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RNA binding protein Pub1p regulates glycerol production and stress tolerance by controlling Gpd1p activity during winemaking

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Abstract Glycerol is a key yeast metabolite in winemaking because it contributes to improve the organoleptic properties of wine. It is also a cellular protective molecule that enhances the tolerance of yeasts to osmotic stress and promotes longevity. Thus, its production increases by genetic manipulation, which is of biotechnological and basic interest. Glycerol is produced by diverting glycolytic glyceraldehyde-3-phosphate through the action of glycerol-3-phosphate dehydrogenase (coded by genes *GPD1* and *GPD2*). Here, we demonstrate that RNA-binding protein Pub1p regulates glycerol production by controlling Gpd1p activity. Its deletion does not alter *GPD1* mRNA levels, but protein levels and enzymatic activity increase, which explains the higher intracellular glycerol concentration and greater tolerance to osmotic stress of the *pub1*Δ mutant. *PUB1* deletion also enhances the activity of nicotinamidase, a longevity-promoting enzyme. Both enzymatic activities are partially located in peroxisomes, and we detected peroxisome formation during wine fermentation. The role of Pub1p in life span control depends on nutrient conditions and is related with the TOR pathway, and a major connection between RNA metabolism and the nutrient signaling response is established.

Keywords *Saccharomyces cerevisiae* · Winemaking · Glycerol · Pub1p · TOR1/Sch9 nutrient signaling pathway · Gpd1p · Peroxisomes

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Introduction

During winemaking, the primary role of wine yeast is to catalyze the rapid, complete, and efficient conversion of grape sugars into mainly ethanol and carbon dioxide. However, other minor but organoleptically important metabolites are also produced, such as glycerol. This polyalcohol is the third major product of yeast alcoholic fermentation in wine production (Pretorius 2000; Remize et al. 2000), which is produced at the beginning of fermentation and remains quite stable until fermentation ends as it is not metabolized when sugars are consumed (Orozco et al. 2012). Obtaining optimal levels of glycerol in wine is a desirable characteristic because it contributes to smoothness and roundness on the palate and enhances flavor (Ribéreau-Gayon et al. 2006), which is why strategies are currently applied to improve its concentration in the final product.

The role of glycerol on very important aspects of yeast physiology such as redox balance (Valadi et al. 2004),

hyperosmotic shock tolerance (Hohmann 2002), oxidative stress protection (Pahlman et al. 2001), and response to heat shock (Siderius et al. 2000) have been studied in great detail. Glycerol is produced by re-routing glycolytic intermediate glyceraldehyde-3-phosphate by the action of glycerol-3-phosphate dehydrogenase, GPDH (coded by genes *HOR1*/*GPD1* and *GPD2*), and glycerol-3-phosphate phosphatase (coded by *RHR2*/*GPP1* and *HOR2*/*GPP2*). Carrier proteins are involved in either active intake of glycerol, such as the Stl1p transporter (Ferreira et al. 2005) or efflux, through aquaglyceroporin channel protein Fps1p (Engel and Stahlberg 2002). Stl1p is a glycerol/H^+ symporter that is highly expressed in response to osmotic shock during exponential growth (Posas et al. 2000; Oliveira et al. 2003). Fps1p mediates glycerol uptake (Oliveira et al. 2003) but also allows glycerol release when the hyperosmotic conditions change (Tamas et al. 2000).

The modern winemaking industry requires yeast strains with improved stress tolerance and enhanced contribution to the organoleptic aspects of the final product. *Saccharomyces cerevisiae* is widely preferred for initiating wine fermentations because of its positive qualities in winemaking, such as stress tolerance and fermentative capacity. Therefore, the development of improved wine yeast strains of *Saccharomyces* is essential in the highly competitive wine market. The great importance of obtaining an industrial strain that produces large amounts of glycerol is illustrated by the number of studies related to the increase of glycerol production in industrial yeast strains (Borneman et al. 2013). There are two main approaches available to achieve this end, which mostly involve the generation of mutants that confer the trait of interest: adaptive evolution and DNA recombinant techniques to obtain genetically modified (GM) strains. Adaptive evolution is a non-GM approach in which genetically stable yeast strains are obtained under a selection pressure. A number of examples illustrate the power of this approach in glycerol overproduction (Kutyna et al. 2012; Tilloy et al. 2014). Unlike adaptive evolution, in GM strains it is possible to direct the genetic modification, which allows to clearly identify phenotypic effects of manipulation and then increase the knowledge on the studied molecular mechanism, although the resulting strains would be difficult to use for industrial purposes in the food industry. A classic example of these engineering techniques is the rerouting of the carbon flux toward glycerol by increasing *GPD1* gene expression (Cambon et al. 2006), which consequently diminishes ethanol yield and NADH availability. This is very important since a large number of quality wines produced by modern winemaking practices frequently contain excess ethanol content. However, the modified strain accumulates considerable amount of acetate and acetoin, which have both a negative sensorial impact on wine. This problem was further solved by deleting *ALD6* and overexpressing *BDH1* genes (Ehsani et al. 2009).

Gpd1p is generally considered a cytosolic protein, but the quantitative mass spectrometry of the *S. cerevisiae* peroxisomal proteome and live cell fluorescence microscopy have identified a pool of Gpd1p in peroxisomes and in other cytoplasmic punctate structures in response to stress (Jung et al. 2010; Kumar et al. 2016). Examinations of the amino acid sequence of Gpd1p have also showed that its N-terminus contains a sequence of amino acids that conforms to the consensus sequence for peroxisomal targeting signal type 2, PTS2 (Platta and Erdmann 2007), and the functionality of this sequence in binding to peroxisomal protein *PEX7* has been confirmed (Jung et al. 2010). Gpd1p dynamically changes its subcellular distribution among the cytosol, peroxisome, and nucleus in accordance with the type of cellular stress. It is primarily cytosolic, but exposure to oleic acid changes its localization to the peroxisome, while exposure of cells to osmotic stress changes it to the nucleus (Jung et al. 2010). Similar dynamics has been found for Pnc1p (Jung et al. 2010; Kumar et al. 2016), another peroxisomal protein implicated in cellular stress and aging (Anderson et al. 2003). Although Pnc1p displays prominent peroxisomal localization, it regulates Sir2p-mediated chromatin silencing and life span extension under caloric restriction and stress conditions (Anderson et al. 2003). Peroxisomes play major roles in oxidative stress in both the generation of, and protection against, cells of reactive oxygen species (Schrader and Fahimi 2006). Although numerous studies have characterized key roles for Gpd1p in osmoregulation and other central metabolic processes, very little attention has been paid to its cellular localization and, in particular, its peroxisomal localization.

We were interested in studying the molecular mechanisms that regulate glycerol production during winemaking in order to characterize possible targets to improve its production, as well as the consequences on yeast stress tolerance and longevity as they can affect industrial performance. Recently, we described a relevant relationship between mRNA regulation and glycerol metabolism by demonstrating that RNA-binding protein Pub1p plays a relevant role in glycerol production as its manipulation is an efficient strategy to increase the concentration of this valuable metabolite (Orozco et al. 2013). Consistently with the elevated glycerol concentration, the *pub1*Δ mutant produces less ethanol and acetate, and no differences in growth or biomass occur during winemaking. These findings demonstrate that *PUB1* genetic manipulation is an efficient strategy to improve the organoleptic properties' quality of wine. It also affects cellular life span, and the mutation extends longevity at the end of vinification. Pub1p regulates the gene expression in different ways by acting in translation and mRNA stability (Duttagupta et al. 2005) and by

taking part in cytoplasmic aggregates known as stress granules (SG) during glucose starvation and under other stress conditions (Balagopal and Parker 2009). In a previous work, we described how SGs formed in late winemaking phases in a Pub1p-dependent manner (Orozco et al. 2013).

It has been demonstrated that glycerol production is required to achieve full chronological life span (CLS) regulation in yeast (Wei et al. 2009), defined as the time during which cells are viable in the stationary phase (Longo et al. 2012). Life span is regulated by many cellular systems, mainly nutrient-sensing pathways PKA and TOR1/Sch9, and the family of deacetylases called sirtuins (Longo et al. 2012). The best known Sir2p is a deacetylase that uses NAD^+ to deacetylate several targets to yield nicotinamide as a product, which is a feedback inhibitor of the enzyme. These systems also control CLS in wine yeast under winemaking conditions (Orozco et al. 2012; Picazo et al. 2015). Glycerol synthesis depends on nutrient signaling pathway TOR1/Sch9 (Wei et al. 2009). In a previous work, we also demonstrated that the chemical inhibition of this pathway with methionine sulfoximine stimulates glycerol production during winemaking and extends CLS (Orozco et al. 2012). Under favorable growth conditions, TORC1 is active and influences virtually all aspects of cell growth. Reducing the nitrogen intake (or dietary restriction, DR) causes the inactivation of TOR1/Sch9, which leads to a metabolic switch to glycerol production which, in turn, and together with the direct regulation of stress resistance systems, causes enhanced cellular protection and life span extension (Wei et al. 2009).

We herein aimed to clarify the role of mRNA-binding protein Pub1p in glycerol metabolism and its connection with the TOR1/Sch9 pathway in terms of life span control. We established an interesting connection between Pub1p and the TOR1/Sch9 pathway during CLS in response to DR. We demonstrated that Pub1p plays a role in glycerol accumulation by modulating the Gpd1p level and, then, GPDH activity, which led to greater tolerance to several stresses in the *pub1*Δ mutant during winemaking. It also regulated the kinetics of peroxisome formation. Pub1p did not regulate the mRNA levels of glycerol metabolism genes, but regulated the mRNA abundance of membrane transporter genes *STL1* and *FPS1*, which were induced in the *pub1*Δ mutant, likely as a result of high glycerol production.

Materials and methods

Yeast strains and growth media

Table 1 lists the industrial wine yeasts used in this work. Industrial wine yeast EC1118 was kindly provided by Lallemand Inc. (Montreal, Canada). Haploid strain C9 was a gift from M. Walker (Walker et al. 2003) and is a haploid derivative of the diploid L2056 commercial wine yeast strain. Gene disruptions were performed by using recyclable selection marker *loxP-kanMX-loxP* from plasmid pUG6 following the protocol of Güldener et al. (1996). The marker was eliminated by transforming with the cre recombinase gene-containing plasmid YEp351-cre-cyh according to Deleneri et al. (2000). For green fluorescent protein (GFP) fusions, plasmid pFA6a-GFP(S65T)-*kanMX6* (Longtine et al. 1998) was used as a template to the C-terminal tagging of the products of the genes *GPD1* and *PNC1*. Yeast transformations were carried out by following the lithium acetate method (Gietz and Woods 2002). Table S1 in the Supplementary Material lists the oligonucleotides employed to amplify deletion cassettes and GFP fusions and to check transformants.

For yeast growth under laboratory conditions, yeast extract peptone dextrose (YPD) medium (1 % yeast extract, 2 % bactopeptone, 2 % glucose) and synthetic complete (SC) medium (0.17 % yeast nitrogen base, 0.5 % ammonium sulfate, 2 % glucose, and 0.2 % dropout mix with all the amino acids) were used (Adams et al. 1998). SC-N 1/25 was like SC, but with 25-fold less ammonium sulfate and amino acids. SC-GLC was like

Table 1 The yeast strains used in this work

Strain	Genotype	Origin
EC1118	Winestrain	Lallemand
EC1118 *pub1*	EC1118 *pub1*::*loxP pub1*::*kanMX*	This work
EC1118 Gpd1-GFP	EC1118 *GPD1*-GFP::*KanMX*	"
EC1118 *pub1* Gpd1-GFP	EC1118 *pub1*::*loxP pub1*:: *loxP GPD1*-GFP::*KanMX*	"
EC1118 *pex12* Gpd1-GFP	EC1118 *pex12*::*loxP pex12*:: *loxP GPD1*-GFP::*KanMX*	"
EC1118 Pnc1-GFP	EC1118 *PNC1*-GFP::*KanMX*	"
EC1118 *pub1* Pnc1-GFP	EC1118 *pub1*::*loxP pub1*:: *loxP PNC1*-GFP::*KanMX*	"
C9	Mat a *ho*::*loxP*	JM Walker
C9 *pub1*	C9 *pub1*::*kanMX*	This work
C9 *tor1*	C9 *tor1*::*kanMX*	"
C9 *tor1pub1*	C9 *tor1*::*loxP pub1*::*kanMX*	"

SC, but with 0.05 % of glucose. Selective plates contained 20 μg mL^{-1} of geneticine or 0.1 μg mL^{-1} of cycloheximide. For the microvinification experiments, synthetic grape juice MS300 was prepared as described by Riou et al. (1997), but with an equimolar amount of glucose and fructose to a final concentration at 20 %.

Grape juice fermentations, stress conditions, and chronological life span measurements

For the microvinification experiments, cells from 2-day cultures in YPD were inoculated at a final concentration of 10^6 cells/mL in filled-in conical centrifuge tubes with 50 mL of MS300. Incubation was done at very low shaking and at 22 °C. Evolution of fermentations was followed by determining cell viability by diluting, plating, and counting colony-forming units (cfu) and sugar consumption, as previously described (Zuzuarregui and del Olmo, 2004). Survival plots were drawn by taking the highest cell viability point (around 3–5 days) as 100 % survival. Stress resistance was tested on winemaking day 3. Ethanol tolerance was measured by adding 10 % ethanol for 1 h. Osmotic stress resistance was tested by adding 1 M of NaCl for 1 h. Oxidative stress tolerance was studied by adding 10 mM of hydrogen peroxide, and thermic tolerance was proved by incubating cells at 46 °C for 15 m.

Under laboratory conditions, the CLS experiments were performed as follows: precultures of selected strains were grown overnight on YPD and inoculated in SC, SC-N, or SC-GLC media at an OD_{600} of 0.1. After day 3 of growth at 30 °C, aliquots were taken, diluted, and plated. Colonies were counted, and the survival percentage was calculated by taking day 3 of growth as 100 % survival. Cell viability was measuring by diluting, plating, and counting cfu.

Determining metabolite and enzymatic activities

Reducing sugars during fermentation was measured by the reaction to dinitro-3,5-salycilic acid (DNS) according to Miller (1959). Glycerol was measured with the kit provided by R-Biopharm (Darmstadt, Germany) following the manufacturer's instructions.

To measure enzymatic activities, cell extracts were prepared under the conditions previously described (Postma et al. 1989), but by breaking cells with one volume of glass beads in FastPrep-25 (MP Biomedicals. Irvine, USA). The enzymatic activity from GPDH was assayed as described by Blomberg and Adler (1989). Then, 5–10-μL aliquots of cell free extracts were incubated in a buffer that contained 20 mM of imidazole-HCl (pH 7.0), 1 mM of dithioerythritol, 1 mM of $MgCl_2$, 0.67 mM of dihydroxyacetone phosphate (DHAP), and 0.09 mM of NADH. The reaction was started by adding DHAP, and it remained linear for at least 2 min. PNC1 activity was carried out according to Ghislain et al. (2002) and by using nicotinamide (NA) as a substrate and measuring the produced ammonia with the Sigma Ammonia Diagnostic Kit (Sigma Chemical Co., St. Louis, MO, USA). Correcting baseline ammonia production was done by subtracting the ammonia produced at each time point in a control sample incubated without the NA substrate. Hydrolysis of NA was proportional to the enzyme concentration and was linear with time. A unit of activity was defined as the amount of enzyme that converted 1 μg mol of substrate NA per minute. Protein concentration was measured by the Bio-Rad (Richmond, USA) protein assay following the manufacturer's instructions.

Western and Northern blots

Western and northern blot analyses were done as described before (Orozco et al. 2013). For the Western blot analyses, the anti-HA and anti-Adh antibodies were obtained from Rockland (Gilberstville, USA). The ECL Western blotting detection system (Amersham. Buckinghamshire, UK) was used following the manufacturer's instructions. Total RNA isolation and formaldehyde denaturing agarose gels and blotting were carried out as previously described (Aranda et al. 1998). The *GPD1*, *GPD2*, *STL1*, and *FPS1* probes were obtained by PCR and digoxigenin labeling using the PCR DIG labeling mix from Roche (Indianapolis, USA) according to the manufacturer's instructions. Probe detection was achieved by chemiluminescence using the DIG Northern Starter and CDP-Star kits by Roche (Indianapolis, USA) and was detected in an LAS-1000 device by Fujifilm.

Microscopy methods

The GFP-labeled cells were directly observed. Cells were visualized with the right filter under a Nikon Eclipse 90i fluorescence microscope.

Results

The long-lived *pub1*Δ mutant produces higher intracellular glycerol levels during winemaking and displays enhanced resistance to stress

In order to gain a better understanding of the larger glycerol production in the media displayed by the *pub1*Δ mutant, we analyzed the profile of intracellular and extracellular accumulation during winemaking in synthetic media compared to parental industrial strain EC1118 (Fig. 1a, b). In the wild type, intracellular glycerol reached its highest value on day 3, when the culture reached saturation and stopped dividing. However, mutant *pub1*Δ rapidly produced this metabolite faster from the beginning, because on day 1 there was 5-fold more glycerol than in the parental strain and glycerol levels remained

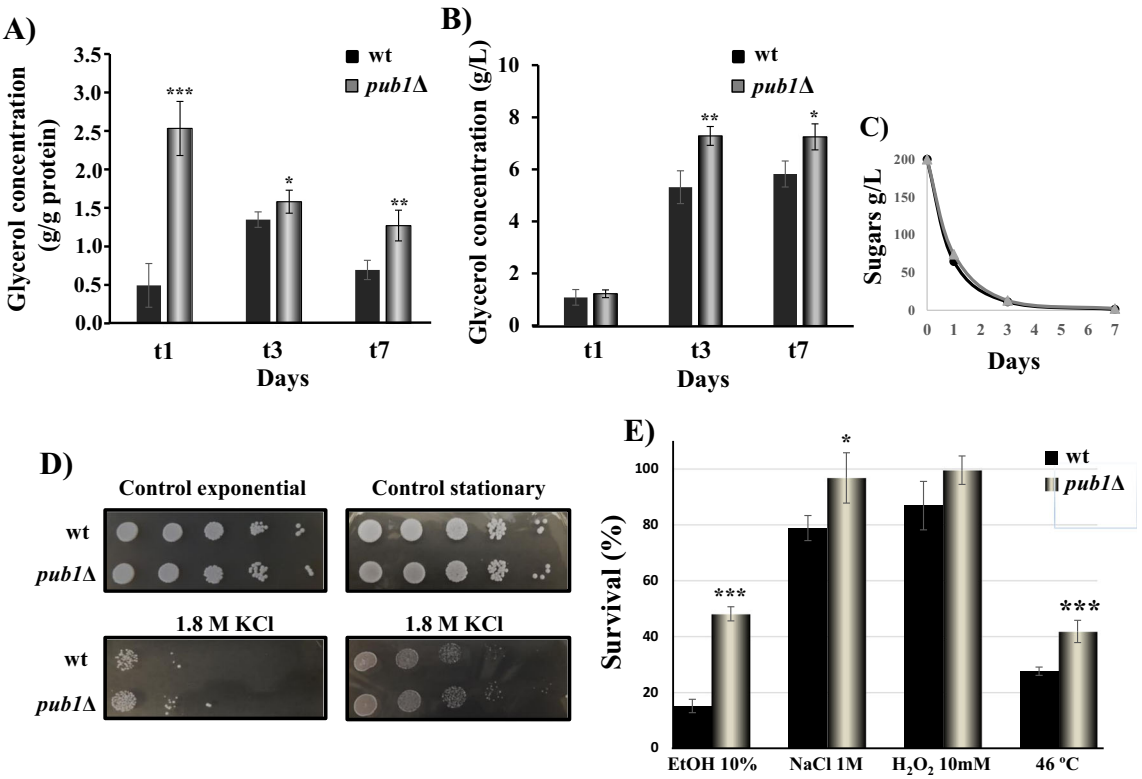

Fig. 1 PUB1 deletion increases the accumulation of intracellular glycerol and improves stress tolerance. Intracellular (**a**) and extracellular (**b**) glycerol concentration of *pub1*Δ and wild-type EC1118 strains on days 1, 3, and 7 of winemaking in MS300. **c** Sugar consumption of both strains during fermentation. **d** Cell viability during osmotic stress in the exponential and stationary phases. Cells were grown on YPD agar plates that either contained KCl 1.8 M, or did not, and were incubated for 48 h at 30 °C. **e** *PUB1* deletion increases stress resistance during winemaking. Cells were grown in MS300 for 3 days and were treated with 10 % ethanol, 1 M of NaCl, and 10 mM of H_2O_2 at 46 °C for 15 min. The survival percentage is relative to untreated cultures. Experiments were done in triplicate, and the mean and standard deviation are provided. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, unpaired *t* test, two-tailed

higher throughout fermentation. In fact, the extracellular concentration of mutant *pub1*Δ at time 3 and 7 days was significantly higher than for the parental strain, as it has been previously described for the C9 *PUB1* deletion (Orozco et al., 2013). It seems that glycerol synthesized is transported out of the cell from the first day. Although there are important differences in glycerol production, both strains have an identical physiological state at the times analyzed because there are no differences in their fermentation rates, as it can be observed in the curves of consumption of sugars (Fig.1c).

In order to test if the elevated glycerol production in the *pub1*Δ mutant conferred greater tolerance to osmotic stress, the exponential and stationary phase cells grown in laboratory medium were challenged by 1.8 M KCl (Fig. 1d). As seen, cells from both the parental and *pub1*Δ mutant strains in the exponential growing phase were more sensitive to osmotic stress, and a slightly increased resistance was observed for the *pub1*Δ mutant in both growth stages. During alcoholic fermentation, yeast cells face several stressful conditions, mainly high osmotic pressure, oxidative stress, increasing ethanol content, and temperature variations, all of which challenge cell viability (Pretorius 2000). We studied the resistance of the *pub1*Δ mutant to these stresses on day 3 of fermentation in synthetic grape juice. Figure 1d shows the percentage of survival of the wild-type and *pub1*Δ mutant after ethanol, osmotic, oxidative, and high-temperature stresses. Significant differences were seen, except for oxidative stress. The *pub1*Δ mutant was more tolerant, especially to a high ethanol concentration, but also to other stresses which occurred during winemaking, which could be due to protection by its high glycerol accumulation.

The expression of the genes involved in glycerol biosynthesis cannot explain the high glycerol levels in the *pub1*Δ mutant during winemaking

In order to test if the higher glycerol production of mutant *pub1*Δ during winemaking was due to transcriptional effects on genes *GPD1* or *GPD2*, we studied the mRNA levels by northern blot analysis (Fig. 2a). The transcript accumulations on days 1, 3, and 7 displayed induction of *GPD1* on day 3, which matched the highest intracellular glycerol levels for the wild type (Fig. 1a), but no differences between strains were detected. However, on day 7, an additional shorter transcript

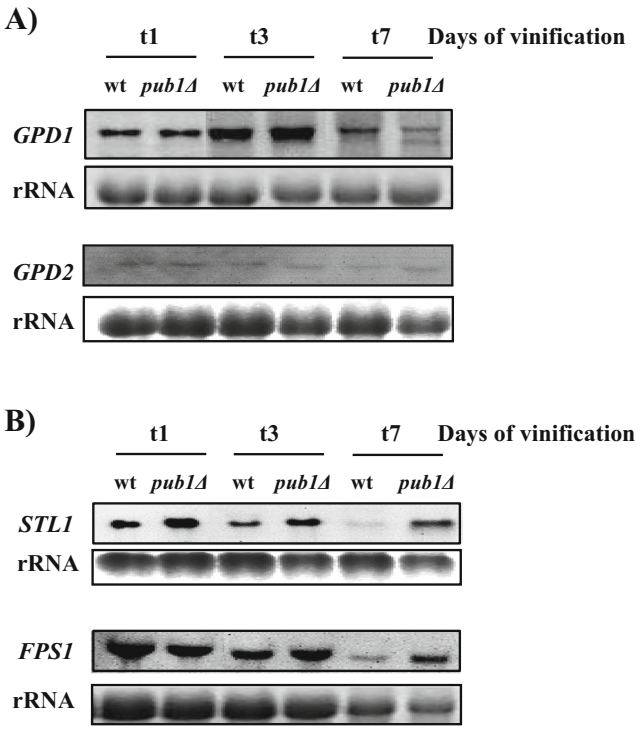

Fig. 2 Expression of the *GPD* genes cannot explain the glycerol accumulation of the long-lived *pub1*Δ strain. **a** *GPD1* and *GPD2* mRNA levels during winemaking in MS300 as measured by the northern blot analyses. **b** *STL1* and *FPS1* mRNA levels as described in panel **a**. rRNA is shown as a loading control

was detected in the *pub1*Δ mutant. This may indicate an unknown role of Pub1 in the start of transcription or in the processing of this particular mRNA.

The *GPD2* expression levels were lower and more constant through vinification, which hints that this isoenzyme is possibly not the most relevant in glycerol production during winemaking. Once again, no differences were found between the wild-type and the *pub1*Δ mutant for the mRNA levels of *GPD2*. We also tested the mRNA level of *GPP1* and *GPP2* coding for the enzymes involved in the second glycerol biosynthesis step, and no difference between strains were found (data not shown). As there were no differences between *GPD1*/*GPD2* and *GPP1*/*GPP2* at the transcriptional level between both strains, the high glycerol accumulation in the mutant was not dependent on the transcriptional regulation of the genes for biosynthetic enzymes. Thus, other unknown mechanisms must exist.

As intracellular and extracellular glycerol accumulation also depends on transport processes across the plasma membrane, we next studied the mRNA levels of the genes that code for the proteins involved in glycerol uptake, as *STL1*, and in efflux, as *FPS1* (Fig. 2b). The wild strain showed higher mRNA levels for these genes in early wine fermentation stages, which decayed and were faintly detectable on day 7. However, the *pub1*Δ mutant showed a more constant level for both mRNAs. With *STL1*, mRNA accumulation was slightly higher than in the wild type on day 1 but was clearly more abundant on days 3 and 7. Any difference over a short time was smaller for *FPS1* mRNA, but a higher level in the *pub1*Δ mutant is also clear for longer times, particularly on day 7. This may be caused by an increased transcription or stability of these mRNAs. We have no data about which transporter was more relevant under normal winemaking conditions, but the increased mRNA level of the genes for both the uptake and efflux systems suggests that cells might sense a more demanding requirement for glycerol transport across the plasma membrane in the absence of Pub1p. This might be the result of a high glycerol biosynthesis level as both intracellular and extracellular glycerol accumulation was greater in the *pub1*Δ mutant.

PUB1 mutant shows increased GPDH activity during winemaking, which is related with an increase in the Gpd1 levels

Next, we studied if the high glycerol accumulation in the *pub1*Δ mutant was caused by the enhanced activity of the biosynthetic enzymes. GPDH is the rate-controlling enzyme of glycerol formation in *S. cerevisiae*. So, we assayed it during winemaking in both the wild-type and *pub1*Δ mutant strains on winemaking days 1, 3, and 7 (Fig. 3a). In the wild-type industrial strain, the maximum occurred on day 3, which correlated with the *GPD1* mRNA accumulation peak (Fig. 2a). Compared to the wild type, mutant *pub1*Δ showed a significant increase in GPDH activity throughout vinification, which is consistent with its high glycerol production.

In order to investigate if the strong GPDH activity observed in the *pub1*Δ mutant was due to a high concentration of the protein, or to verify if further regulatory mechanisms that act post-translationally were involved, we tagged the main isoenzyme Gpd1p with influenza virus hemagglutinin epitope (Gpd1p-3HA) to detect it by Western blot analysis in the winemaking experiments (Fig. 3b, c). Gpd1p protein levels were higher at day 1 of fermentation (Fig. 3b) and remained high also at longer times (Fig. 3c). This is in fact in agreement with the maximal activity detected for the enzyme and is also consistent with the activity measurements, where the *pub1*Δ mutant showed a higher GPDH activity than did the wild type. Therefore, Pub1p plays a role in the regulation of glycerol production through effects on the protein Gpd1p level, likely by increasing the translation efficiency on its mRNA.

Gpd1p is generally considered a cytosolic protein, but the peroxisomal localization of a pool of Gpd1p has also been described. So, we compared the subcellular distribution of the enzyme in both the wild-type and the *pub1*Δ mutant as it might be related to the differences observed in GPDH activity. For this purpose, we tested the formation of peroxisomes during winemaking and the possible recruitment of

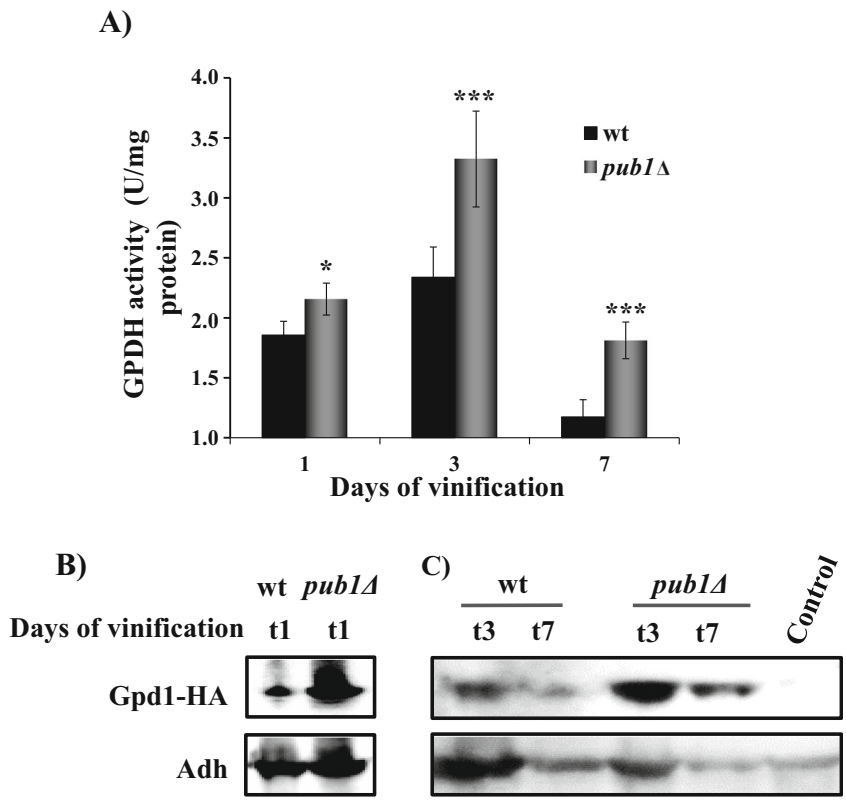

Fig. 3 PUB1 deletion increases GPDH activity and Gpd1p levels during winemaking. **a** Specific enzymatic activities of the *pub1*Δ and wild-type EC1118 strains at days 1, 3, and 7 of synthetic grape juice fermentation. Experiments were done in triplicate, and the mean and standard deviation are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, unpaired *t* test, two-tailed. **b** Western blotting of Gpd1p-HA at day 1 and **c** at days 3 and 7 of synthetic grape juice fermentation in the *pub1*Δ mutant strain and its parental EC1118. Immunodetection of Adhp was used as a loading control

the enzyme into them. We tagged Gpd1p with GFP (Gpd1p-GFP) in both strains. Cells from several time points of fermentation in synthetic grape juice were observed under fluorescence microscopy (Fig. 4). In the wild-type strain (Fig. 4a), Gpd1p was localized in peroxisomes since day 4 and remained there until day 7. Interestingly in the *pub1*Δ mutant (Fig. 4b), Gpd1p was recruited into peroxisomes earlier since granules were visible on day 1 of fermentation and remained in them until day 7. Fluorescence intensity was stronger than in the wild-type strain, especially at the initial time point. Therefore, Pub1p was not necessary for peroxisome biogenesis, but its deletion could induce these organelles to form earlier. The analysis of Gpd1p localization in a deletion mutant of gene *PEX12* (Fig. 4c), which codes for a peroxisomal protein, and which displayed impaired peroxisome formation, indicated that the protein aggregates that formed during winemaking were true peroxisomes.

Extended longevity of the *pub1*Δ mutant depends on growth conditions and is mediated by the TOR1/Sch9 pathway

As similar regulation and localization dynamics have been described for Gpd1p and the nicotinamidase Pnc1p, which are implicated during cellular stress and aging, we next proceeded to investigate Pnc1p activity and peroxisomal localization during winemaking in both the wild-type and *pub1*Δ mutant strains. As observed for Gpd1p, the *pub1*Δ mutant showed a significant increase in nicotinamidase activity at both the tested winemaking time points, particularly on day 7 (Fig. 5a). Regarding its subcellular localization (Fig. 5b), Pnc1p was also recruited into peroxisomes during winemaking, as is happens with Gpd1p. Although this localization is not dependent on Pub1p, more aggregates appeared by day 7 in the *pub1*Δ mutant.

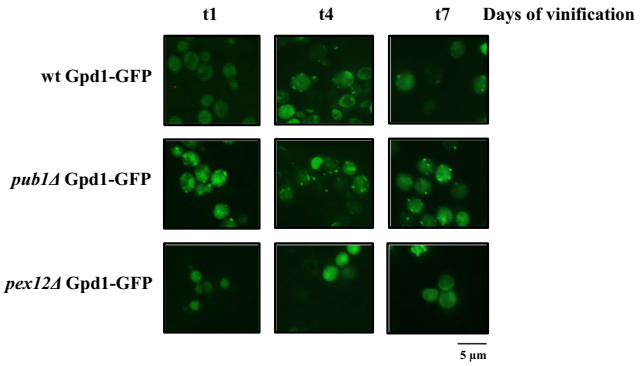

Fig. 4 Pub1p controls the cellular localization of Gpd1p into peroxisomes during winemaking. Localization of the Gpd1p tagged with GFP by fluorescence microscopy in the wild-type EC1118 strain and mutants *pub1*Δ and *pex12*Δ. Cells were collected on days 1, 4, and 7 of winemaking in MS300

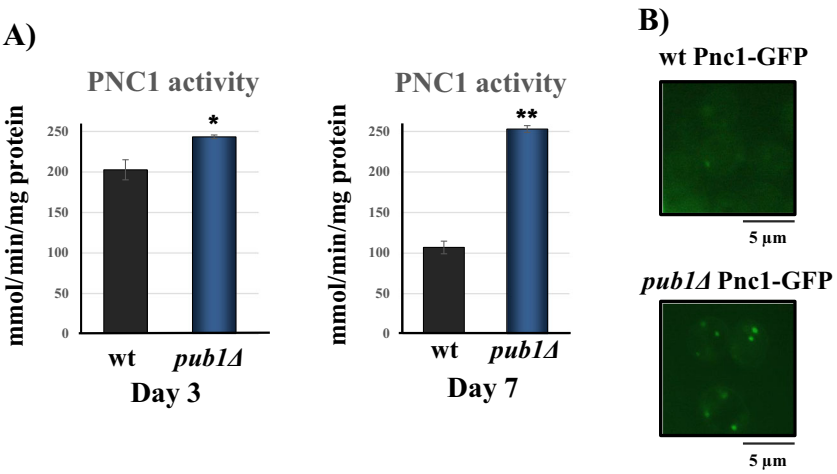

Fig. 5 Pub1p regulates activity and cellular localization into peroxisomes of Pnc1p during winemaking. **a** Specific enzymatic activities of *pub1*Δ and wild-type EC1118 strains on days 3 and 7 of synthetic grape juice fermentation. Experiments were done in triplicate, and the mean and standard deviation are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, unpaired *t* test, two-tailed. **b** Localization of the Pnc1p tagged with GFP by fluorescence microscopy in the wild-type EC1118 strain and *pub1*Δ at 3 days of winemaking

The effects on Pcn1p activity caused by the mutation of *PUB1* could be related to the prolonged life span displayed by the mutant strain in laboratory medium SC and during winemaking in synthetic must. In order to explore this interesting question, we studied the effect of growth under two different nutritionally limited laboratory conditions on CLS in the *pub1*Δ mutant. To this end, a SC medium that contained only 0.05 % of glucose (SC-GLC) and a medium that comprised 25-fold less ammonium sulfate and amino acids (SC-N) were used to conduct the CLS experiments with the wild-type and *pub1*Δ mutant strains. Upon glucose scarcity (Fig. 6a), the mutation of *PUB1* caused a slight increase in CLS. Therefore, Pub1p did not seem to play a relevant role in controlling longevity under caloric restriction conditions. Interestingly with nitrogen scarcity (Fig. 6b), the *pub1*Δ mutant displayed a shortened CLS when compared to the parental strain. Under these nitrogen-limiting conditions, mutant strain growth was very slow, so the time point that corresponded to day 7 was taken as 100 % survival instead of day 3. These results indicate that Pub1p is required to control CLS in a nutrient-dependent manner. As the TOR1/Sch9 pathway was the main mechanism to transduce nitrogen starvation signals, we analyzed the effect of combining the deletion of gene *TOR1* with *PUB1* deletion by assaying CLS in SC at a low nitrogen concentration (Fig. 6c). To help construct double mutants, we used the haploid wine strain C9 for this experiment. Both single mutants displayed shortened life spans under these conditions, and this effect was more dramatic for the *tor1*Δ mutant. Therefore, both proteins played a positive role in longevity under nitrogen-limiting growth conditions. The double mutant did not show an additive effect, but intermediate behavior, which suggests that Pub1p and Tor1p are not independent players in yeast life spans. The CLS phenotype of the double mutant suggests that Pub1p could mediate some of Tor1p's roles in aging as the deletion of *PUB1* partially compensated the effect of *TOR1* deletion.

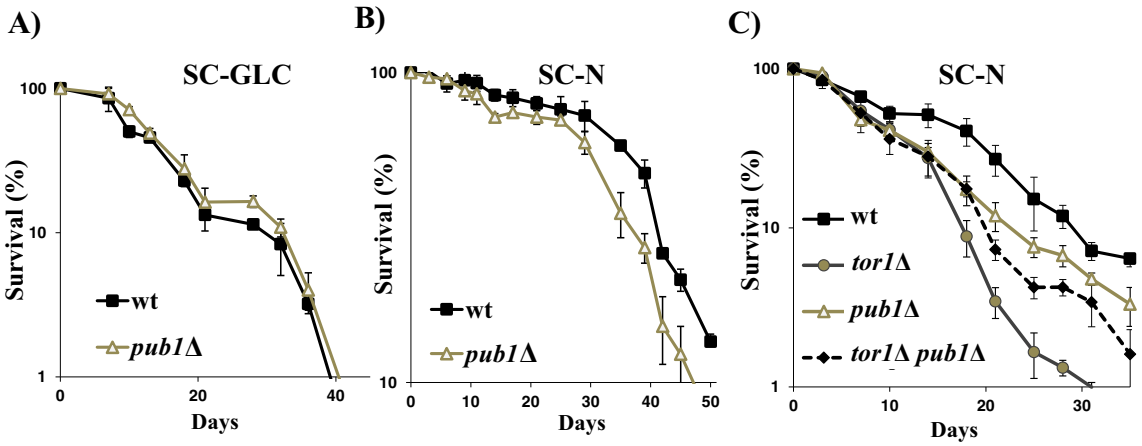

Fig. 6 Extended longevity of the *PUB1* mutant depends on growth conditions and is mediated by the TOR1/Sch9 pathway. a Survival curves in low glucose (SC-GLC) for the *pub1*Δ mutant in wine strain C9. Cell number on day 3 after inoculation was considered to have 100 % viability. **b** Survival curves in low nitrogen (SC-N). In this case, the cell number on day 7 after inoculation was considered to be 100 %. **c** Survival curves under the same conditions as in panel **b** in mutants *tor1*Δ, *pub1*Δ, and *tor1*Δ*pub1*Δ. Experiments were performed in triplicate. *Error bars* show the standard deviation

Discussion

Increased glycerol concentration in wine contributes to improving the organoleptic quality of the final product and to the stress tolerance of yeast during fermentation. There are several strategies that increase the amount of glycerol during wine fermentation, but direct genetic manipulation allows us to perfectly know the genetic modification and molecular mechanism of its metabolic regulation and thus provides further information about the physiological behavior of yeast during its industrial performance. The genetic manipulation of RNA-binding protein Pub1p is an efficient strategy to increase the glycerol concentration in the medium during winemaking. In this work, we described how *PUB1* deletion also leads to elevated intracellular glycerol accumulation. Pub1p plays a role in glycerol synthesis by modulating the accumulation of the main isozyme of glycerol-3-P dehydrogenase, Gpd1p (Fig. 3), which is the rate-controlling enzyme of glycerol formation in *S. cerevisiae*. The regulatory effect has to occur at the post-transcriptional level as the *pub1*Δ mutant shows no differences at the mRNA level of the genes involved in glycerol biosynthesis (*GPD1* and *GPD2* or *GPP1* and *GPP2*). Instead, it shows a significant increase in mRNA accumulation for the genes that codify two different proteins, described as glycerol carriers *FPS1* and *STL1*. The simultaneous activation of these two glycerol carriers suggests that the overaccumulation of this metabolite both intracellularly and extracellularly causes the miss-sensing of the osmolarity signal. Hence, both glycerol efflux, mediated by Fsp1p, and glycerol uptake, mediated by Stl1p, occur. We currently have no data on the relative contribution of both transport systems on the balance of glycerol on both sides of the cell membrane. However, a higher expression of both transporters would match increased export and import of glycerol to give rise to an increase in both intracellular and extracellular concentrations and the partial diversion of the glycolytic flux to glycerol formation. But neither synthetic nor natural grape have glycerol initially since it is produced by the yeast during fermentation. For this reason, the high intracellular accumulation of glycerol in mutant *PUB1* at the beginning of fermentation cannot be explained by differences in *STL1* expression. So, this higher expression may arise as a result of glycerol overaccumulation of the mutant, as produced by increased Gpd1p activity (Fig. 3a), which matches the increased amount of its main isozyme Gpd1p (Fig. 3b). These data suggest that Pub1p suppresses Gpd1p translation (Melamed et al. 2008), as it does with other proteins such as human *CALM2* or *CASP8* (Lopez de Silanes et al. 2005).

Together with Gpd1p, another peroxisomal protein, Pnc1p, implicates this organelle in the NAD^+ metabolism. Pnc1p provides a source of nicotinic acid through its nicotinamidase activity, which regulates nuclear Sir2p-mediated chromatin silencing and life span extension under caloric restriction and stress conditions (Anderson et al. 2003). The Pnc1p-mediated conversion of NA into nicotinic acid (Ghislain et al. 2002) relieves the inhibition of Sir2p, which leads to an increase in both histone deacetylation and silencing (Gallo et al. 2004). Together with Gpd1p high activity, which produces an elevated glycerol concentration in the mutant of *PUB1*, Pnc1p activity seems to play a positive role in the stress response and longevity during winemaking. Glycerol production, in addition to the regulation of stress resistance mechanisms, optimizes life span extension in mutants deficient in TOR1/Sch9 pathway activity (Wei et al. 2009). Reduced nitrogen intake causes the inactivation of the TOR1/Sch9 pathway, which leads to the metabolic switch to glycerol production which, together with the direct regulation of stress resistance systems, causes enhanced cellular protection and life span extension. Yet, this effect of the *tor1*Δ mutant on CLS is dependent on the nitrogen concentration of the media because under nitrogen scarcity conditions, the mutant does not show increased longevity, which also happens with the *pub1*Δ mutant. A previous work has demonstrated that the *sch9*Δ mutant shows a shortened life span (Picazo et al. 2015) under the same growth conditions, and also during grape juice fermentation, which indicates that nutritional unbalances are key factors for the impact of TOR1/Sch9 mutations. It is noteworthy how this aspect relates the regulatory function of RNA-binding protein Pub1p on the TOR1/Sch9 signaling pathway, which is an interesting connection that would be worth characterizing in the metabolic context.

Pub1p takes part in cytoplasmic aggregates, known as stress granules, during glucose starvation and at the end of winemaking (Orozco et al. 2013). Pub1p can also repress Gpd1p translation by triggering its aggregation into SGs. Nonetheless, we herein describe how Pub1p regulates the peroxisomal accumulation of Gpd1p during winemaking, but in the opposite way to SG formation as the *pub1*Δ mutant is unable to form these aggregates at the end of fermentation. We demonstrated that Pub1p plays a role in Gpd1p localization in peroxisomes, although this fact may merely reflect a mislocalization due to increased levels of the protein. The possible role of Pub1p in peroxisome formation needs to be closely studied. During winemaking, the *pub1*Δ mutant accumulates higher Gpd1p levels in peroxisomes, which are organelles in which fatty acids are broken down through β-oxidation. Peroxisomes play major roles in oxidative stress in both the generation and protection of cells from reactive oxygen species (Schrader and Fahimi 2006). This link to cellular stress has also implicated peroxisomes in aging. Although Gpd1p is not required for peroxisomal β-oxidation, it has been suggested that it may form part of a glycerol 3-phosphate shuttle to regenerate NAD^+ from NADH produced by β-oxidation (Valadi et al. 2004). However, those cells that lack Gpd1p do not show growth defects in the media that contain oleic acid. Thus, peroxisomal Gpd1p does not seem

to play a primary role in peroxisomal NAD^+ regeneration. It therefore remains unclear why Gpd1p localizes to peroxisomes. This scenario might reflect a mechanism to control Gpd1p activity by enhancing its stability or protecting it from degradation by simply sequestering enzymes into peroxisomes, where Gpd1p seems completely active. It has been demonstrated that the dynamic distribution of Gpd1p among different cellular locations provides a mechanism for cells to respond to cellular stress. Indeed, when Gpd1p localization alters, cell growth is dramatically compromised when cells are challenged with both oleic acid and osmotic stress (Jung et al. 2010). Our results coincide with the functional and regulatory relevance of Gpd1p localization because the mainly peroxisomal localization of Gpd1 in the *pub1*Δ mutant provides higher glycerol production and enhanced stress resistance during winemaking.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of interest The authors have declared that no competing interests exist.

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