerevisiae* under such conditions, indicating that it plays only a minor role in CSRE-dependent regulation. This has been attributed to the different binding affinities of Cat8 and Sip4 to the promoter elements, with Sip4 showing a higher specificity for certain elements than Cat8 (Roth et al., 2004; Vincent and Carlson, 1998).