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RESEARCH ARTICLE OPEN ACCESS

Peroxiredoxin Tsa1 Regulates the Activity of Trehalose Metabolism-Related Enzymes During Wine Yeast Biomass Propagation

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Received: 19 March 2025 | **Revised:** 15 April 2025 | **Accepted:** 23 April 2025

Funding: This work was supported by Maria Zambrano postdoc contract (ZA21-068) from the Spanish Ministry of Universities. Ministerio de Ciencia e Innovación, PID2021-122370OB-I00. Generalitat Valenciana, CIGE/2023/32. Cost Action TRANSLACORE CA21154. V.G. is supported by Atracció de Talent predoctoral contract from the Universitat de València.

Keywords: biomass propagation | molasses | trehalase | trehalose | Tsa1 | wine yeast

ABSTRACT

Trehalose metabolism plays a crucial role in yeast stress tolerance during biomass propagation and dehydration, but its regulatory mechanisms under these industrial conditions remain incompletely understood. This study analyses the role of an antioxidant enzyme, the cytosolic peroxiredoxin Tsa1, in modulating trehalose metabolism in *Saccharomyces cerevisiae* wine strains during biomass production in molasses. Through comparative analyses in three commercial genetic backgrounds (L2056, T73, EC1118), we demonstrate that *TSA1* deletion generally leads to increased intracellular trehalose accumulation despite phenotypic variability among strains. Enzymatic assays revealed that Tsa1 does not regulate trehalose synthesis by altering glycolytic/gluconeogenic flux through pyruvate kinase. However, the deletion of *TSA1* resulted in increased oxidation of trehalose synthesis enzymes, as well as enhanced activity of trehalose-6-phosphate synthase and the trehalases Nth1 and Ath1, suggesting the involvement of peroxiredoxin in the futile cycle of trehalose synthesis and degradation. Scaling up the yeast biomass propagation process to semi-industrial conditions confirmed these findings, with increased trehalose levels in the *tsa1*Δ mutant correlating with enhanced desiccation resistance of the resulting biomass. These results highlight a novel Tsa1-dependent regulatory mechanism governing trehalose metabolism beyond its canonical antioxidant role. Understanding this pathway provides new insights into optimising yeast biomass propagation for industrial applications.

1 | Introduction

The yeast *Saccharomyces cerevisiae* is widely used in biotechnology and the food industry, such as wine production. To reduce the risk of sluggish fermentations and ensure process reproducibility, modern winemaking practices predominantly involve the inoculation of grape must with selected yeast strains, primarily *S. cerevisiae*, in the form of active dry yeast (ADY) (Pérez-Torrado et al. 2015). These commercial starter yeasts are produced using sugar beet or sugarcane molasses as substrates, which are by-products of the sugar industry. Those are therefore cost-effective and have a high sugar content (65%–75% sucrose). At an industrial scale, yeast biomass propagation is performed in a series of increasingly large bioreactors with controlled aeration, following two different cultivation phases. In the initial batch phase, yeast cells are grown in nutrient-supplemented molasses to promote rapid fermentative growth until the sucrose is depleted and the ethanol produced is fully consumed. This phase is followed by fed-batch cultivation, where limited feeding maintains low sucrose concentration, driving the yeast into

respiratory metabolism and thereby maximising biomass yield. Finally, the yeast biomass is dehydrated to produce ADY with a moisture content below 8%, ensuring enhanced product stability for long-term storage (Pretorius 2000; Pérez-Torrado et al. 2015).

During this process, the intense aeration required for the transition from fermentative to respiratory metabolism, coupled with the endogenous respiratory activity of the yeast, results in the substantial production of reactive oxygen species (ROS). These oxidative byproducts impose significant oxidative stress on yeast cells during both biomass propagation and dehydration, ultimately affecting the biomass yield and the technological performance of ADY (Matallana and Aranda 2017). In this line, previous studies have reported the induction of genes associated with oxidative stress during biomass propagation (Pérez-Torrado et al. 2005; Gómez-Pastor et al. 2010a) and dehydration (Garre et al. 2010). Cellular responses to oxidative damage are triggered when ROS levels exceed the detoxification capacity of the yeast antioxidant defence systems (Morano et al. 2012), causing damage to biological molecules such as lipid peroxidation. In addition to some antioxidant enzymes like catalases and superoxide dismutases, *S. cerevisiae* possesses glutaredoxin and thioredoxin systems, which maintain cellular redox balance by repairing oxidative damage to key cysteines in a variety of proteins (Herrero et al. 2008). Under the aforementioned industrial conditions, the cytosolic thioredoxin system has been the most studied (Gómez-Pastor et al. 2012). At the core of this system is Tsa1, the principal cytosolic thioredoxin peroxidase or peroxiredoxin, which detoxifies hydrogen peroxide. The catalytic mechanism of Tsa1 involves the oxidation of its peroxidatic cysteine (C48) by hydrogen peroxide to form a sulfenic acid, which condenses with the resolving cysteine (C171) to form a disulfide bond. This bond is subsequently reduced by the cytosolic thioredoxins Trx1 and Trx2 cysteines to form another disulfide bond in them, which are regenerated by thioredoxin reductase (Trr1) through electron transfer from NADPH. Beyond its peroxide detoxification function, Tsa1 also acts as a molecular chaperone when excessively oxidised (Hanzén et al. 2016). Previous work from our group has shown that all components of the cytosolic peroxiredoxin-thioredoxin-thioredoxin reductase system influence the regulation of yeast metabolism (Picazo et al. 2019, 2018). Notably, Tsa1 plays a role in regulating acetic acid and trehalose metabolism during biomass propagation in molasses (Garrigós et al. 2020, 2025).

Trehalose is a non-reducing disaccharide consisting of two glucose molecules. It serves as a stress protectant (against cold, heat, desiccation, osmotic, and oxidative stress) and a signalling molecule (Elbein et al. 2003; Garre et al. 2010). In fact, high levels of trehalose during wine yeast propagation have been described as a marker of improved yeast performance after dehydration (Gamero-Sandemetrio et al. 2014). In *S. cerevisiae*, trehalose synthesis takes place in the cytosol and involves two enzymatic activities: trehalose-6-phosphate synthase (Tps1), which catalyses the formation of trehalose-6-phosphate (T6P) from glucose-6-phosphate and UDP-glucose, and trehalose-phosphatase (Tps2), which dephosphorylates T6P to produce trehalose. Trehalose mobilisation, on the other hand, relies on two trehalase activities. Neutral trehalase activity is primarily attributed to Nth1, a cytosolic protein responsible for intracellular trehalose degradation. Acid trehalase activity, attributed to Ath1, is associated with vacuolar or periplasmic locations and is mainly involved in the breakdown of extracellular trehalose (Eleutherio et al. 2015).

Understanding the role of Tsa1 in yeast physiology and trehalose metabolism will be useful for identifying key regulatory mechanisms and novel players involved in the industrial production of wine yeasts. The aim of this study is to further characterise our preliminary findings on the role of Tsa1 in trehalose accumulation (Garrigós et al. 2020) and to perform a temporal dissection of Tsa1-regulated trehalose metabolism through laboratory-scale simulations of biomass propagation. We found that *TSA1* deletion resulted in increased activity of trehalose-6-phosphate synthase (Tps1) and the trehalases Nth1 and Ath1, ultimately leading to elevated intracellular trehalose levels during the biomass propagation process. This phenotype persisted at a semi-industrial scale. Furthermore, this accumulation of intracellular trehalose in the absence of peroxiredoxin was associated with higher yeast viability following dehydration.

2 | Material and Methods

2.1 | Yeast Strains and Growth Conditions

The yeast strains herein used are listed in Table S1. Deletion of the *TSA1* gene in the wine yeasts EC1118, L2056 and T73 was performed by CRISPR-Cas9 using the pRCC-K plasmid, a gift from Eckhard Boles (Addgene plasmid #81191), and following the protocol described in (Generoso et al. 2016). Plasmid pFA6a-13Myc-kanMX6 (Longtine et al. 1998) was used as a PCR template for the Ct Myc-epitope tagging of *TPS1*, *TPS2* and *NTH1* genes in strains T73 and T73 *tsa1*Δ. Yeast transformations were carried out using the lithium acetate method (Gietz and Woods 2002). For standard growth purposes, yeast cultures were grown at 30°C in rich YPD medium (1% yeast extract, 2% peptone, 2% glucose). Solid media were prepared by supplementing with 2% agar, and geneticin at a concentration of 200 mg/L was used for selecting *kan*MX transformants. The genetic modification of the selected resistant colonies was verified by PCR. The list of genes subjected to genetic manipulation in this study, along with their corresponding functions, is provided in Table S2.

Precultures for biomass propagation were prepared in liquid YPD medium and incubated for 24 h. Bench-top biomass propagation experiments in flasks were conducted using sugar beet molasses adjusted to 60 g/L sucrose for standard molasses, or 20 g/L sucrose for diluted molasses. Sugar beet or cane molasses are the usual substrates for yeast biomass production because they are cheap and rich in sugars in the form of sucrose, but nitrogen sources and vitamins have to be added. In both cases, the molasses were autoclaved for 20 min at 121°C and supplemented with autoclaved 7.5 g/L $(NH_4)_2SO_4$, 3.5 g/L KH_2PO_4, 0.75 g/L $MgSO_4$, and filtered 10 mL/L vitamin solution (Torrellas et al. 2020). Cultures were incubated at 30°C with constant shaking (180 rpm) for 72 h, allowing for the study of the exponential growth phase, diauxic shift, and stationary phase.

Industrial simulations of yeast biomass propagation were carried out in a 5 L ez2-Control bioreactor (Applikon Biotechnology,

Netherlands) equipped with proportional, integral and derivative (PID) control units for pH, temperature, oxygen and agitation speed. During the batch phase, the bioreactor contained 3 L of autoclaved and supplemented molasses medium (60 g/L sucrose) and was inoculated with an initial OD_{600} of 0.1 from YPD precultures. Antifoam 204 (Sigma) was added at 0.05% (v/v). Cells were grown at 30°C with shaking. Dissolved oxygen was measured with an electrode and maintained at 20% by a PID control system that allowed automatic modification of stirring speed between 300 and 500 rpm. For the fed-batch phase, the bioreactor was fed with molasses at 100 g/L sucrose, maintaining the respiratory metabolism of the yeast. The initial pH was 4.5, varying freely during the batch phase and maintained at 4.5 during the fed-batch phase by the automatic addition of 1 M NaOH or 42.5% H_3PO_4. Cell growth was monitored by measuring OD (600 nm).

2.2 | Biomass Dehydration and Rehydration Conditions

Yeast biomass was separated by centrifugation at 1792× g, followed by multiple washes with sterile distilled water to remove residual molasses. The resulting biomass paste was shaped into thin strands with syringes and placed inside a tabletop fluid bed dryer (Sherwood Scientific, Cambridge, UK). Dehydration was carried out using an airflow rate of 2.5 m^3/min at 37°C for 50 min, the time required to achieve a final moisture content of 8%, as determined by weight loss for T73 (Torrellas et al. 2020). The dried yeast biomass was subsequently stored at 4°C until further analysis. For rehydration, the dry yeast biomass was suspended in sterile distilled water (1:9 biomass-to-water ratio) and incubated at 37°C for 10 min, followed by gentle agitation at 140 rpm for an additional 10 min.

To evaluate cell viability, fresh biomass (before dehydration) and rehydrated biomass were diluted in sterile distilled water, plated on YPD agar, and incubated at 30°C for 24 h. Colony-forming units (CFU) were then enumerated, and the survival percentage was determined by setting the CFU count of fresh biomass as the 100% survival reference.

2.3 | Metabolite Determination

Sucrose determination was performed in 50 mM sodium acetate buffer pH 5.0 containing invertase (Sigma, USA). The samples were then incubated at 30°C for 10 min. The glucose released by the reaction was determined by the glucose oxidase-peroxidase method. The enzymatic quantification of ethanol was performed by spectrophotometric detection at 340 nm of NADH formed during the oxidation of ethanol to acetaldehyde by the alcohol dehydrogenase enzyme as described (Torrellas et al. 2023).

Intracellular trehalose and glycogen levels were quantified as described by Gamero-Sandemetrio et al. (2014) and Parrou and François (1997), using 100 mg of cells. Cells were resuspended in 250 mM Na_2CO_3 and incubated at 95°C for 4 h. Then, they were centrifuged at 12,000 rpm for 30 s. The supernatant was recovered and incubated overnight with 8.4 mU of commercial trehalase (Sigma) at 37°C for trehalose determination, or with amyloglucosidase (1.2 U/mL) (from *Aspergillus niger*, Sigma, 1000 U/mL) at 57°C for glycogen determination. The released glucose was measured by the glucose-oxidase-peroxidase method (Torrellas et al. 2023).

2.4 | Enzymatic Activities

Cell-free extracts for enzymatic analysis were prepared from 100 mg of cells collected by centrifugation, washed with sterile water to remove any residual molasses, and subsequently resuspended in the specific lysis buffer for each enzymatic activity. Cells were disrupted with glass beads using a Precellys Evolution homogeniser (Bertin). Protein concentration was measured using the DC Protein Assay (BioRad), following the manufacturer's protocol. For pyruvate kinase activity determination, lysis buffer contained 100 mM 2-morpholinoethanesulfonic (MES) acid pH 7.0, 200 mM KCl, 15 mM MgCl2, 5% glycerol and COMPLETE Mini protease inhibitor (Sigma Aldrich). The activity was measured as previously described (Jurica et al. 1998; Irokawa et al. 2016).

For trehalose-6 phosphate synthase (TPS) activity, cell-free extracts were obtained in cold lysis buffer (25 mM MES pH 7.1). TPS activity was measured by a discontinuous procedure as described previously (Argüelles et al. 1993). One unit of T-6P synthase activity produced 1 nmol of UDP per min at 37°C.

For neutral and acid trehalase activities, cell-free extracts were obtained using 10 mM MES (pH 6.0) as the extraction buffer. Both enzymatic activities were measured as described above, with the pH of the incubation buffer adjusted to selectively favour either neutral or acid trehalase activity (Pedreño et al. 2002; San Miguel and Argüelles 1994). The glucose concentration was determined in the supernatants using the glucose oxidase/peroxidase assay.

2.5 | NEM-mPEG Assay and Western Blot

The oxidation state of the cysteines in Tps1 and Tps2 proteins was studied in cells expressing Myc-tagged versions of Tps1 or Tps2, by the NEM-mPEG assay following the steps described by Sjölander et al. (2020). To follow the protein levels of Tps1, Tps2 and Nth1, cells expressing Myc-tagged versions of these proteins were lysed using glass beads, and whole-cell extracts were prepared in lysis buffer containing 1 M Tris–HCl (pH 7.5), 5 M NaCl, 1 M $MgCl_2$, 10% (v/v) NP40, 0.1 M PMSF, and a commercial protease inhibitor tablet (COMPLETE Mini, EDTA-free; Roche). In both assays, protein concentration was measured using the DC Protein Assay (BioRad), following the manufacturer's protocol. Samples were subsequently separated by SDS-PAGE using an Invitrogen mini-gel system and transferred to PVDF membranes using a Novex semi-dry transfer system (Invitrogen, Carlsbad, CA, USA). Immunodetection was performed with the anti-Myc (Santa Cruz Biotechnology) antibody. Anti-Pgk1 (Invitrogen) was used as the loading control. Signal detection was carried out using the ECL Western Blotting Detection System (GE Healthcare). Relative quantification of the protein bands in the resulting images was performed with ImageJ.

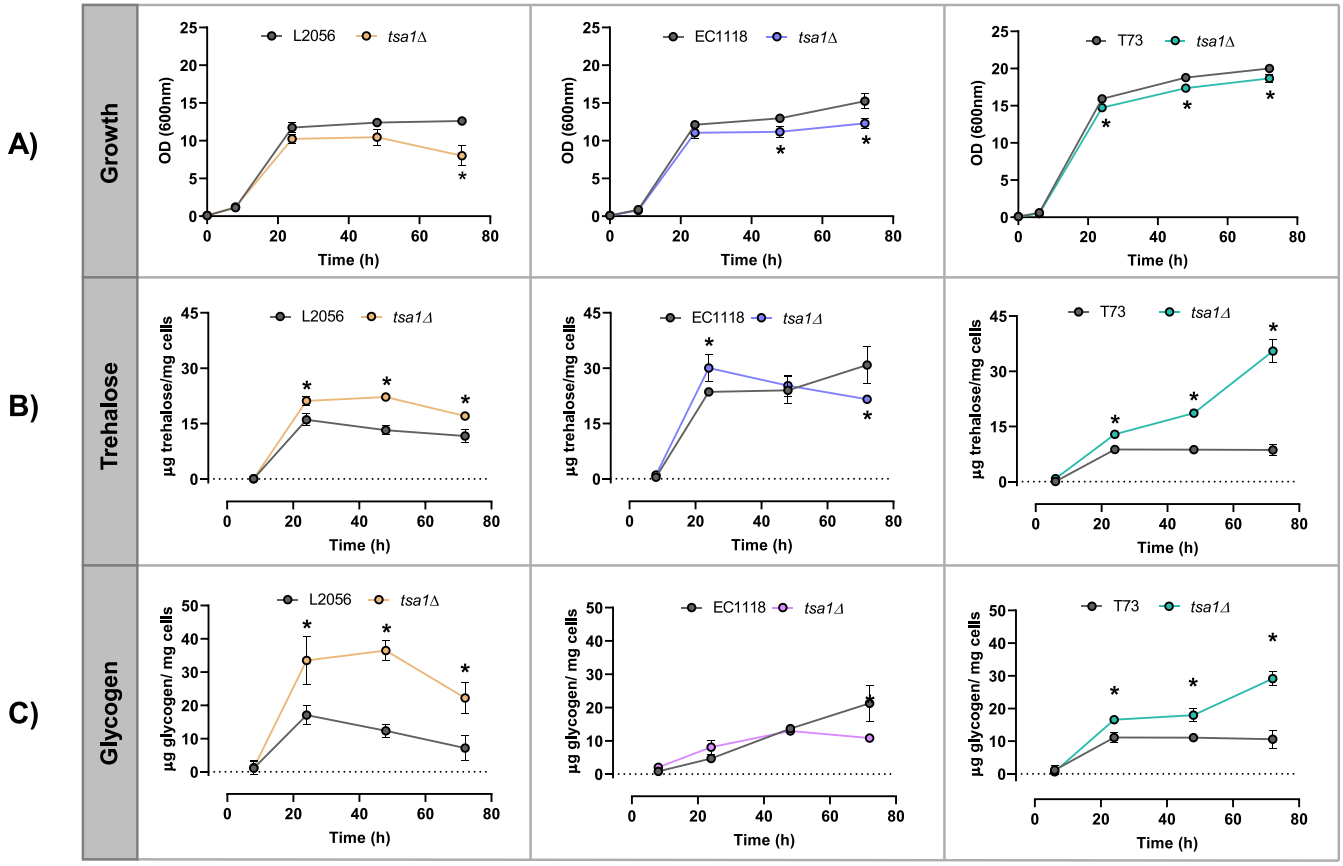

FIGURE 1 | Effect of *TSA1* deletion in different genetic backgrounds in molasses. (A) Growth measured as OD_{600}. (B) Intracellular trehalose and (C) intracellular glycogen accumulation profiles of L2056, EC1118, and T73 wine strains and their respective *tsa1*Δ mutants during biomass propagation in flasks, using 6% (w/v) sucrose molasses as the substrate. The experiments were carried out in triplicate, and the average and standard deviation are provided. Significant differences (*$p < 0.05$, Student's *t*-test) between the *tsa1*Δ mutants and their parental strains at each time point are shown.

3 | Results

3.1 | Effect of *TSA1* Deletion in Different Wine Strains During Growth in Natural Molasses

Preliminary findings from our research group, using the diploid wine strain L2056, demonstrated that the cytosolic peroxiredoxin Tsa1 significantly impacts intracellular trehalose and glycogen accumulation after 24 h of growth in sugar beet molasses (Garrigós et al. 2020). However, wine yeasts exhibit substantial phenotypic diversity (Vallejo et al. 2020a; Su et al. 2021), which is also reflected in the role of Tsa1 in yeast growth and acetic acid metabolism (Garrigós et al. 2025). To investigate this further, a CRISPR-Cas9 method was developed to delete this gene in the commercial wine strains L2056, T73 and EC1118. These strains were selected based on their established phylogenetic divergence (Borneman et al. 2016), allowing us to assess the impact of Tsa1 across the natural genetic diversity present among industrial yeast strains. Moreover, this comparative analysis will facilitate the identification of the most suitable genetic background for exploring the time-course impact of Tsa1 on trehalose metabolism during growth under industrial biomass propagation conditions. Additionally, two types of molasses were tested for this purpose: 6% (w/v) sucrose molasses, commonly employed at the industrial level (Pérez-Torrado et al. 2005; Garre et al. 2010), and 2% (w/v) sucrose diluted molasses, which has been shown in previous studies to maintain biomass yield in *S. cerevisiae* wine strains while reducing substrate expenditure (Schnierda et al. 2014; Torrellas et al. 2023). The experiments were conducted in flasks to allow the analysis of multiple strains and growth conditions.

First, the growth and trehalose accumulation in 6% (w/v) sucrose molasses were studied (Figure 1). *TSA1* deletion did not significantly affect the growth profile in any of the genetic backgrounds. However, the *tsa1*Δ mutants exhibited a consistently lower cell density at some points in the stationary phase (Figure 1A). After 24 h of growth, *TSA1* deletion led to increased intracellular trehalose levels in all genetic backgrounds. These levels remained elevated in L2056 *tsa1*Δ throughout growth, further increased in T73 *tsa1*Δ, but decreased in EC1118 *tsa1*Δ at 72 h (Figure 1B). Since Tsa1 has also been reported to influence intracellular glycogen accumulation (Garrigós et al. 2020), we additionally analysed fluctuations in glycogen levels to further understand its role in carbohydrate reserve dynamics during biomass propagation (Figure 1C). The T73 *tsa1*Δ mutant exhibited higher glycogen accumulation, with levels up to 1.5-fold higher than the wt at 24 h, 1.6-fold at 48 h, and 2.7-fold at 72 h. The *TSA1* deletion in L2056 also led to increased glycogen accumulation after 24 h of growth, whereas this effect was not observed in the EC1118 genetic background (Figure 1C),

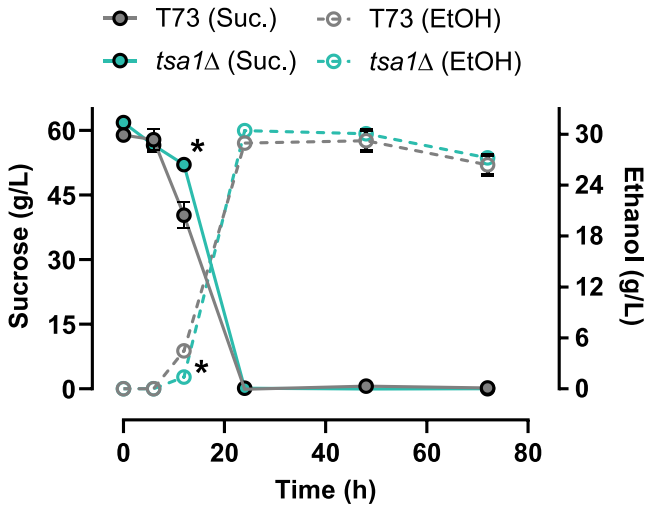

FIGURE 2 | Sucrose consumption and ethanol production profiles of the T73 and T73 *tsa1*Δ mutant strains during yeast biomass propagation in flasks. 6% (w/v) sucrose molasses were used as substrate. The experiments were carried out in triplicate, and the average and standard deviation are provided. Significant differences (*$p < 0.05$, Student's *t*-test) between the *tsa1*Δ mutant and the parental strain at each time point are shown. EtOH, ethanol; Suc, sucrose.

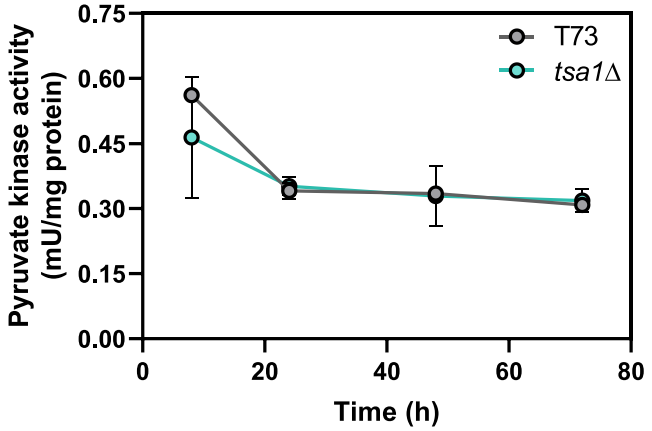

FIGURE 3 | Effect of Tsa1 on pyruvate kinase activity in molasses. Pyruvate kinase activity in T73 and the *tsa1*Δ mutant during biomass propagation in flasks, using 6% (w/v) sucrose molasses as the substrate. The experiments were carried out in triplicate, and the average and standard deviation are provided. Significant differences (*$p < 0.05$, Student's *t*-test) between the *tsa1*Δ mutant and the wild-type strain at each time point are shown.

consistent with previous findings for trehalose (Figure 1B). Therefore, the impact of Tsa1 on carbohydrate metabolism appears to be less pronounced in the EC1118 genetic background in these conditions.

During wine yeast biomass propagation in diluted molasses (Figure S1), *TSA1* gene deletion had a less pronounced impact on growth across all genetic backgrounds. The L2056 *tsa1*Δ mutant showed a growth defect that, although evident from the early stages, became more pronounced over time. In contrast, in the T73 and EC1118 genetic backgrounds, *TSA1* deletion had minimal impact on growth (Figure S1A). Additionally, the L2056 *tsa1*Δ mutant exhibited increased intracellular trehalose accumulation after 24 h of growth but failed to reach the glycogen and trehalose levels observed in the wild-type strain during the stationary growth phase (Figure S1B,C). In T73 and EC1118, this phenotype was not consistently maintained. In these genetic backgrounds, *tsa1*Δ mutants exhibited higher intracellular trehalose levels throughout growth, whereas glycogen accumulation was only increased in the T73 *tsa1*Δ mutant at 24 h.

Given the phenotypic variability among strains and conditions, the T73 genetic background was selected for further experiments, as the trehalose accumulation phenotype of the *tsa1*Δ mutant was stable across different substrates. Regarding growth conditions, 6% sucrose molasses was selected, as it is the commonly used industrial substrate and showed the greatest consistency among strains in terms of the *TSA1* deletion effect. Since the *tsa1*Δ mutant exhibited a growth defect compared to its parental strain T73 from 24 h onward, we investigated whether this was due to differences in sucrose consumption and ethanol production efficiency. As shown in Figure 2, although both strains fully consumed the sucrose by 24 h, the *tsa1*Δ mutant showed slower sucrose uptake at 12 h, resulting in lower ethanol production at that point. This slight delay in sucrose utilisation may have contributed to the mutant's slower growth, which persisted beyond 72 h. Notably, neither strain was able to efficiently consume the ethanol produced. After 72 h of growth, both strains achieved only a 10% reduction in ethanol levels compared to the maximum recorded at 24 h. This indicates that, during biomass propagation in molasses in flask, the conditions are insufficient to induce a complete shift to respiratory metabolism in yeast, probably due to poor aeration levels.

3.2 | Tsa1 Does Not Exert Control Over Pyruvate Kinase Activity During Yeast Biomass Propagation

A previous study has shown that Tsa1 improves the efficiency of gluconeogenesis by physically interacting with pyruvate kinase (Cdc19) and inhibiting its activity during the diauxic shift, thus favouring the transition from glycolysis to gluconeogenesis (Irokawa et al. 2016). Trehalose is accumulated after the diauxic shift when the gluconeogenic flux is activated (Lillie and Pringle 1980). Therefore, if this mechanism applies, *TSA1* deletion should decrease the levels of this protective disaccharide. However, we have shown that during yeast biomass propagation in molasses, trehalose accumulation is induced in T73 *tsa1*Δ mutant wine strains (Figure 1B). To determine whether this phenotype was due to the effect of Tsa1 on pyruvate kinase activity, we cultured T73 and *tsa1*Δ strains in flasks with molasses and monitored this enzymatic activity during 72 h of growth (Figure 3). As expected, pyruvate kinase activity was higher in both strains at 8 h of growth when sucrose was still available. After 24 h, once sucrose was consumed, there was a decrease in this enzymatic activity, which remained constant after 72 h. This highlights the redirection of metabolic fluxes towards gluconeogenesis. However, *TSA1* deletion did not affect pyruvate kinase activity, indicating that Tsa1 does not exert control over Cdc19 in wine strains and under biomass propagation conditions in molasses (Figure 3).

The *tsa1*Δ mutants in the L2056 and EC1118 strains mirrored this phenotype, so this mechanism is conserved in all wine strains tested (Figure S2). Overall, these findings suggest that Tsa1 represses trehalose and glycogen accumulation independently of Cdc19 activity during the propagation of wine yeast biomass in molasses.

3.3 | Tsa1 Regulates the Activity and Oxidation Status of the Main Enzymes Involved in Trehalose Synthesis

Since Tsa1 does not indirectly influence trehalose metabolism by controlling a key enzyme involved in glycolytic/gluconeogenic fluxes, we sought to investigate whether Tsa1 directly affects the abundance of the primary enzymes involved in trehalose synthesis, Tps1 and Tps2. To address this, we monitored the protein levels of Tps1 and Tps2 during growth in molasses medium, using tagged versions of these proteins.

As expected, the Tps1 (Figure 4A) and Tps2 (Figure 4B) protein levels increased in a coordinated way after 24 h in both the wild-type and *tsa1*Δ strains, coinciding with the complete sucrose consumption and the onset of the diauxic shift (Winderickx et al. 1996). Tps1 abundance was higher in the *tsa1*Δ mutant at the very start of growth, just slightly higher throughout the latter growth stages, except at 72 h. On the other hand, Tps2 followed an opposite trend, their levels always being slightly lower in the T73 *tsa1*Δ strain. However, these slight differences in Tps1 and Tps2 abundance do not explain the pronounced trehalose accumulation in the mutant (Figure 1B). Furthermore, trehalose levels in the *tsa1*Δ mutant steadily increased during growth, while Tps1 and Tps2 protein levels peaked at 24 h and then gradually declined, becoming visibly lower by 72 h. This lack of correlation between trehalose levels and protein abundance in the mutant strain prompted us to evaluate trehalose-6-phosphate synthase (TPS) activity, which catalyses the initial step in trehalose biosynthesis. As expected, TPS activity increased following the diauxic shift (Figure 4C). Importantly, this enzymatic activity was 1.57-fold higher in the *tsa1*Δ mutant after 12 h, with further increases observed at 24 h (1.58-fold) and 48 h (1.66-fold) compared to the wild-type strain. By 72 h, the activity levels in both strains converged, probably due to the significantly reduced abundance of Tps1 protein in the mutant. Therefore, the trehalose levels at 72 h in *tsa1*Δ must result from the accumulation of high enzymatic synthesis activity at earlier time points. These findings suggest that, during yeast biomass propagation, trehalose accumulation in the mutant is driven primarily by the derepression of Tsa1-dependent TPS activity, rather than by the abundance of Tps1 and Tps2 proteins.

Previous studies have underscored the critical role of oxidative stress defence mechanisms during yeast biomass propagation. Specifically, Tsa1 is induced during this process (Gómez-Pastor et al. 2010a), and the overexpression of the cytosolic thioredoxin *TRX2* was explored as a strategy to enhance the yeast antioxidant defence and fermentative capacity (Gómez-Pastor et al. 2012). Consistent with these findings, deletion of *TSA1* has been shown to increase intracellular ROS levels (Garrigós et al. 2020), which is expected to affect protein oxidation and associated cellular damage. To further explore this phenomenon, we investigated the oxidation levels of Tps1 and Tps2 in both wild type and *tsa1*Δ strains. We employed a methodology that enables differential labelling of reduced and oxidised cysteine (Cys) residues using N-ethylmaleimide (NEM) and methoxy polyethylene glycol (mPEG) 5000, respectively. The mPEG reagent binds specifically to oxidised Cys residues, resulting in reduced electrophoretic mobility proportional to the number of modified residues. Our analysis revealed that Tps1 oxidation levels increased during biomass propagation, with significantly higher oxidation observed in the *tsa1*Δ mutant (Figure 4D). This finding suggests that the hyperoxidation of key cysteines in the absence of Tsa1 seems to increase TPS activity. In contrast, Tps2 oxidation was less pronounced overall; however, a notable increase in oxidation was detected in the *tsa1*Δ mutant, particularly at 24 h (Figure 4E). Therefore, Tsa1 contributes to both the activity and the oxidative state of the key enzymes involved in trehalose synthesis.

3.4 | Tsa1 Has an Impact on Trehalose Degradation Enzymes

To comprehensively assess the role of Tsa1 in trehalose metabolism, we extended our investigation to its impact on degradation. Due to the cytosolic location of Tsa1, the most likely target is the neutral trehalase Nth1 (Nwaka and Holzer 1997). To investigate Nth1, a labelled version of this protein was used to monitor its abundance during biomass propagation in molasses. Under laboratory conditions, Nth1 activity has been shown to peak during the early phases of growth, becoming inactive during the stationary phase (Dengler et al. 2021). However, the present study is the first to provide a temporal analysis of Nth1 protein levels and activity throughout growth in molasses. As shown in Figure 5A, Nth1 levels in the wild-type strain remained relatively stable, with a slight increase after 24 h, coinciding with sucrose depletion. In contrast, the *tsa1*Δ mutant exhibited significantly elevated Nth1 abundance compared to the wild-type strain under post-diauxic conditions, with differences becoming more pronounced after 24 h. This increase in Nth1 protein levels correlated strongly with its enzymatic activity (Figure 5B). In the wild-type strain, a slight rise in Nth1 activity was observed during the stationary growth phase. However, in the *tsa1*Δ mutant, neutral trehalase activity was triggered after 24 h and progressively increased to levels up to 14-fold higher than those of the wild-type strain at the end of growth. These findings demonstrate that Nth1 activity is not inhibited following the diauxic shift during biomass propagation in molasses. Moreover, Tsa1 regulates both the protein levels and activity of Nth1 under these conditions.

As Nth1 activity was induced during the stationary phase, we also examined the activity of the acidic trehalase, Ath1 (Figure 5C). Previous studies have shown that acidic trehalase activity gradually increases when yeast cells pass through the diauxic phase in glucose-based medium, reaching its maximum levels upon entering the stationary phase, where it is able to mobilise intracellular trehalose (Garre et al. 2009). As expected, Ath1 activity increased slightly after sucrose depletion in the medium (Figure 5C). However, this induction was markedly higher in the *tsa1*Δ mutant, with Ath1 activity peaking at the end of the stationary phase and reaching levels up to 16-fold

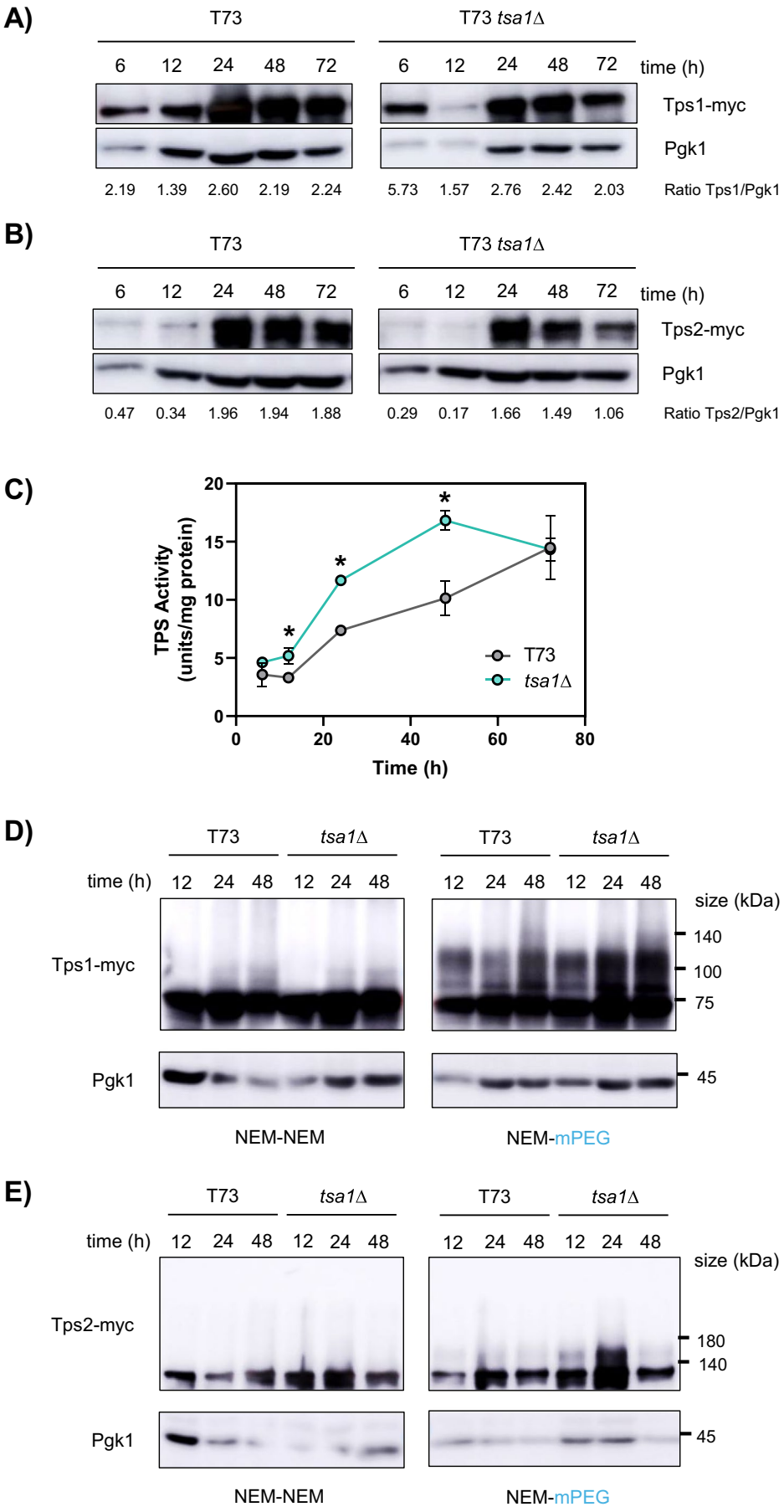

FIGURE 4 | Legend on next page.

FIGURE 4 | Analysis of the impact of Tsa1 on Tps1 and Tps2 in molasses. Biomass propagation in flasks with 6% (w/v) sucrose molasses was conducted using the T73 and T73 *tsa1*Δ strains, which included the 13myc-tagged versions of Tps1 and Tps2. This setup facilitated monitoring (A) Tps1 and (B) Tps2 protein levels throughout the propagation process. Detection of the 13myc-tagged proteins was performed using an anti-Myc antibody. Pgk1 was used as a loading control and was detected with a Pgk1-specific antibody. (C) Trehalose-6-phosphate synthase (TPS) activity was measured from T73 and T73 *tsa1*Δ (non-tagged Tps1/2 strains) cultures in molasses medium. The oxidation status of (D) Tps1 and (E) Tps2 from cultures described in (A, B) was conducted using the NEM-mPEG assay. The experiments were carried out in triplicate, and the average and standard deviation are provided. Significant differences (*$p < 0.05$, Student's *t*-test) between the *tsa1*Δ mutant and the wild-type strain at each time point are shown.

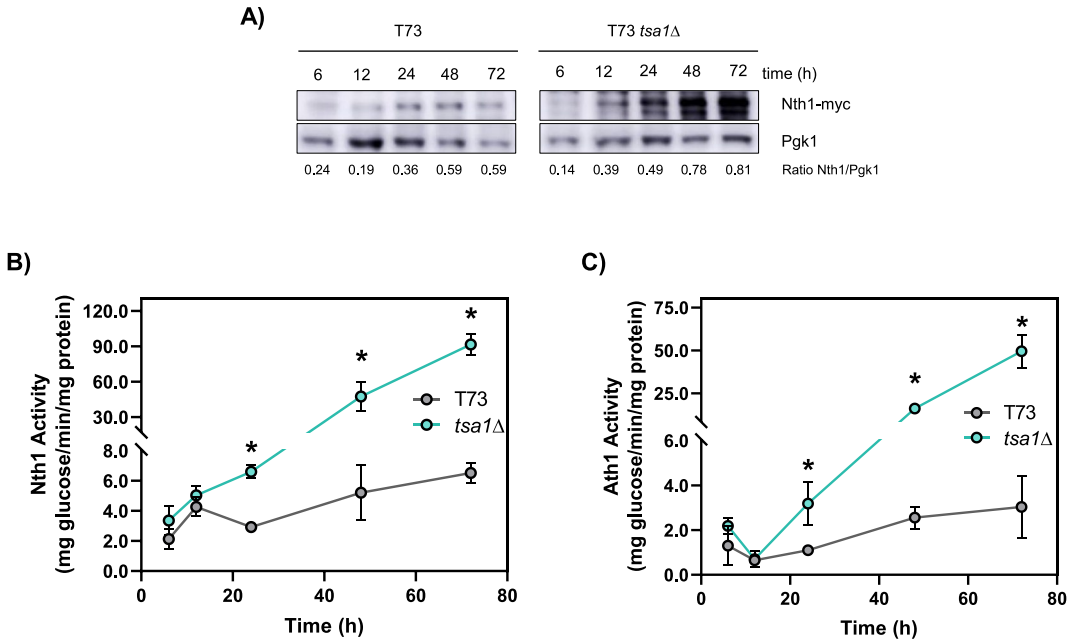

FIGURE 5 | Analysis of the Impact of Tsa1 on Nth1 and Ath1 in molasses. Biomass propagation in flasks with 6% (w/v) sucrose molasses was conducted using the T73 and T73 *tsa1*Δ strains, which included the 13myc-tagged versions of Nth1 for monitoring (A) Nth1 protein levels throughout the propagation process. (B) Nth1 activity and (C) Ath1 activity were measured from T73 and T73 *tsa1*Δ (non-tagged Nth1 strains) cultures in molasses. The experiments were carried out in triplicate, and the average and standard deviation are provided. Significant differences (*$p < 0.05$, Student's *t*-test) between the *tsa1*Δ mutant and the wild-type strain at each time point are shown.

higher than in the wild-type strain. These results suggest that Tsa1 represses the activity of both neutral and acidic trehalases in a coordinated way in post-diauxic conditions.

3.5 | Regulation of Trehalose Metabolism by Tsa1 Is Maintained at a Semi-Industrial Scale

Biomass propagation in flasks is a useful approach for studying yeast growth in molasses under batch conditions and investigating the metabolic changes that take place following the diauxic shift. However, at an industrial scale, once the yeast metabolises the ethanol produced through respiration, the process transitions to a fed-batch phase. During this stage, controlled feeding and aeration of the bioreactor or fermentation tank are implemented to maintain yeast respiratory metabolism and maximise biomass production.

In this work, we found that during biomass propagation in flasks, yeasts were unable to respire the ethanol produced due to insufficient aeration (Figure 2). This underscores the need to scale the process up to a bioreactor to study the impact of Tsa1 on trehalose metabolism under enforced respiration and during the transition from batch to fed-batch stages. Using a 5 L bioreactor, we reproduced the biomass propagation process in molasses on a semi-industrial scale. As shown in Figure 6A, both the wild-type and the *tsa1*Δ mutant strain achieved optical densities (OD at 600 nm) up to four times higher than those observed during flask-based growth. Analysis of the growth curve revealed that *TSA1* deletion resulted in a small growth defect, consistent with previous observations in flask cultures (Figure 1A) and bioreactor using synthetic molasses as a substrate (Garrigós et al. 2020). This growth defect became evident at approximately 10 h, during the exponential growth phase, likely due to the reduced efficiency in carbon source uptake and delayed establishment of fermentative metabolism of the mutant. This was reflected in a very slight delay in sucrose consumption and subsequent lag in ethanol production (Figure 6B), although the overall profile is highly similar. The growth defect became more pronounced in the post-diauxic phase, once sucrose was depleted. As shown in Figure 6B, yeast cells adopted a respiratory metabolism after 24 h to metabolise the ethanol produced during fermentation. Under optimal aeration conditions, both wild-type and *tsa1*Δ strains completely consumed ethanol by 48 h, transitioning into the fed-batch phase, which continued until 72 h. During the *fed-batch* stage, no ethanol production was observed, confirming

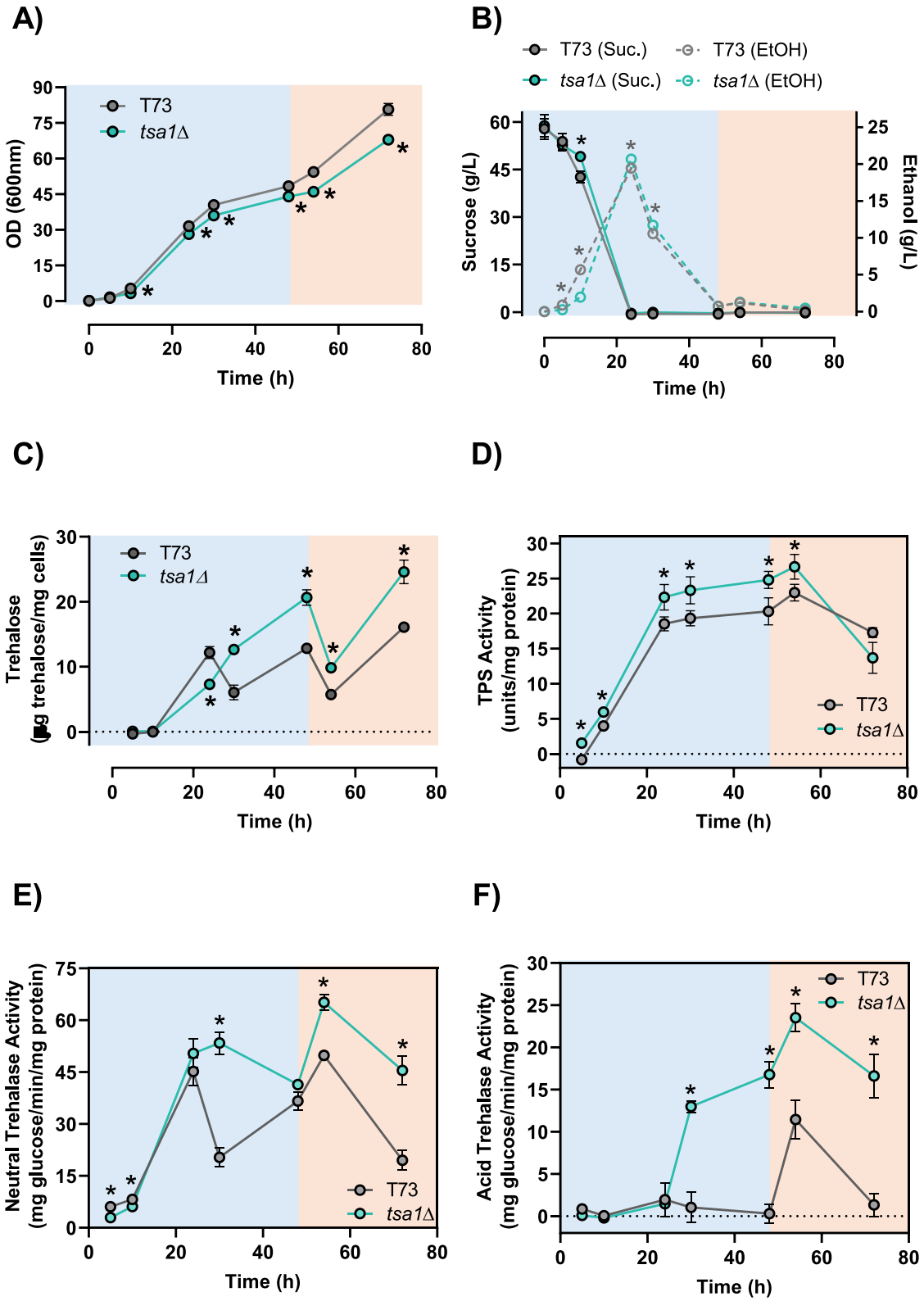

FIGURE 6 | Validation of Tsa1 as a regulator of trehalose metabolism in bioreactor. The T73 and T73 *tsa1*Δ strains were cultured using a batch/fed-batch approach. The batch phase employed 6% (w/v) sucrose molasses (blue background), while the fed-batch stage, which started after 48 h, used 10% (w/v) sucrose molasses (orange background). (A) Growth measured as OD_{600}. (B) Sucrose consumption and ethanol production. (C) Intracellular trehalose accumulation. (D) Trehalose-6-phosphate synthase (TPS) activity. (E) Nth1 activity. (F) Ath1 activity. The measurements were carried out in triplicate, and the average and standard deviation are provided. Significant differences (*$p < 0.05$, Student's t-test) between the *tsa1*Δ mutant and the wild-type strain at each time point are shown.

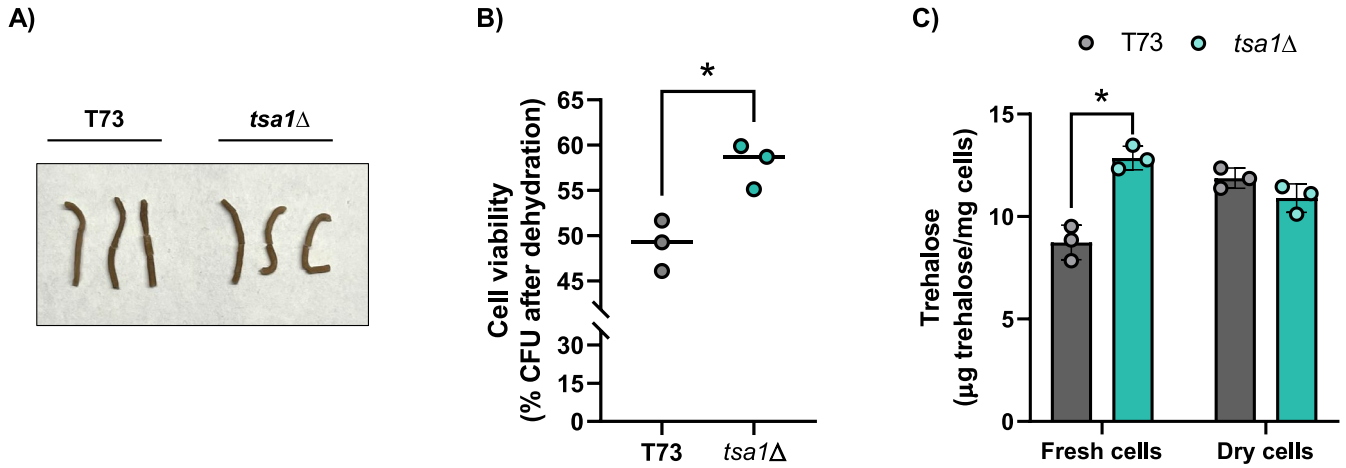

FIGURE 7 | Effect of *TSA1* deletion in ADY production. (A) Appearance of Active Dry Yeast at 8% moisture content. (B) Cell viability of ADY, considering 100% the cell viability of wet biomass. (C) Intracellular trehalose content from fresh and dry cells after growth in flasks, using 6% (w/v) sucrose molasses as the substrate, and comparing T73 and T73 *tsa1*Δ mutant strains. The experiments were carried out in triplicate, and the average and standard deviation are provided. Significant differences (*$p < 0.05$, Student's *t*-test) between the *tsa1*Δ mutant and the wild-type strain are shown.

that both strains maintained their respiratory metabolism. These results demonstrate that proper aeration in the bioreactor not only maximised biomass production in both strains but also allowed detailed analysis of their respiratory metabolism. Importantly, these findings confirmed that Tsa1 has a more pronounced impact during the post-diauxic growth phase, highlighting its role in detoxifying reactive oxygen species (ROS), a by-product of respiration.

To study the involvement of Tsa1 in trehalose metabolism under bioreactor growth conditions, we focused on analysing key enzymatic activities, as previous results indicated that protein abundance alone was not a reliable indicator (Figure 4). Regarding trehalose levels, the wild-type strain accumulated more intracellular trehalose during the first 24 h, likely due to its earlier consumption of sucrose (Figure 6C). However, once respiratory metabolism was fully activated, the *tsa1*Δ mutant exhibited higher intracellular trehalose levels, with concentrations 1.6-fold higher than wild-type at 48 h and 1.5-fold higher at the end of the process. This increase was likely driven by enhanced TPS activity in the *tsa1*Δ mutant throughout growth (Figure 6D). Although TPS activity was higher in the *tsa1*Δ mutant, its overall profile was similar to that of the wild-type strain. As the yeast fermented sucrose from the medium, TPS activity increased until it stabilised at 24 h, coinciding with the transition to respiratory metabolism, and remained stable until the end of the batch phase. At the onset of the fed-batch phase, both the wild-type and *tsa1*Δ strains displayed a slight increase in TPS activity, which then significantly decreased by 72 h when the yeast experienced complete nutrient depletion. Further analysis of neutral and acidic trehalase activities revealed that the deletion of *TSA1* led to the derepression of these enzymes in the bioreactor, mainly under respiratory conditions. At the start of the batch phase, Nth1 activity was slightly higher in the wild-type strain. However, this difference disappeared after 24 h, when both strains had consumed the available sucrose and shifted to respiratory metabolism (Figure 6E). At 30 h, while the wild-type strain experienced a decrease in neutral trehalase activity, the *tsa1*Δ mutant maintained levels up to 2.6-fold higher. Throughout the fed-batch phase, both strains exhibited an initial increase in neutral trehalase activity upon detecting sucrose in the medium, followed by a decline after 72 h, coinciding with nutrient depletion. Nevertheless, Nth1 activity remained significantly higher in the *tsa1*Δ mutant during the fed-batch stage. In contrast, Ath1 activity peaked at 51 h in the wild-type strain during the fed-batch phase (Figure 6F). The *tsa1*Δ mutant, however, exhibited a derepression of acid trehalase activity from 30 h onward, reaching levels 13-fold higher than the wild-type strain. Ath1 activity remained high in the mutant throughout respiratory metabolism, peaking at 51 h. Despite this, trehalose levels increased again at the end of the process, reaching their maximum in both strains. These findings confirm that Tsa1 regulates trehalose metabolism on a semi-industrial scale by controlling both its synthesis and degradation activities.

3.6 | Tsa1-Dependent Trehalose Accumulation Confers Increased Resistance to Desiccation

Multiple studies have demonstrated the critical role of trehalose in yeast survival during the dehydration process (Garre et al. 2010; Tapia and Koshland 2014; Tapia et al. 2015). Additionally, evidence confirms the induction of oxidative stress response genes during yeast dehydration, underscoring the importance of redox defence systems (Pérez-Torrado et al. 2005; Garre et al. 2010). As *TSA1* deletion increased intracellular trehalose levels, we aimed to determine whether Tsa1 plays a more significant role as an antioxidant protein or as a repressor of trehalose accumulation during dehydration.

To address this question, both the T73 wild-type strain and the *tsa1*Δ mutant were grown in molasses using flask cultures, and cells were harvested after 24 h of growth (Torrellas et al. 2020). To simulate the industrial dehydration process, a bench-top fluid bed dryer was used to produce ADY with approximately 8% residual humidity (Figure 7A). Cell viability after dehydration was then assessed. The *tsa1*Δ mutant displayed viability values upwards of 58%, which was higher than the 49% of viable cells in the wild-type strain (Figure 7B). This was probably due to the higher levels of trehalose present in the mutant before

drying, with a slight mobilisation in the dried cells that equalised the trehalose levels to those of the T73 strain (Figure 7C). These results suggest that trehalose during dehydration is more relevant than the oxidative stress defence provided by the cytosolic peroxiredoxin Tsa1.

4 | Discussion

Since wine production is a seasonal process, modern oenology requires large quantities of wine yeast starters, typically of the species *S. cerevisiae*, for massive and timely use after harvest. To ensure the stability of this yeast biomass during long-term storage, the desired wine strains undergo a biomass propagation process followed by dehydration to produce ADY (Gonzalez and Morales 2022). As a result, wine yeasts must adapt, both physiologically and metabolically, to three biotechnological processes involving different environmental and nutritional conditions: biomass propagation, drying, and wine fermentation (Matallana and Aranda 2017). There is extensive knowledge on the behaviour of the *Saccharomyces* strains during wine fermentation (Moimenta et al. 2023; Minebois et al. 2021; Vallejo et al. 2020b) and, to a growing extent, during dehydration (Calahan et al. 2011; Gamero-Sandemetrio et al. 2014, 2019). However, studies on the yeast's behaviour and response during biomass production remain scarce, prompting our group to focus on this process for years. During this stage, yeast cells are exposed to intense oxidative stress, which damages cellular components and leads to a variety of detrimental effects, including protein carbonylation, lipid peroxidation, DNA damage, and a reduction in antioxidant molecules such as reduced glutathione (Morano et al. 2012; Gómez-Pastor et al. 2012). To counteract this stress, yeast relies on its endogenous antioxidant defence systems. In this context, the overexpression of oxidative stress response genes, such as *TRX2*, has been explored as a strategy to enhance tolerance to oxidative stress, leading to increased biomass yields and improved ADY fermentative performance (Gómez-Pastor et al. 2010b, 2012). Alternatively, the supplementation of food-grade oils with established antioxidant properties, such as argan oil, has also been tested during yeast biomass propagation, resulting in reduced oxidative damage and enhanced fermentative capacity of the yeast (Gamero-Sandemetrio et al. 2019). Trehalose has also been demonstrated to be an effective protective agent against oxidative damage (Herdeiro et al. 2006; Landolfo et al. 2008). Recent studies from our group have demonstrated that the cytosolic peroxiredoxin Tsa1 influences trehalose accumulation during yeast biomass propagation in molasses (Garrigós et al. 2020). Understanding the regulatory mechanisms controlling trehalose metabolism in wine yeasts under industrial conditions can be used as a potential biotechnology improvement tool. Therefore, in this work, we analysed the molecular causes behind this regulation.

In this study, we performed a temporal dissection of the role of Tsa1 in regulating enzymes involved in trehalose metabolism during wine yeast biomass propagation in molasses. Our results revealed variability between wine strains and growth conditions. Metabolic differences between strains were expected, given the differential activation of their signalling pathways (Vallejo et al. 2020a; Scott et al. 2023) and the cytosolic peroxiredoxin-thioredoxin-thioredoxin reductase system itself (Garrigós et al. 2025). Furthermore, in line with our results, variability in intracellular trehalose levels has been observed among *S. cerevisiae* wild strains, which led to studying the contribution of the enzymes involved in both its synthesis (Chen et al. 2022) and degradation (Chen et al. 2024). At the same time, the variability observed across different substrates and growth conditions was also unsurprising. On one hand, during growth on diluted molasses (2% (w/v) sucrose), the yeast experienced lower initial osmotic stress compared to 6% (w/v) sucrose molasses, which may contribute to trehalose accumulation (Pérez-Torrado et al. 2005; Eleutherio et al. 1997). On the other hand, flask agitation did not replicate the aeration levels achieved in the bioreactor, leading to differences in oxidative stress derived from respiratory metabolism. This, in turn, impacts both the cytosolic thioredoxin system and trehalose synthesis genes (Gómez-Pastor et al. 2010a). Therefore, conducting a preliminary screening of genetic backgrounds and culture conditions was of great relevance to optimise the analysis of the impact of Tsa1 on trehalose metabolism.

Molasses 6% (w/v) in sucrose was the culture medium that resulted in the most homogeneous phenotype among wine strains in terms of trehalose accumulation, and has previously been shown to simulate at laboratory scale the results obtained in the production industry (Garre et al. 2010). Generally, in shake flasks, *TSA1* deletion led to higher levels of intracellular trehalose during yeast biomass propagation (Figure 1B), consistent with semi-industrial scale bioreactor results (Figure 6C). Trehalose is a multifunctional molecule that enhances tolerance to desiccation (Tapia et al. 2015). In fact, high trehalose levels during yeast propagation have been identified as a marker of improved yeast performance after dehydration (Gamero-Sandemetrio et al. 2014). The *tsa1*Δ mutant demonstrated greater viability after dehydration (Figure 7B), despite being exposed to elevated ROS levels (Garrigós et al. 2020) and increased protein oxidative damage (Figure 4D,E). This finding indicates that under these conditions, trehalose provides greater protection than the antioxidant function of Tsa1, suggesting that trehalose may have additional roles, for instance, acting as a chemical chaperone preventing cytosolic protein aggregation (Tapia and Koshland 2014). Furthermore, the *tsa1*Δ mutant may compensate for the peroxiredoxin deficit by activating other antioxidant systems, such as those based on glutathione (Garrigós et al. 2020).

Tsa1 influence in metabolism has been described to be direct, modulating specific metabolic enzymes, or indirect, controlling signalling pathways. Previous studies demonstrated an example of direct regulation of Tsa1 on intracellular trehalose accumulation by repressing pyruvate kinase activity, redirecting metabolic flux towards gluconeogenesis (Irokawa et al. 2016). However, we observed the opposite phenotype and did not detect the influence of Tsa1 on pyruvate kinase under conditions of biomass propagation in molasses (Figure 3 and Figure S2). Therefore, Tsa1 may be influencing trehalose metabolism indirectly through other pathways, such as protein kinase A (PKA)-dependent regulation. This kinase belongs to the Ras/cAMP/PKA pathway, which responds to glucose availability (Conrad et al. 2014). Under high glucose conditions, PKA becomes active and phosphorylates several targets, such as Nth1, enhancing its activity, or inhibiting others, including the general stress

response transcription factors Msn2/4, thereby repressing the expression of STRE-dependent genes like *TPS1*, *TPS2* or *NTH1* (Estruch 2000). Interestingly, it has been described that Tsa1 interacts with the regulatory subunit (Bcy1) and one of the catalytic subunits (Tpk1) of PKA, decreasing its activity (Roger et al. 2020; Kritsiligkou et al. 2021). Based on these findings, it could be hypothesised that the deletion of *TSA1* might lead to reduced intracellular trehalose levels due to increased PKA activity, although direct evidence is lacking. This indirect mechanism could also account for the observed accumulation of glycogen, as PKA regulates the expression of *GSY1/2* genes, which encode enzymes involved in glycogen synthesis, through the transcription factors Msn2/4. Another plausible explanation for the increased trehalose and glycogen levels in the *tsa1*Δ mutant is the potential accumulation of UDP-glucose, a shared precursor for the biosynthesis of both protective carbohydrates. All these observations raise several intriguing questions: Could the molecular mechanisms involving Tsa1 in laboratory strains differ from those in wine strains? Could molasses influence the regulatory role of Tsa1?

Alternatively, we demonstrated that Tsa1 influences the activity of key enzymes involved in trehalose synthesis and degradation during yeast biomass propagation, both in flask cultures and at a semi-industrial scale (Figures 4–6). Previous studies had highlighted the genetic interaction of *TSA1* with the genes encoding the trehalose-6-phosphate synthase (*TPS1*) and phosphatase (*TPS2*) (Hanzén et al. 2016), as well as its protein interaction with Tps2 and the regulatory subunits of the trehalose synthase complex (Tps3, Tsl1) (Seisenbacher et al. 2025). The fact that there is higher oxidation of the biosynthetic enzymes in the *tsa1*Δ mutant suggests an unknown regulatory mechanism that may influence folding, aggregation or another similar way to increase enzymatic activity that probably is based in the formation and release of disulfide bonds in key cysteines of such proteins (Figure 4). However, no direct interactions between Tsa1 and trehalases have been described. This limitation diverted the effort to investigate the oxidation state of Nth1 or Ath1. Although a direct modulation of enzyme activity cannot be completely ruled out, the most plausible explanation is that Tsa1 influences trehalase activity through an indirect mechanism. This is particularly evident for Ath1, whose distinct subcellular location makes direct regulation by Tsa1 unlikely, suggesting the possible existence of a shared indirect regulatory mechanism governing the activity of both Nth1 and Ath1. As Nth1 levels are increased in the *tsa1*Δ mutant (Figure 5B), an impact of *NTH1* gene expression by PKA modulation may be a suitable explanation. As mentioned above, our results revealed that *TSA1* deletion increased TPS activity, which likely explains the high levels of intracellular trehalose. Interestingly, the *tsa1*Δ mutant also exhibited increased neutral and acid trehalase activities. The coordinated expression of genes involved in both trehalose synthesis and certain degradation enzymes has been previously demonstrated under stress conditions (Parrou et al. 1997), pointing to the existence of a futile cycle of trehalose synthesis and degradation during heat shock (Hottiger et al. 1987) in which Tsa1 could be involved. This cycle, despite consuming energy, is believed to help the cells quickly adapt to environmental changes by balancing metabolic fluxes and maintaining cellular homeostasis. It allows the cell to quickly shift metabolic flux in either direction without needing to synthesise or degrade enzymes; therefore, it provides metabolic readiness for fluctuating conditions, such as nutrient availability or stress. As Tsa1 directly interacts with most synthesis enzymes, which are more abundant than trehalases (Ho et al. 2018), a pronounced and more direct derepression of trehalose synthesis may be occurring in the *tsa1*Δ mutant, thereby explaining its elevated intracellular trehalose levels. Moreover, the observation that *TSA1* deletion increases Ath1 activity in molasses, even in the absence of extracellular trehalose, suggests enhanced mobilisation of intracellular trehalose to the vacuole, where it is degraded by the acid trehalase. Overall, this study underscores the role of Tsa1 as a novel regulator of trehalose metabolism during the biomass propagation of *S. cerevisiae* wine strains in molasses. This regulatory function of Tsa1 is achieved by modulating the activity of enzymes involved in both trehalose synthesis and degradation. The accumulation of trehalose caused by *TSA1* manipulation causes an increase of cell viability after drying (Figure 7). That is a technological improvement that can lead to more robust yeast starters in the form of ADY that would improve the speed of grape juice fermentation. Understanding this process at the molecular level may help develop strain selection methods or strain improvement by Adaptive Laboratory Evolution that may lead to yeast starters with improved biotechnological performance. Further work to unveil the mechanisms of action of Tsa1 will be addressed in the future.

Author Contributions

Víctor Garrigós: conceptualization, formal analysis, investigation, validation, visualization, writing – original draft, writing – review and editing. **Emilia Matallana:** conceptualization, supervision, project administration, funding acquisition, writing – review and editing. **Cecilia Picazo:** conceptualization, supervision, funding acquisition, writing – review and editing. **Agustín Aranda:** conceptualization, supervision, formal analysis, funding acquisition, writing – review and editing, project administration.

Acknowledgements

The authors would like to acknowledge the financial support from the Spanish Ministry of Science and Innovation through grant PID2021-122370OB-I00 (co-financed by FEDER funds) to A.A. and E.M., from the Generalitat Valenciana through Emergentes grant number CIGE/2023/32 to C.P., and from the Cost Action TRANSLACORE CA21154 to C.P. V.G. was supported by a predoctoral grant from the University of Valencia (Atracció de Talent Program), and C.P. was supported by a Maria Zambrano postdoc contract (ZA21-068) from the Spanish Ministry of Universities.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.