Wine yeast sirtuins and Gcn5p control aging and metabolism in a natural growth medium

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Running title: Wine yeast as a model system to study life span

Abstract

Grape juice fermentation by wine yeast is an interesting model to understand aging under conditions closer to those in nature. Grape juice is rich in sugars and, unlike laboratory conditions, the limiting factor for yeast growth is nitrogen. We tested the effect of deleting sirtuins and several acetyltransferases to find that the role of many of these proteins during grape juice fermentation is the opposite to that under standard laboratory aging conditions using synthetic complete media. For instance, *SIR2* deletion extends maximum chronological lifespan in wine yeasts grown under laboratory conditions, but shortens it in winemaking. Deletions of sirtuin *HST2* and acetyltransferase *GCN5* have the opposite effect to *SIR2* mutation in both media. Acetic acid, a well known pro-aging compound in laboratory conditions, does not play a determinant role on aging during wine fermentation. We discovered that *gcn5*Δ mutant strain displays strongly increased aldehyde dehydrogenase Ald6p activity, caused by blocking of Ald6p degradation by autophagy under nitrogen limitation conditions, leading to acetic acid accumulation. We describe how nitrogen limitation and TOR inhibition extend the chronological lifespan under winemaking conditions and how the TOR-dependent control of aging partially depends on the Gcn5p function.

Key words: wine yeast, sirtuins, Gcn5p, chronological lifespan, Ald6p, autophagy.

1. Introduction

The budding yeast *Saccharomyces cerevisiae* is a widely used model of aging (Bitterman et al., 2003; Kaeberlein, 2010). The yeast NAD^+-dependent histone deacetylase Sir2p gave its name to a family of proteins called sirtuins (of which there are four other members in yeast, Hst1-4p) that have a relevant function on lifespan in yeast. Its impact on aging is complex, as replicative life span (RLS), measured as the times a mother cell divides to produce a daughter cell, is extended by the enhanced Sir2p function, while chronological life span (CLS), measured as survival after growth cessation, is reduced by the Sir2p function (Longo and Kennedy, 2006). The effect on CLS has been explained as based on the repression of alcohol dehydrogenase isoenzyme Adh2p by Sir2p (Fabrizio et al., 2005). This enzyme converts ethanol produced by fermentation back into acetaldehyde to obtain energy by respiration through its transformation to acetic acid first, and finally to acetyl-CoA. It was then established that ethanol is a pro-aging factor that shortens long-term longevity. There is increasing evidence that the acetylation-deacetylation machinery may similarly control metabolism by the direct modification of metabolic enzymes. In yeast, the best characterized example is the deacetylation by Sir2p of gluconeogenic enzyme phosphoenolpyruvate carboxykinase (Lin et al., 2009). Sirtuins have been shown to regulate acetyl-CoA synthetase in mammals and, as indirect evidence, the yeast double mutant *hst3*Δ *hst4*Δ is unable to grow on acetate as a carbon source (Starai et al., 2003).

The best known link between aging and metabolism is the fact that reduced food intake without malnutrition (dietary restriction, or DR) increases both types of longevity (Fontana et al., 2010; Lin et al., 2000; Sinclair, 2005). The nutrient-sensing pathways TOR/Sch9p and RAS/PKA are at the heart of this control. When high levels of nutrients

are present in the environment, these pathways promote growth and repress anti-aging mechanisms like stress response and autophagy (see below), which are necessary for long-term survival. In yeast, reducing glucose concentration from the standard 2% in laboratory media to 0.5-0.05% is the preferred way to test DR (Longo and Kennedy, 2006). Less is known about the effect of DR caused by low levels of nitrogen sources. It has been established that lowering the amount of nonessential amino acids extends RLS (Jiang et al., 2000). The situation during CLS is more complex. Lowering the amount of ammonium in the medium extends CLS (Fabrizio et al., 2004) as does using media with a lower amino acids concentration (Matecic et al., 2010). However in a different assay, lowering amino acid concentration did not extend CLS and a ten-fold increase in amino acids reduced it (Murakami et al., 2008). The mechanisms involved in the effect of amino acid concentration on longevity remain poorly understood. Many genes linked to CLS by global analyses are also involved in nitrogen metabolism and are regulated by TOR (Powers et al., 2006). TOR inhibition by rapamycin and L-methionine sulfoximine triggers life extension. A specific effect of TOR/Sch9 on yeast CLS implies that their deletion leads to an accumulation of glycerol, which acts as an anti-aging factor (Wei et al., 2009). On the contrary, the accumulation of acetic acid and concomitant acidification of the medium have been associated with shortened CLS (Burtner et al., 2009).

TOR also regulates autophagy, a highly relevant event taking place during nutrient starvation (Powers et al., 2006). Macroautophagy is the process by which parts of the cytoplasm and organelles (e.g., mitochondria and peroxisomes) are engulfed by a double membrane to form an autophagosome, which then fuses to the vacuole to be degraded and to recycle their components (Nakatogawa et al., 2009). Nitrogen starvation triggers autophagy, and cytosolic aldehyde dehydrogenase Ald6p has been

used as a marker of this process(Onodera and Ohsumi, 2004). Autophagy is an essential process for normal aging for most conditions (Kaeberlein, 2010).

The biotechnological application of yeasts is important given its ability to perform alcoholic fermentation. Yeasts are used in industry to produce bread, beer, wine and bioethanol. *S. cerevisiae* strains, devoted to winemaking, show some genetic differences to the strains used in the laboratory (Bisson et al., 2007). They present high levels of heterozygosity, chromosomal polymorphisms, rearrangements and karyotype instability. Most are roughly diploid, but there is a high degree of aneuploidy. Commercial wine yeasts are prototrophs, i.e., they can produce all their amino acids from a single nitrogen source (Fleet, 1993), while laboratory strains generally carry mutations in the genes involved in amino acid or nitrogen-base biosynthesis, which are used as transformation markers. Wine yeasts are more robust and tend to be more stress-tolerant to the environmental challenges they face during their industrial task, while laboratory strains are unable to complete grape juice fermentation successfully (Rachidi et al., 2000).

Grape juice is a very different complex medium to standard laboratory mediums as it is rich in sugars (20-25%, equimolar mixture of glucose and fructose) and its pH is lower (pH 3.0-3.8) (Ribéreau-Gayon et al., 2006). Yeasts are commonly added to grape juice in the form of active dry yeast. After a lag phase, cells start dividing, and growth stops after 6-7 divisions when sugar concentration is still high. It is generally believed that the limiting factor for growth under winemaking conditions is nitrogen (Bisson et al., 2007; Ribéreau-Gayon et al., 2006). Cells metabolize all sugars by fermentation with no further divisions and, for unknown reasons, they do not consume the ethanol produced. The death phase under these conditions has not been studied in detail. Some authors claim that cells start dying when sugars are consumed (Boulton et al., 1996),

while others affirm that cell viability starts dropping when sugars are still present (Ribéreau-Gayon et al., 2006). According to our experience, both situations occur and depend on the strain and environmental conditions, i.e., temperature (Cardona et al., 2007).

This work studied the genetic determinants of aging during grape juice fermentation by wine yeasts. We believe that analyzing this process in a medium closer to those found by unicellular fungi in nature could provide useful information. Besides, the fact that industrial strains have no deletions in the genes involved in nitrogen metabolism renders them useful for studying the role of nitrogen restriction on aging. We found that the role of well-known age-related proteins, like Sir2p, on chronological aging depends on growth conditions, and that the effect of their deletion is positive or negative on lifespan in different growth media. We defined acetyltransferase Gcn5p as a key factor in controlling nitrogen-dependent autophagy, and also in acetic acid levels resulting from cytosolic aldehyde dehydrogenase regulation by autophagy.

2. Materials and methods

2.1. Yeast strains and media.

Industrial wine yeasts L2056 and EC1118 were kindly provided by Lallemand Inc. (Montreal, Canada). Haploid strain C9 (Mat a, *ho*::*loxP*) was a gift from Michelle Walker (Walker et al., 2003). Gene disruptions were performed by using the recyclable selection marker *loxP-kanMX-loxP* from plasmid pUG6 according to the protocol of (Guldener et al., 1996). The marker was eliminated by transforming with the cre recombinase-containing plasmid YEp351-cre-cyh according to (Delneri et al., 2000).

Supplementary Table 1 lists the oligonucleotides employed to amplify deletion cassettes and to check transformants. Supplementary Table 2 contains the resulting strains.

For yeast growth, YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) was used. SC medium contained 0.17% yeast nitrogen base, 0.5% ammonium sulphate, 2% glucose and 0.2% drop-out mix with all amino acids (Adams et al., 1998). SD-N is as SC without ammonium sulphate and amino acids. SC N 1/25 is as SC with 25-fold less amonium sulphate and amino acids. Solid plates contained 2% agar and 20 μg mL^{-1} geneticine or 0.1 μg mL^{-1} cycloheximide. Red grape juice (Bobal variety) was a gift from Bodegas Murviedro and was sterilized overnight with 500 μg/L of dimethyl dicarbonate. Synthetic grape juice MS300 was prepared as described by (Riou et al., 1997) but with an equimolar amount of glucose and fructose at 10% instead of glucose at 20%.

2.2. Yeast growth and chronological life span measurements

Under laboratory conditions, the CLS experiments were performed as follows: precultures of selected strains were grown overnight on YPD and inoculated in SC media at an OD_{600} of 0.1. After day 3 of growth at 30ºC, aliquots were taken, diluted and plated. Colonies were counted and percentage of survival was calculated taking day 3 of growth as 100% survival.

For the microvinification experiments, cells from the 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 grape juice. Incubation was done at very low shaking (22ºC for natural juice, 24ºC for synthetic grape juice). Evolution of vinifications was followed by determining cell viability and sugar consumption, as previously described (Zuzuarregui

and del Olmo, 2004). Survival plots were drawn by taking the highest cell viability point (around 2-4 days) as 100% survival.

2.3. Metabolite and enzymatic activities determinations and Western blotting

Reducing sugars during fermentation was measured by the reaction to DNS (dinitro-3.5-salycilic acid) according to Miller (1959). Ethanol, acetic acid, glycerol and acetaldehyde were measured with the kits provided by r-Biopharm, and ammonia with a kit provided by Sigma, following the manufacturer's instructions.

To measure enzymatic activities, cells extracts were prepared under the conditions previously described (Postma et al., 1989), but by breaking cells with one volume of glass beads in a Fast-Prep 25 (MP Biomedicals). Enzymatic activities from pyruvate decarboxylase, acetyl-CoA synthetase and alcohol dehydrogenase were performed according to Postma (1989), and aldehyde dehydrogenase activities according to previous work (Aranda and del Olmo Ml, 2003). Protein concentration was measured by the Bio-Rad Protein assay, following the manufacturer's instructions.

To conduct the Western blot analysis, SDS-PAGE were done in an Invitrogen mini-gel device and were blotted on PVDF membranes. The anti-ADH and anti-ALDH antibodies came from Rockland (Gilberstville, USA). The ECL Western Blotting detection system (Amersham) was used for detection, following the manufacturer´s instructions.

3. Results

3.1. Roles of wine yeast sirtuins and acetyltransferases in chronological lifespan under laboratory conditions.

In order to study the role of the acetylation/deacetylation machinery in CLS regulation on prototrophic yeast under laboratory conditions, we first constructed several deletion mutants in haploid strain C9, derived from commercial strain L2056 (Walker *et al.* 2003). This enabled us to easily construct multiple mutants using the recyclable *kan*MX marker (Güldener et al., 1996). Mutant strains were grown in complete synthetic media (SC) with all 20 amino acids and 2% glucose (Adams et al., 1998). Cell viability on day 3 was taken as 100% survival (Fig. 1). First we tested the deletion of *SIR2* in this background (Fig. 1A), which shortened the mean lifespan. Yet the deletion mutant displayed extended maximum longevity, as described in laboratory strains under DR conditions (Fabrizio et al., 2005). In RLS, the antagonist of Sir2p has been described as acetyltransferase Sas2p (Dang et al., 2009). However, *SAS2* deletion showed a similar behavior to *SIR2* deletion with an extended maximum lifespan (Fig. 1A), indicating that they do not play opposite roles in such conditions. The double mutant behaved differently to simple deletions and displayed a shorter maximum CLS, indicating that both proteins control different aspects of chronological aging.

Next we tested the effect on CLS of deleting other members of the yeast sirtuin family, *HST1-4*. *HST1* deletion showed no effect on maximum life span in this condition, although it causes a shortened mean life span (Fig. 1B). *HST2* deletion reduced maximum CLS, unlike *HST3* and *HST4* which actually increased maximum CLS (Fig. 1B). Those results do not agree with those obtained in laboratory strains

(Eisenberg et al., 2009; Smith et al., 2007). As *SIR2*, *HST3* and *HST4* deletions increased longevity under these conditions, we tested double mutants *hst3*Δ*hst4*Δ, *hst3*Δ*sir2*Δ and *hst4*Δ*sir2*Δ (Supplementary Fig. 1). Additional deletion of *HST3* and *HST4* in the *sir2*Δ mutant slightly increased maximum life span, suggesting that their effect may be additive, although the Sir2p function seems more relevant. The *hst3*Δ*hst4*Δ combination clearly extended maximum CLS more than parental strains, indicating that they could be redundant, like in laboratory strains (Starai et al., 2003). We failed to construct the triple mutant from all three double mutants, suggesting that deletion of all sirtuins is lethal in this genetic background. All five sirtuins were deleted in the laboratory strains, but only in a *fob1*Δ background (Murakami *et al.* 2008).

Finally, we tested the deletion of other proteins involved in deacetylation/acetylation. Gcn5p is a histone acetyltransferase whose deletion has been reported to decrease CLS in laboratory strains (Eisenberg et al., 2009; Laschober et al., 2010), which was also the case for mean and maximum life spans in wine yeast (Fig. 1C). In previous global studies (Powers et al., 2006; Eisenberg et al., 2009), deacetylase Hda1p, Gcn5p's counterpart in many histone acetylation events (Millar and Grunstein, 2006), had the opposite effect, and this occurred under our conditions (Fig 1C). Spt10p, an acetyltransferase that acetylates the H3K56 residue, which is deacetylated by Hst3p and Hst4p, had a very short maximum life span (Fig. 1C), as occurs under laboratory conditions (Eisenberg *et al.* 2009). Thus, deletions of sirtuin family members and acetyltransferases mostly play similar roles between wine and laboratory yeasts when tested in laboratory media.

3.2. The roles of Sir2p, Hst2p, Gcn5p and Spt10p on chronological lifespan during wine fermentation are the opposite to laboratory conditions.

The availability of mutants on age-controlling genes in wine yeast enabled us to test the effect of these mutations on the growth conditions found by yeast in nature, such as grape juice fermentation. We chose three sirtuins (*SIR2*, *HST2* and *HST3*) and three deacetylases (*SAS2*, *GCN5* and *SPT10*) to be deleted in the diploid commercial strain L2056, parental of strain C9. Cells were inoculated from a 2-day preculture on YPD to red grape juice, initially at 10^6 cells/ml, a widely used inoculation ratio in industry. Under these conditions, cells usually undergo 6-7 divisions to reach saturation (e.g., Fig. 2A, L2056 divides by 6.96 times on average). Grape must is very rich in sugar. It has been well established that the carbon source is not the limiting growth factor (Bisson et al., 2007; Fleet, 1993) as it stops at high sugars concentrations (i.e., around 10% sugars in Fig. 2A). Under our conditions, ammonium is depleted from grape juice after just one day (indicated by the arrow in Fig. 2A).

When *SIR2* deletion was tested for grape juice fermentation, it consumed sugars at a similar ratio, but gave a different growth and death pattern than parental strain L2056 (Fig 2A). It reached higher viability, but it was lower at the end of fermentation. To provide a clearer picture of the aging profile, viability was made relative to its highest value, reached in this case on day 3 (see Fig. 2B), showing a clear defect in mean and maximum CLS in the *sir2*Δ mutant. The same plotting type is shown for the remaining mutants tested in the following figures. To rule out the strain effect, we tested the mutation on haploid strain C9 (Supplementary Fig. 2A) and on EC1118, another commercial strain (Supplementary Fig. 2B). In both cases, *SIR2* deletion shortened CLS. Therefore in chronological aging with natural grape juice, Sir2p plays a positive

role, similar to that observed during RLS under laboratory conditions, but the opposite to that observed in CLS. We tested the deletion of *HST2* and *HST3*, which gave opposite results in laboratory media (see Fig 1B). The *hst2*Δ mutant displayed increased mean and maximum CLS (Fig. 2C). Once again, this is the opposite result to that seen under laboratory conditions (Fig. 1B). However, *HST3* deletion slightly increased maximum CLS (Fig 2C), the same result that was seen in the SC medium.

Next we tested the effect of deleting acetyltransferases (Fig. 2D). The *sas2*Δ mutant had no relevant effect on maximum CLS under winemaking conditions (Fig. 2D). *GCN5* deletion led to an extended maximum lifespan (Fig. 2D), and a similar but less pronounced effect was observed for *SPT10* deletion (Fig. 2D). For these two acetyltransferases, behavior was again the opposite to that under SC conditions, suggesting that the determinants of aging for both experimental conditions substantially differ. Therefore, the role of the acetylation/deacetylation machinery is complex and highly growth medium-dependent.

3.3. Metabolism is controlled by the acetylation/deacetylation machinery in wine yeast.

Previously, ethanol and acetic acid have been reported to have a negative influence on CLS (Fabrizio et al. 2005, Burtner et al., 2009), while glycerol has a positive one (Wei et al., 2009). We previously described how acetaldehyde is a stress inducer which also causes the transcriptional repression of *SIR2* and *HST3* (Aranda and del Olmo, 2004). To test the relevance of these metabolites under our conditions, we measured their concentrations when fermentation ended (when the concentration of the remaining sugars is below 2 g/L) in several previously described mutants. Figure 3A

shows their concentration in relation to the wild-type strain. Large amounts of ethanol are produced after grape juice fermentation under these conditions (around 11-12%), but there were no big differences in the final ethanol concentrations (Fig. 3A). The *hst3*Δ mutant significantly produced less ethanol (11.678±0.227 vs 12.346±0.192 g/100 mL), while *sir2*Δ produces more (11.472±0.317 vs 10.897±0.299 g/100 mL). There is an inverse correlation, be it a poor one, between maximum life span and ethanol production (Supplementary Fig. 3A), confirming the relevance of this metabolite on aging.

Acetic acid is produced in the 0.2-0.4 g/L range by the parental strain under the testing conditions. We detected higher variability among the mutants, where the *sir2*Δ mutant produced less acetate, and *hst2*Δ, *hst3*Δ and *gcn5*Δ mutants produced more, especially *gcn5*Δ (2.5 fold increase; Fig. 3A). Under these growth conditions, acetate did not appear to be a pro-aging factor as those mutants producing more acetate live longer (Fig. 2), and the *sir2*Δ mutant displayed a shorter CLS despite producing less acetate. Acetate's effect on aging has been linked to the medium's pH (Burtner et al., 2009). Grape juice is a highly buffered medium as it contains large amounts of tartaric, malic and citric acids whose pH remains low. pH remains stable during wine fermentation, and drops slightly from 3.2 to around 3.0; even mutation *gcn5*Δ, which produces the most acetate, showed no significant pH variation (Supplementary Fig. 3B). Therefore, acetate does not appear to be the key player in chronological aging in grape juice.

We also examined the final concentrations of glycerol, a molecule that induces a lengthened life span (Fig. 3A). The wild-type strain gave around 5.5-6.5 g/L under these conditions. We noted a slight significant increase in the *sas2*Δ mutant and a greater increase in *GCN5* and *SPT10* deletions. Although high glycerol production in the *gcn5*Δ

deletion strain may contribute to its extended CLS, there is no correlation between maximum life span and final glycerol concentration for the whole of the mutants (Supplementary Fig. 3C), although if *hst2*Δ mutant is not taken into account a poor positive correlation is shown. Finally, acetaldehyde production between strains was similar, falling in the 30-40 mg/L range, except for *GCN5* deletion which showed increased acetaldehyde production (Supplementary Fig. 3D).

As *GCN5* deletion had the greatest impact on metabolism, with a huge increase in acetic acid and a moderate, yet significant, increase in glycerol, we measured the production of both metabolites during wine fermentations (Supplementary Fig. 3E). Regarding acetate, the *gcn5*Δ mutant produced more from the start, and levels remained high and stable for the rest of fermentation. Glycerol production was lower on the first days of vinification for deletion mutant *gcn5*Δ, but reached a higher value when fermentation ended. It is noteworthy that, as in industrial winemaking processes, none of these metabolites was assimilated by cells after completing fermentation (data not shown). We measured acetic acid for the *gcn5*Δ mutant on day 1 of aging in laboratory medium SC under the conditions shown in Figure 1C, and found that acetic acid also significantly increased (Fig. 3B). Therefore, high acetic acid production may contribute to the shorter lifespan of the *gcn5*Δ mutant under laboratory conditions (Fig. 1C). Glycerol concentration under the same conditions showed no differences between the mutant and its parental strain (Fig. 3C).

Given the relevance of acetic acid and ethanol on aging, we investigated the activity of the enzymes involved in the two-carbon metabolism (Supplementary Fig. 4A) after 5 days of fermentation in a synthetic grape juice in two mutants sirtuin mutants, *sir2* Δ and *hst3*Δ, and two acetyltransferases, *gcn5*Δ and *sas2*Δ. This medium was selected because polyphenols in wine (i.e., resveratrol and quercetin) have been

reported to potentially alter the activities of enzymes such as aldehyde dehydrogenases (Kitson et al., 2001); Orozco *et al.*, in preparation). We observed the same aging pattern for the mutants tested in natural and synthetic grape juices (i.e., compare *gcn5*Δ mutant in Fig. 2D and Fig. 6). The first step in the alcoholic fermentation is the breaking the pyruvate molecule produced by glycolysis into acetaldehyde and CO_2 by pyruvate decarboxylase (PDC). We found no significant differences in the mutants tested (Supplementary Fig, 4B), although *hst3*Δ and *gcn5*Δ displayed slightly greater activities, which might explain the higher acetaldehyde levels in the *gcn5*Δ mutant (Supplementary Fig. 3B). Acetaldehyde is mainly transformed into ethanol by alcohol dehydrogenases (ADH) during fermentation, but some can be transformed into acetate by aldehyde dehydrogenases (ALDH; Aranda and del Olmo Ml, 2003). Regarding ADH activity, the *hst3*Δ mutant shows a significantly increased activity (Fig. 4A), while *sir2*Δ mutant has a slightly increased activity. This could reflect the fact that the *sir2*Δ mutant exhibited greater activity of Adh2p (Fabrizio et al., 2005), an isoenzyme devoted to respiration of ethanol. This enhanced activity might explain the higher ethanol concentration noted in the *sir2*Δ mutant, which seems to be the case for the *hst3*Δ mutant, suggesting a more complex metabolic control pattern.

The main ALDH activity when growing cells in glucose is cytosolic K^+-dependent Ald6p isoenzyme, while mitochondrial Mg^+-dependent Ald4p seems to play a during respiration (Aranda and del Olmo Ml, 2003; Remize et al., 2000). Ald6p activity remarkably increased and Ald4p activity decreased in the *gcn5*Δ mutant (Fig. 4B and C). This greater activity of cytosolic ALDH may explain the higher acetate levels produced by the *gcn5*Δ mutant, alcoholic fermentation being a cytosolic process. The *sas2*Δ mutant also showed a defect in Ald4p, but with no change in acetic acid concentration (Fig. 3A). Finally, acetate can be assimilated by forming acetyl-CoA by

acetyl-CoA synthetases (ACS). The single mutations tested had no significant effect on this particular activity (Supplementary Fig. 4C), and there is likely some redundancy in the control of those enzymes by sirtuins which cannot be detected in individual mutants.

Since *gcn5*Δ substantially changed ALDH activity during winemaking, we measured this activity during aging in laboratory media SC (time 1 day in Figure 1C) and we also found increased Ald6p activity, although less intense than that observed during fermentation (Fig. 4D), plus diminished Ald4p activity (Fig. 4E). Therefore, the metabolic control of ALDH takes place under a variety of growth conditions. Given the greater glycerol formation in the *gcn5*Δ mutant (Fig. 3A), we also looked for differences in the first and limiting step of glycerol biosynthesis, glycerol-3-phosphate dehydrogenase. We found no significant differences in the activity between this mutant and its parental strain (Supplementary Fig. 4D). This analysis shows a complex enzymatic regulation network which may affect the final by-products of metabolism in a way we do not fully understand, indicating that Gcn5p is a key factor in metabolic control.

3.4. **Gcn5p controls cytosolic aldehyde dehydrogenase by autophagy.**

Given the marked changes in ALDH activity and acetate concentration in the *gcn5*Δ mutant during winemaking, we analyzed the Ald4/6p levels during fermentation by Western blot. Both enzymes can be detected by using an anti-ALDH antibody (Onodera and Ohsumi, 2004), an approach that has been used successfully on wine yeasts (Cebollero and Gonzalez, 2006). Ald4p levels were maintained during fermentation in the parental strain and the *gcn5*Δ mutant (Fig. 5A). This enzyme has been reported to be glucose-repressible and, despite the high levels on day 1 of

fermentation, we failed to detect any Ald4p activity (data not shown), suggesting a posttranslational control of its activity. Despite the lower levels of Ald4p activity on day 5 (Fig. 4C), strain *gcn5*Δ presented similar levels of this protein, indicating that Gcn5p may be involved in its posttranslational regulation. Protein levels of the major cytosolic isoform, Ald6p, dropped sharply during wine fermentation, and were almost undetectable on day 3 (Fig. 5A). However, Ald6p was still present in the *GCN5* deletion strain, although faint. Therefore, major differences in enzyme activity (Fig. 4B) reflect differences in protein levels. We failed to detect any direct acetylation of ALDH after immunoprecipitation (data not shown), so these proteins may be regulated indirectly by Gcn5p action.

Ald6p has been identified as a selective marker for autophagy during nitrogen starvation (Onodera and Oshumi, 2004). Thus, the sharp drop in its levels during grape juice fermentation could reflect the low nitrogen conditions that yeasts face. We performed an autophagy experiment using a standard procedure consisting in a change from a rich medium, YPD, to a minimal medium lacking any nitrogen source SD-N. Under these conditions, Ald4p, whose intensity even increased after nitrogen depletion (data not shown), made the interpretation of the results difficult. We tagged Ald6p C-terminally with a Myc tag, but this modification blocked protein degradation (data not shown). Thus, to exclude the Ald4p signal, we deleted its gene. In the wild-type strain, we saw the expected sharp decline in protein levels after switching to low nitrogen conditions, which markedly lowered after 15 hours, becoming almost absent after 72 h (Fig. 5B). However in the *gcn5*Δ deletion strain we found high Ald6p levels during this period, indicating a defective degradation of this protein by the autophagic pathway. Gcn5p could affect gene *ALD6*'s mRNA levels; indeed, the basal levels of *ALD6* transcripts at time 0 were slightly higher in the *gcn5*Δ mutant (Supplementary Fig. 5),

but rapidly disappeared in both cases after nitrogen depletion, reflecting a post-transcriptional event.

Autophagy is a process whose failure shortens lifespan for most conditions and model organisms tested, including yeast. As expected, deletion of gene *ATG7*, required for autophagososme formation (Nakatokaga et al., 2009) in diploid strain L2056, shortened maximum CLS during aging in laboratory medium SC (Supplementary Fig. 6), as it did in laboratory strains (Alvers et al., 2009). Under these conditions, and as observed in haploid wine strain C9 (Fig.1C), *GCN5* deletion also shortened CLS, but to a greater extent (Supplementary Fig. 6), while *atg7*Δ *gcn5*Δ double mutant shortened CLS even more, suggesting that Gcn5p does not control life span exclusively by controlling autophagy. As previously observed, *GCN5* deletion extended maximum lifespan under winemaking conditions (Fig. 5C). Surprisingly, *atg7*Δ mutant also showed increased longevity throughout winemaking, extending both mean and maximum lif spans, suggesting that it may be a detrimental process for longevity for these conditions. It is interesting to note that double mutant *atg7*Δ *gcn5*Δ behaved like simple mutant *gcn5*Δ, indicating that Gcn5p may be a key regulator of the autophagic machinery in this environment.

3.5. **Nitrogen concentration plays a role in aging on laboratory media**

Grape juice has a lower nitrogen/carbon source than the standard laboratory medium SC. In order to further explore the relationship between CLS in laboratory conditions and nitrogen concentration, a SC medium containing 25 fold less ammonium sulphate and amino acids (SC N 1/25) was prepared. This medium contained a total of 53.6 mg of assimilable nitrogen/L, a ratio similar to the one found in natural grape

juices (Fleet, 1993). In this medium, wine strain L2056 reached a lower cell density when compared to SC medium (SC N1/25=4.2x10^7 CFU/mL, SC =8.5x10^7 CFU/mL) after 3 days of growth (the time point usually taken as time=0). Cells in SC started dying after this time point (Fig. 6A). However, in SC N 1/25 medium cells still grew up to day 4 (7 days after inoculation) to reach a 121% viability (Fig. 6A), dying afterwards. Both cultures reached a similar viability after 14 days. Therefore, the initial nitrogen concentration changed the death profile of a wild type strain. To compare the effect of the most interesting mutations we have previously analysed in this limiting medium, we took 7 days after inoculation as saturation point (Fig. 6B). Deletion of *SIR2* caused a shortened CLS, as happened in grape juice fermentation (Fig. 2B). The deletion of another sirtuin, *HST2*, that had the opposite effect during growth on grape juice (Fig. 2C), had a death profile similar to the wild type, with only a faint increase in maximum longevity. The *gcn5*Δ mutation extended CLS in low nitrogen medium as it did in grape juice fermentation (Fig. 2D). Finally, the autophagy defective *atg7*Δ mutant has a decreased mean CLS (as it happened in SC; Supplementary Fig. 6), but it showed an extended maximum life span, as it happened in grape juice (Fig. 5C). Therefore, low nitrogen SC medium mimics partially grape juice regarding the effect of some mutations on life span, indicating that low nitrogen is one, but not the only, cause of the differences between the aging profile on laboratory and natural media.

3.6. TOR role in lifespan during grape juice fermentation

After characterizing the function of Gcn5p on aging and nitrogen starvation-driven autophagy, we tested a key pathway's role in controlling nutrient response and autophagy, the TOR pathway, and its relationship with Gcn5p. First, we analyzed the

effect of TOR inhibition on winemaking conditions. To do this, we added to natural grape juice L-methionine sulfoximine (MSX), an inhibitor of glutamine synthetase that causes intracellular starvation for glutamine that triggers partial TOR inhibition (Crespo et al., 2002). This repression does not affect general stress response transcription factors Msn2/4p. We previously described that overexpression of Msn2p in early grape juice fermentation stages inhibits growth (Cardona et al., 2007). Hence, we believe that this kind of TOR inhibition, which has been proven to extend CLS (Powers et al., 2006), could be more appropriate under our conditions. Indeed TOR inhibition extended mean and maximum lif spans CLS of wine yeast during natural grape juice fermentation (Supplementary Fig. 7A). So this essential pathway on aging control also works under our conditions as expected. It has been established that the TOR/Sch9p pathway controls glycerol production. MSX addition indeed increases glycerol after fermentation, similarly to *GCN5* deletion (see Supplementary Fig. 7B).

In order to gain more insight into the role of DR caused by nitrogen starvation, we repeated the same experiment using synthetic grape juice and altered the total amount of nitrogen (Figure 7A). We tested a rich medium with 300 mg/L of assimilable nitrogen (MS300) in the form of amino acids and ammonium, and a limiting juice with a quarter of this nitrogen amount (MS75). We observed how longevity abruptly increased when applying nitrogen restriction (Fig. 6A). MSX also extended lifespan when added to MS300, but to a lower extent than nitrogen starvation, indicating that additional pathways are acting in the DR condition. MSX also slightly extended longevity in MS75 (Fig. 7A), which implies that it activates different pathways which may, at least partially, work in parallel to achieve lifespan extension. *GCN5* deletion increased maximum life span in synthetic grape juice MS300 (Fig. 7B), similarly to natural grape juice (Fig. 2D). The mutant was still sensitive to the CLS extension

caused by nitrogen starvation. Thus, Gcn5p is not the key regulator of this pathway. However, the *gcn5*Δ mutant was insensitive to the effect of MSX on maxium life span at high and low nitrogen (Fig. 7B), indicating that the effects caused by TOR inhibition on aging are, at least partially, channelled through this protein.

4. **Discussions**

For a considerable time now, laboratory strains of *S. cerevisiae* have been cultivated under optimal nutrients and temperature conditions. Microorganisms adapt to the laboratory environment and lose protective mechanisms against more harmful and variable natural conditions (Palkova, 2004). It has been recently shown that a vineyard yeast strain has an aging behaviour in laboratory conditions similar to that of laboratory strains (Murakami et al., 2011). This paper aims to study the genetic determinants of CLS on wine yeast in a natural growth medium: grape juice. Under laboratory conditions, our results show that when using a synthetic defined medium SC, wine yeasts mainly display the expected behavior according to previous studies. Mutations in the genes involved in histone acetylation (*GCN5*) or deacetylation (*HDA1*) and autophagy (*ATG7*) give the expected outcome (Figs. 1 and Supplementary Fig. 5). As expected, inhibition of the TOR pathway during winemaking also extends CLS (Fig. 7A). Therefore, the central genetic mechanisms of aging are not lost during laboratory-based yeast domestication. However, we still note some differences in the behavior of other mutants; e.g., sirtuins. Sir2p has been associated with blocking extreme lifespan extension under DR conditions, such as incubation in water, but not in SC medium (Fabrizio et al., 2005). In our case, *sir2*Δ moderately extended maximum CLS in SC (Fig. 1A). Smith *et al.* (2007) found that *hst1*Δ and *hst3*Δ mutants had shorter maximum

life span, while that of *hst2*Δ and *hst4*Δ mutants was normal. We described how *HST2* deletion shortens lifespan, while *HST3* and *HST4* knockouts extend it (Fig. 1B). These minor differences may suffice to avoid adaptation to harsh natural environments. Hence, we believe it is interesting to study yeast in conditions closer to nature, as those in the wine industry. For instance, cocoa polyphenols induce the expression of *HST3* (Martorell et al., 2011), and wine polyphenols can modulate the expression or requirement of different sirtuins during the aging process. Besides, wine yeasts carry no mutations to be used as selection markers. Prototrophy may avoid the unpredictable behavior caused by mutations in amino acid biosynthetic pathways, particularly in nitrogen deficient media. For instance, there is evidence that restoring *LEU2* prototrophy in a laboratory strain extends CLS (Alvers et al., 2009). Life span in higher eukaryotes is also determined by amino acid intake, for instance, a methionine deficient diet extends maximum life span in mice (Miller et al., 2005).

The most striking result obtained under winemaking conditions is the opposite role that some proteins play when compared to standard laboratory conditions (see Supplementary Table 3); i.e., *SIR2* deletion, which causes maximum chronological lifespan extension in laboratory media (Fig. 1A), shortens it in grape juice fermentation (Fig. 2B). Therefore, its role is similar to what happens in RLS under laboratory conditions. The opposite is true for deletions in genes *HST2*, *GCN5*, *SPT10* and *ATG7* as these deletions shorten CLS under laboratory conditions, but extend it under grape juice conditions. This may be explained by: differences in medium composition when growth commences, the environment where aging occurs, or different oxygen concentrations.

When the process commences, medium composition differs substantially from standard laboratory media (see Introduction). Differences in the limiting nutrient for

growth surely affect the ways that cells enter a nondividing state and their longevity within it. Surprisingly, calorie restriction abolishes the effect of mutations in autophagy like *atg7*Δ (Matecic et al., 2010), suggesting that this process is highly dependent on environmental factors. Moreover, *ATG7* deletion has been seen to shorten lifespan in synthetic-defined media, but has no effect on a richer medium; e.g., YPD (Alvers et al., 2009). If YPD is a complex medium containing yeast extract, red grape juice is even more complex and richer. The effect of *GCN5* deletion is the same in natural and synthetic grape juice (Fig. 2 and 7), therefore the relative high sugar/low nitrogen composition of these media seems to be determinant for the different outcome of the mutation regarding longevity. That was proven for *GCN5* and *SIR2* deletion mutants using a SC medium with very low nitrogen (Fig. 6). In those conditions, the effect of deletions of *SIR2* and *GCN5* genes is similar to the one observed in grape juice, although that is only partially the case for *ATG7* deletion (that only extends maximum CLS in low nitrogen SC medium, although it shortens mean CLS). Therefore, there is not a single environmental stimulus that controls all mechanisms involved on aging in the same way. Under winemaking conditions, autophagy may be not necessary, and may even prove detrimental, even though those are conditions of nitrogen starvation as seen by the degradation of a marker of nitrogen-dependent autophagy, such as Ald6p (Fig. 5A). It will be necessary to identify autophagy targets under winemaking conditions to fully understand its impact on aging. In the low nitrogen medium SC N1/25, autophagy helps initially cells to maintain high mean life span, as expected on starvation conditions, although it shortens maximum life span, suggesting a negative role in the latter stages of life similar to found in winemaking conditions, maybe through apoptosis induction.

Conditions at the end of winemaking differ considerably from those in a typical laboratory stationary phase. When fermentation finalizes, there is a highly energetic environment with large amounts of unconsumed ethanol, together with glycerol and acetic acid. Under laboratory conditions, however, all these carbon sources are used by yeast after glucose consumption. The traditional explanation is that high ethanol concentration blocks membrane transport. However, there are no significantly different tolerances to ethanol shock between the mutants tested (data not shown). Moreover, glycerol accumulation might prove relevant for these conditions. *GCN5* deletion and TOR inhibition significantly increase in extracellular glycerol (Supplementary Fig. 7B), although there is not a clear correlation for other mutations tested, such *hst2*Δ (Supplementary Fig.3C). The effects may seem relatively small but, in absolute terms, this increase is around 1 g/L, while differences of 40 mg/L under laboratory conditions trigger major extensions in CLS (Wei et al., 2009). Glycerol is produced to protect against hyperosmotic stress caused by high sugar concentrations when fermentation commences, and it has been shown that a hyperosmotic stress causes CLS extension (Smith et al., 2007; Murakami et al., 2008) maybe through a mechanism involving glycerol biosynthesis.

Finally, fermentation conditions involve low oxygen concentration as oxygen is displaced by high CO_2 production, and mitochondrial activity probably differs much from highly aerated laboratory conditions. Gcn5p is a key factor in the signaling pathway which controls nuclear transcription depending on mitochondrial dysfunction, called the retrograde response. Gcn5p is required for retrograde response-dependent RLS extension (Kim et al., 2004). Mitochondrial status has been recently reported to control the autophagic process (Graef and Nunnari, 2011). Therefore, differences in

mitochondrial status may lead to the activation or repression of different pathways and be relevant for lifespan.

We found that *GCN5* deletion blocks cytosolic aldehyde dehydrogenase Ald6p degradation by autophagy during nitrogen starvation (Fig. 5B). Gcn5p and histone deacetylases Hda1p and Rpd3p have been related to the autophagic control of recombination protein Sae2p (Robert et al., 2011). Given this failure to degrade Ald6p, the amount of Ald6p increases during fermentation, with greater enzymatic activity and more acetic acid. During aging under laboratory conditions, enzymatic activity and acetic acid concentration also increase (to a lower extent), which may explain the mutant's shorter lifespan. This fact is probably the cause of high acetic acid production by mutants defective in autophagy, as previously described (Matecic et al., 2010). However during grape juice fermentation, the deletion mutant is long-lived and this may be caused by grape juice being highly buffered and pH suffers minor variations during the winemaking process (Supplementary Fig. 3E). We also detected a lower activity of mitochondrial isozyme Ald4p (Fig. 4C). *ALD4* deletion has been shown to strongly extend CLS under laboratory conditions (Laschober et al., 2010). Under grape juice fermentation conditions, high acetic acid levels in mitochondria may be detrimental, while *GCN5* deletion may alleviate this lowering Ald4p activity. We failed to detect the direct acetylation of immunoprecipitated aldehyde dehydrogenase (data not shown). Possibly, Gcn5p may act either indirectly by acetylating a regulatory protein or via a different mechanism.

Finally, we attempted to better understand the TOR regulation of lifespan by nitrogen in grape juice fermentation using wine yeast. As the strain tested is a prototroph, we expected to see an improved picture. We used an indirect TOR repression by employing glutamine synthetase inhibitor L-methionine sulfoximine

(MSX; Crespo et al.,2002), and we expected to see only the TOR effects related to the nitrogen starvation as it has not been seen to affect the general stress response and r-protein transcription. Given our conditions, MSX extends lifespan, as described for laboratory conditions (Powers et al., 2004), but to a lower extent than total nitrogen starvation. This indicates that it acts on a subset of the responses caused by nitrogen deficiency (Figure 6A). *gcn5*Δ mutant is insensitive to life span extension by MSX, suggesting that this inhibitor's effects are channelled through this protein. MSX activates transcription factors Gln3p, Rtg1p and Rtg3p (Crespo; et al., 2002). It is well-known that the heterodimer Rtg1/3p-mediated retrograde response is controlled by Gcn5p (Kim et al., 2004). Apparently, therefore, the function of transcription factor Gln3p in the transcription of nitrogen-regulated genes depends on Gcn5p under aging conditions.

5. **Conclusions**

The role of sirtuins Sir2p and Hst2p, acetyltransfersase Gcn5p and autophagy machinery protein Atg7p on chronological life span during high sugar and low nitrogen grape juice fermentation is the opposite to that under standard laboratory aging conditions using synthetic complete media of low glucose and high nitrogen concentrations. Gcn5p has an impact on lifespan, autophagy and TOR signalling in both grape juice and laboratory media.

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Figure legends

Fig. 1. Deletion of sirtuins and acetyltransferases has similar effects on CLS in wine yeast compared to laboratory yeast under laboratory conditions. The CLS assays in the SC medium were performed by making serial dilutions, plating an aliquot and counting colonies. CFU (colony forming units)/mL were made in relation to values from day 3 post-inoculation, and were considered 100% survival. (A) Survival curves of the mutants carrying *SIR2* and *SAS2* deletions. (B) Survival curves of the mutants carrying single deletions on sirtuins *HST1-4*. (C) Survival curves of the deletion mutants in acetyltransferase genes. Experiments were done in triplicate, showing the mean and standard deviation. *$p<0.05$, **$p<0.01$, unpaired t-test, two-tailed.

Fig. 2. Deletions of *SIR2*, *HST2*, *GCN5* and *SPT10* have opposite effects under winemaking conditions. (A) Grape juice fermentation of *sir2*Δ mutant and its parental strain L2056. Cell viability measured as CFU/mL and total sugars are indicated along time. (B) Survival curve of *sir2*Δ mutant in winemaking conditions. Cell viability on day 3 of Figure (A) was taken as 100% survival. (C) Survival curves of sirtuin mutants *hst2*Δ and *hst3*Δ. (D) Survival curves of acetyltrasferases mutants *sas2*Δ, *gcn5*Δ and *spt10*Δ. Experiments were done in triplicate, showing the mean and standard deviation.

Fig. 3. *GCN5* deletion impacts metabolite production. (A). Concentrations of ethanol, acetic acid and glycerol when finalizing grape juice fermentations of several mutants of sirtuins and acetyltransferases shown in Fig. 2. Values were made relative to wild type strain L2056. (B) Acetic acid production of *gcn5*Δ on day 1 of aging on SC medium from Fig. 1D. (C) Glycerol production for the same conditions as (B). Experiments

were done in triplicate, showing the mean and standard deviation. *p<0.05, **p<0.01, ***p<0.005, unpaired t-test, two-tailed.

Fig. 4. Gcn5p is a key factor in aldehyde dehydrogenase activity. Enzymatic activities of selected mutants on day 5 of synthetic grape juice fermentation. Extracts were assayed spectrophotometrically for different enzymatic activities made relative to the total protein concentration. (A) Alcohol dehydrogenase activity (B) Cytosolic aldehyde dehydrogenase activity. (C) Mitochondrial aldehyde dehydrogenase activity. (E) Acetyl-CoA synthetase activity. (D) Cytosolic and (E) mitochondrial aldehyde dehydrogenase activities from aging cultures in SC. Experiments were done in triplicate, showing the mean and standard deviation. *p<0.05, **p<0.001, ***p<0.005, unpaired t-test, two-tailed.

Fig. 5. Gcn5p controls Ald6p degradation by autophagy. (A) Western blotting of ALDH during grape juice fermentation in the *gcn5*Δ mutant strain and its parental L2056. An ADH antibody was used as a loading control. (B) Western blotting of ALDH detection during nitrogen starvation conditions in SD-N media. C9 *ald4*Δ–derived strains were used. ADH antibody detection was used as a loading control. (C) Survival curves of L2056-derived mutants on grape juice fermentation. Conditions as in Fig. 2.

Fig. 6. Low-nitrogen medium mimics partially grape juice. (A) CLS assays of wine yeast L2056 in the SC medium and the low nitrogen SC N 1/25 medium were performed as in Fig. 1, taking day 3 post-inoculation as 100% survival. (B) Survival curves of the mutants on strain L2056 carrying *SIR2*, *HST2*, *GCN5* and *ATG7* deletions

in SC N 1/25 medium. Day 7 after inoculation was considered 100% survival. . Experiments were done in triplicate, showing the mean and standard deviation.

Fig. 7. TOR controls life span under winemaking conditions. Survival curves of wild-type L2056 (A) and *gcn5*Δ mutant (B) strains in synthetic grape juice (MS) containing high (MS300) or low (MS75) amounts of total nitrogen. MSX was also added at the 400 mM concentration in these fermentations. Conditions as in Fig 2. Experiments were done in triplicate, showing the mean and standard deviation.

Fig. 1

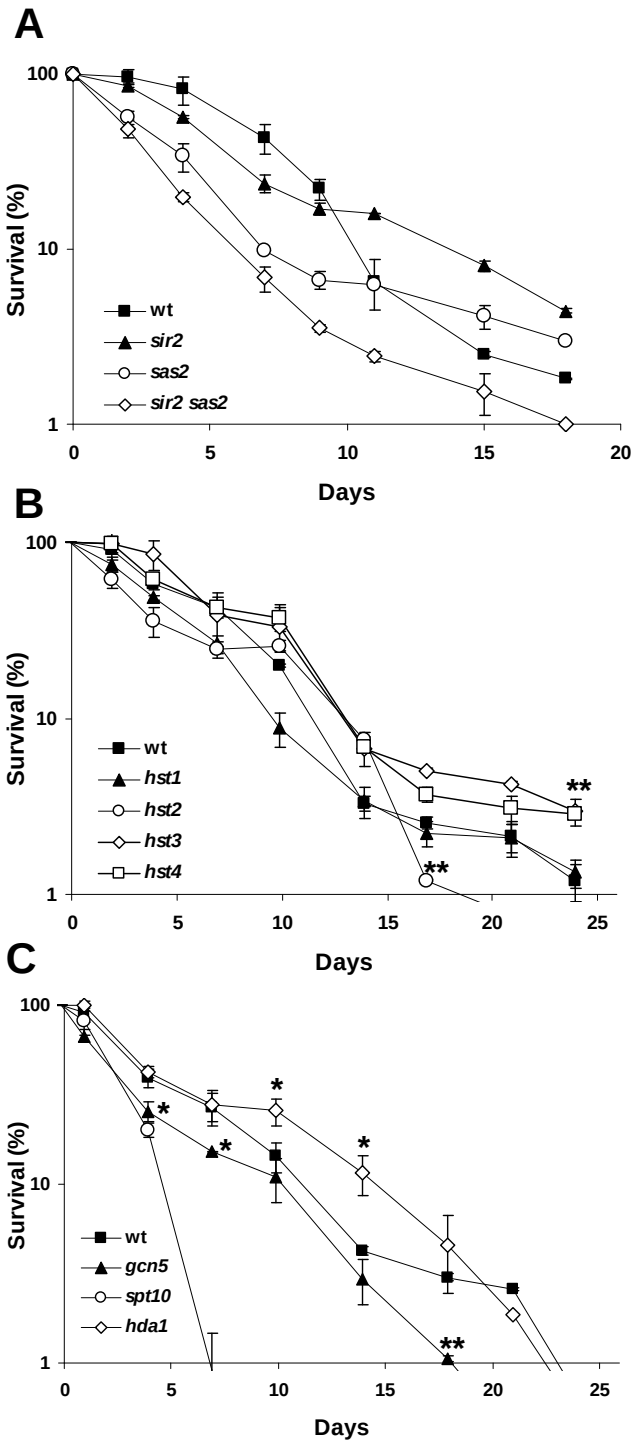
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Fig. 2

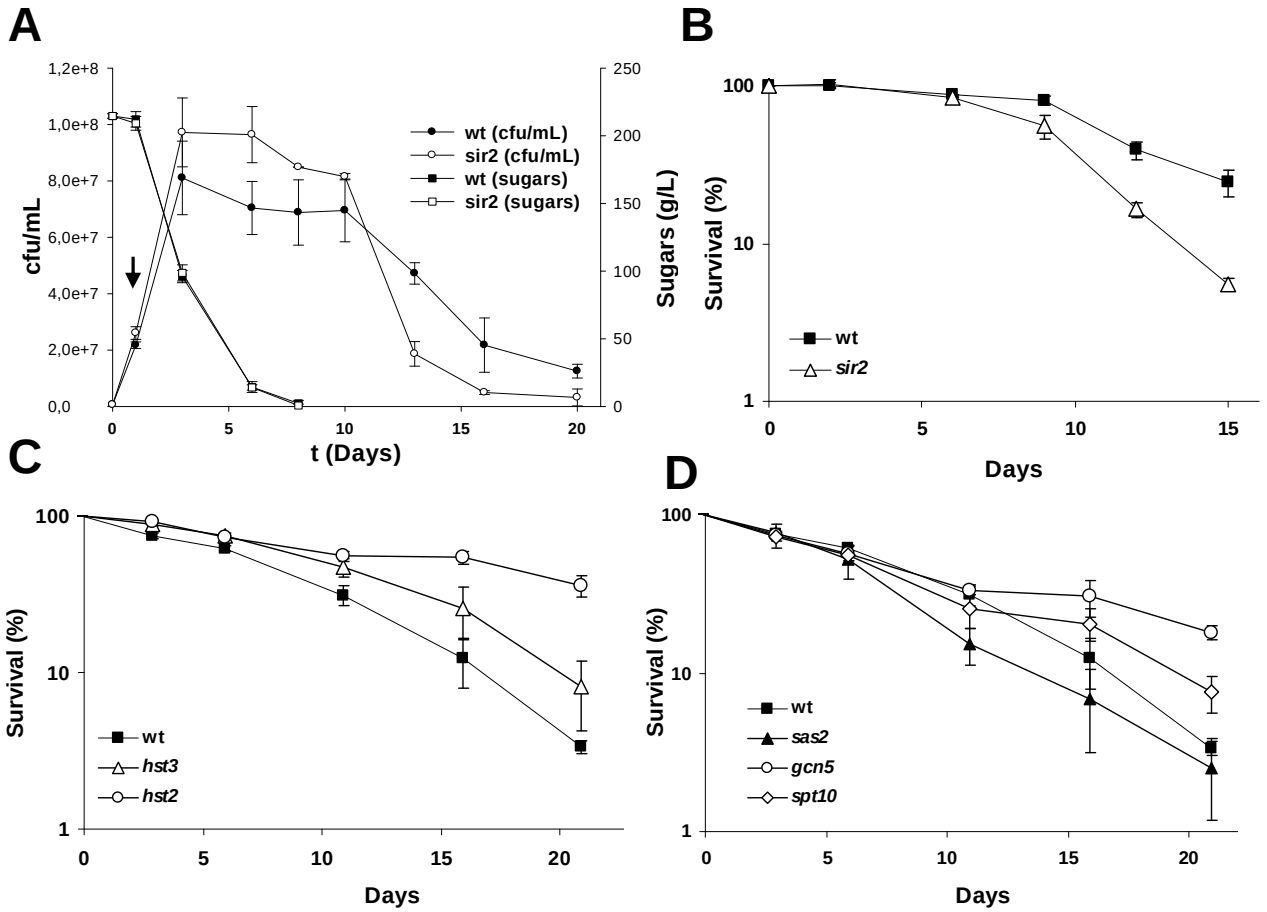
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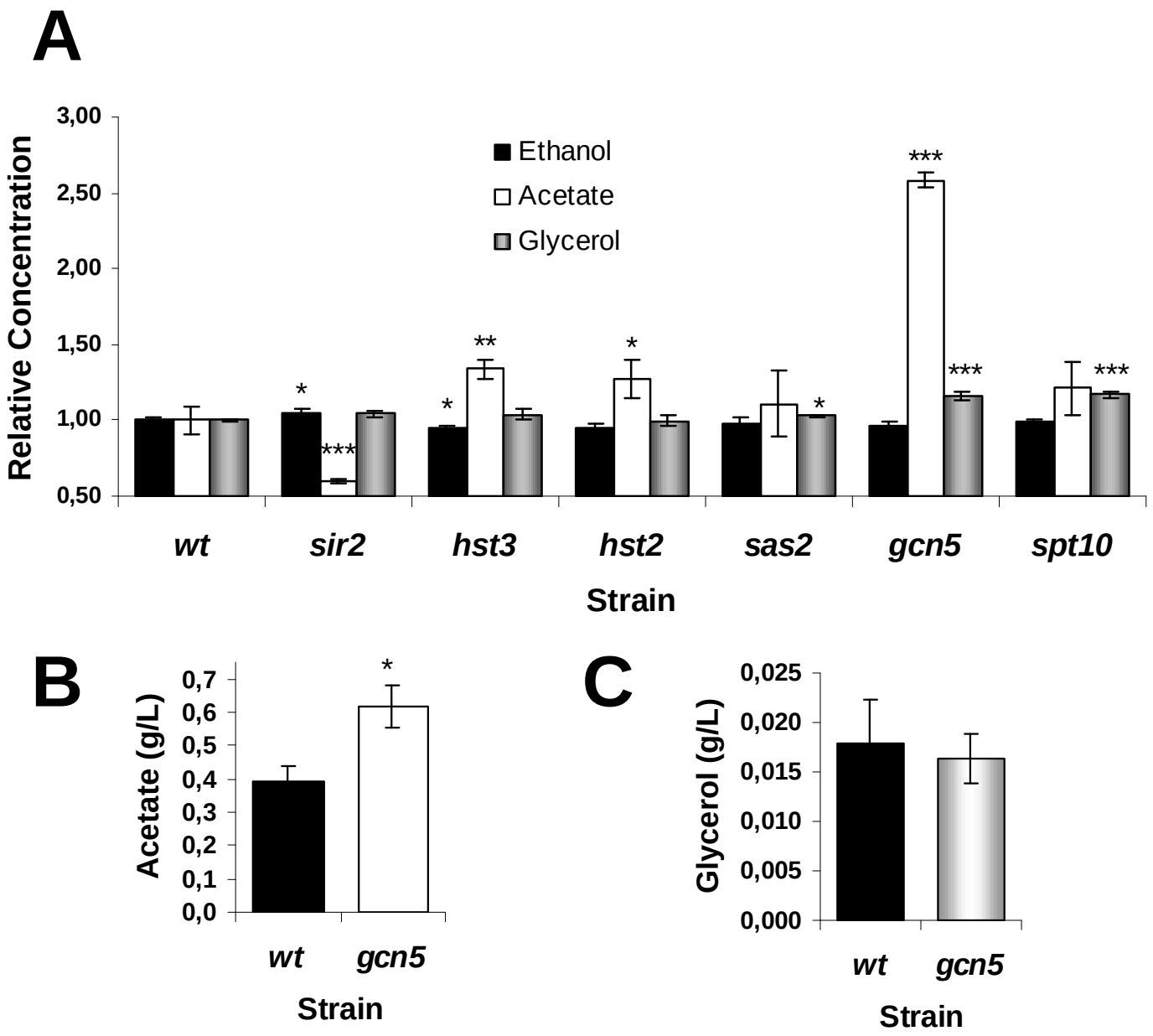
Fig. 3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3,00
2,50
2,00
1,50
1,00
0,50
Ethanol
Acetate
Glycerol
*
***
*
**
*
*
***
***
***
wt
sir2
hst3
hst2
sas2
gcn5
spt10
Strain
B
Acetate (g/L)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
*
wt
gcn5
Strain
C
Glycerol (g/L)
0,025
0,020
0,015
0,010
0,005
0,000
wt
gcn5
Strain

Fig. 4

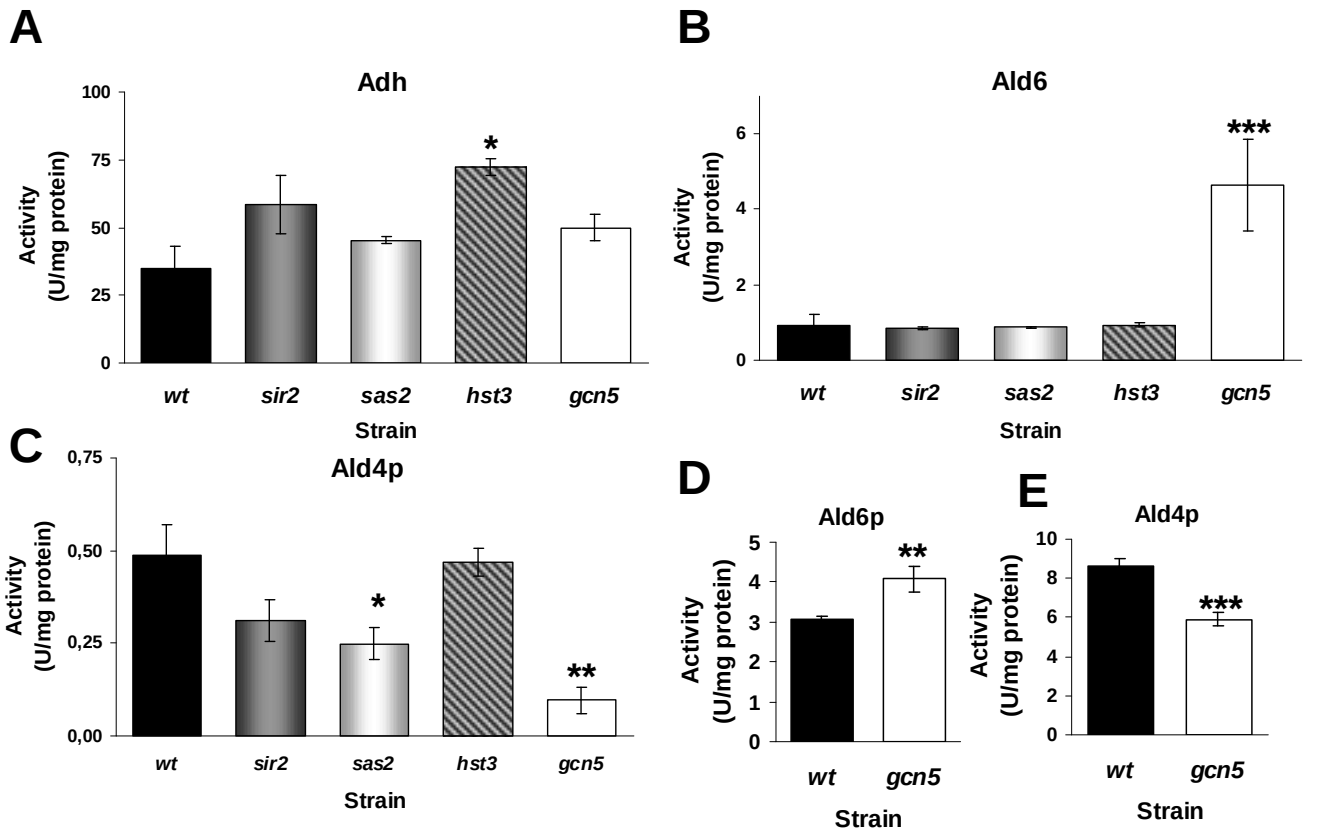

A
Adh
Activity (U/mg protein)
100
75
50
25
0
*
wt
sir2
sas2
hst3
gcn5
Strain
B
Ald6
Activity (U/mg protein)
6
4
2
0
***
wt
sir2
sas2
hst3
gcn5
Strain
C
Ald4p
Activity (U/mg protein)
0,75
0,50
0,25
0,00
*
**
wt
sir2
sas2
hst3
gcn5
Strain
D
Ald6p
Activity (U/mg protein)
5
4
3
2
1
0
**
wt
gcn5
Strain
E
Ald4p
Activity (U/mg protein)
10
8
6
4
2
0
***
wt
gcn5
Strain

Fig. 5

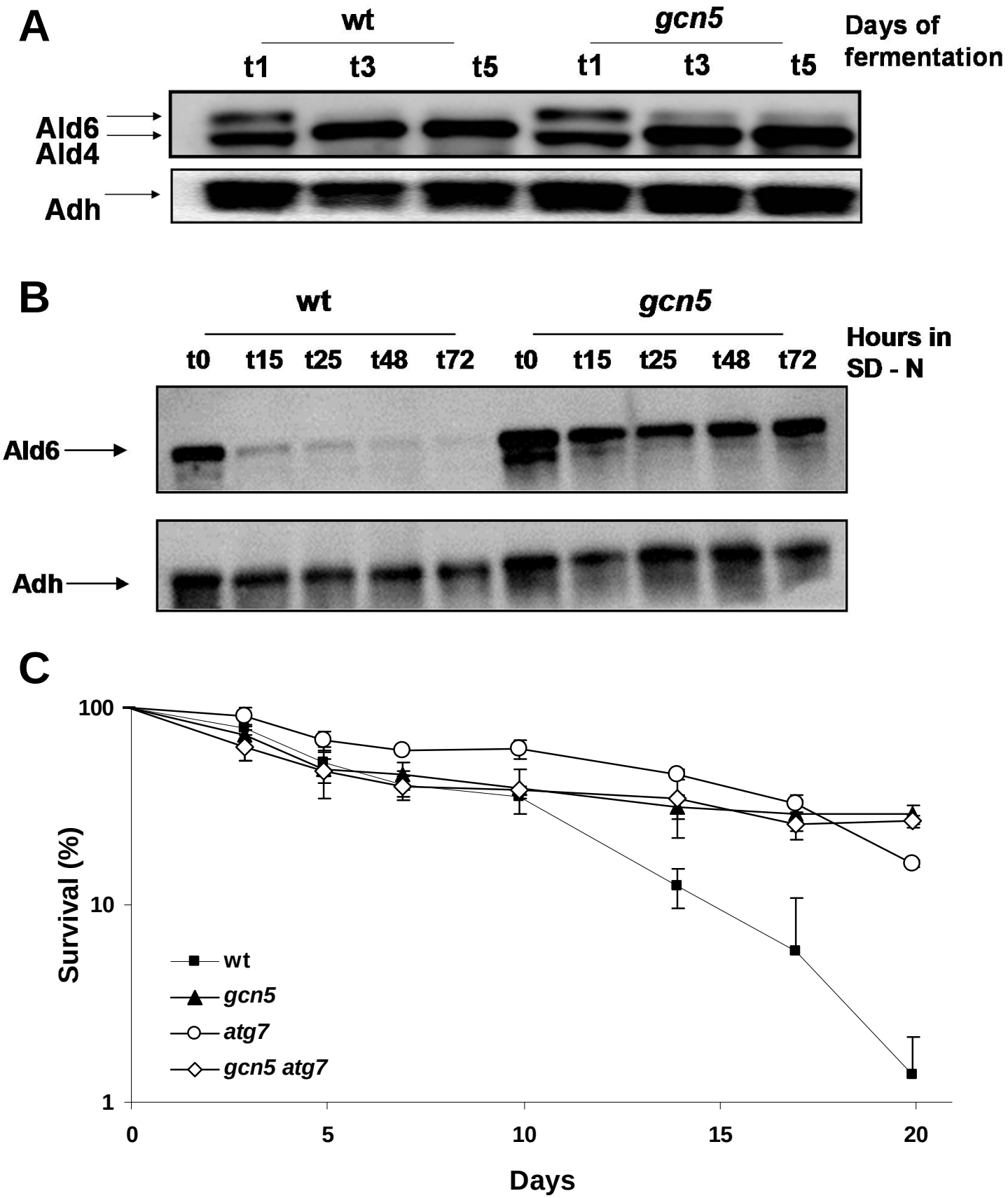

A
wt
gcn5
Days of fermentation
t1
t3
t5
t1
t3
t5
Ald6
Ald4
Adh
B
wt
gcn5
Hours in SD - N
t0
t15
t25
t48
t72
t0
t15
t25
t48
t72
Ald6
Adh
C
100
10
1
Survival (%)
wt
gcn5
atg7
gcn5 atg7
0
5
10
15
20
Days

Fig. 6

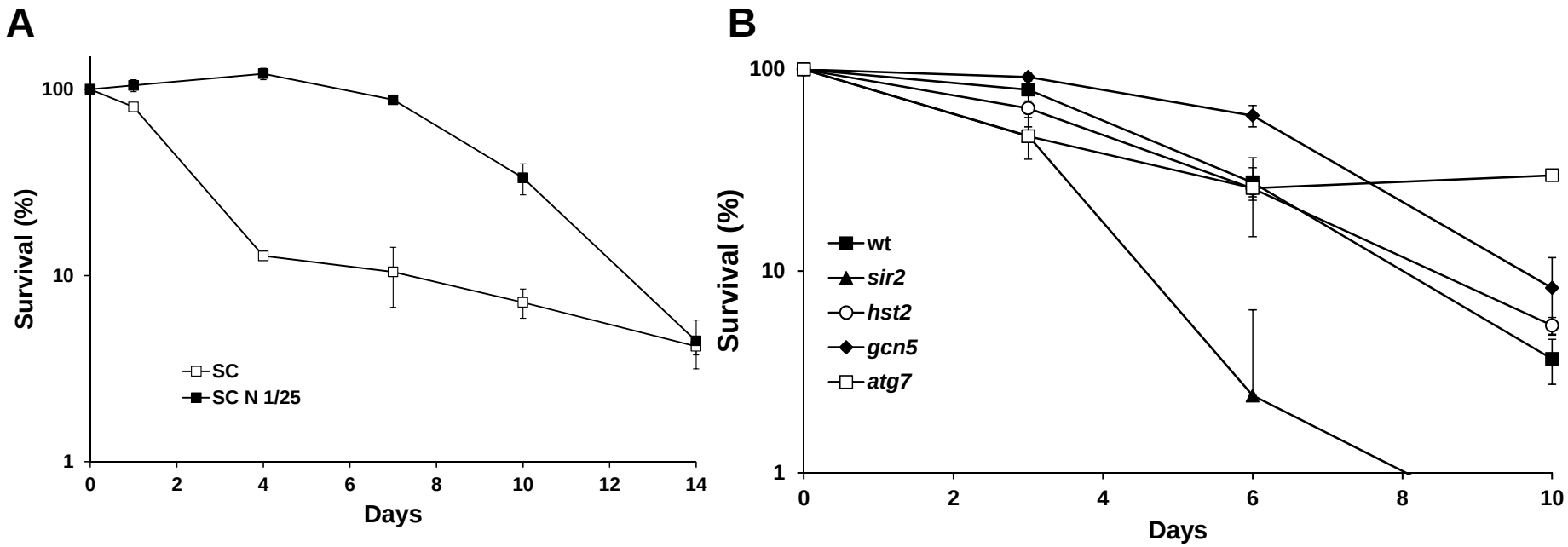
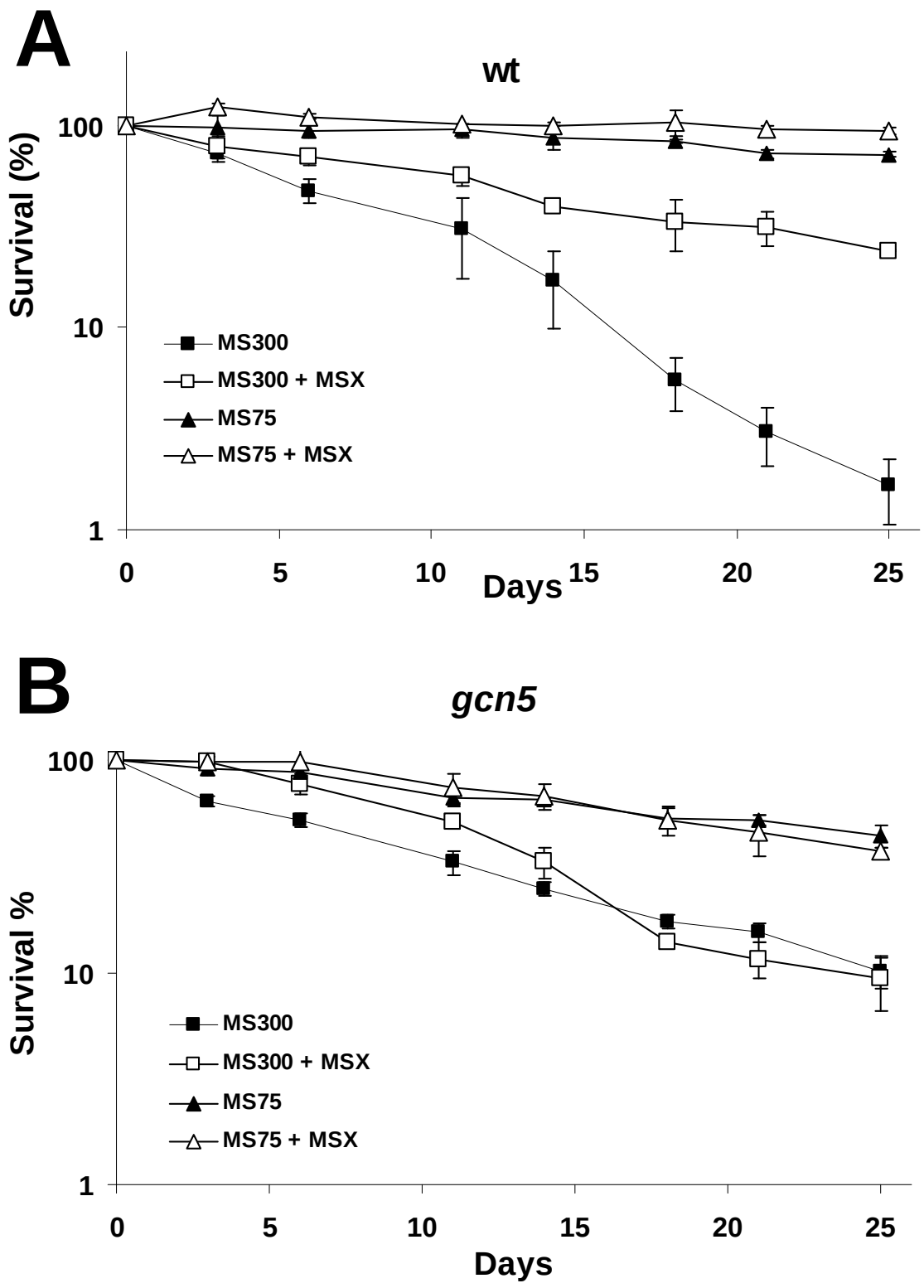

A
100
10
1
Survival (%)
0
2
4
6
8
10
12
14
Days
SC
SC N 1/25
B
100
10
1
Survival (%)
0
2
4
6
8
10
Days
wt
sir2
hst2
gcn5
atg7

Fig. 6


A
wt
Survival (%)
100
10
1
MS300
MS300 + MSX
MS75
MS75 + MSX
0
5
10
15
20
25
Days
B
gcn5
Survival %
100
10
1
MS300
MS300 + MSX
MS75
MS75 + MSX
0
5
10
15
20
25
Days