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Chromatin conformations of *HSP12* during transcriptional activation in the *Saccharomyces cerevisiae* stationary phase

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ABSTRACT

During evolution, living cells have developed sophisticated molecular and physiological processes to cope with a variety of stressors. These mechanisms, which collectively constitute the Environmental Stress Response, involve the activation/repression of hundreds of genes that are regulated to respond rapidly and effectively to protect the cell. The main stressors include sudden increases in environmental temperature and osmolarity, exposure to heavy metals, nutrient limitation, ROS accumulation, and protein-damaging events. The growth stages of the yeast *S. cerevisiae* proceed from the exponential to the diauxic phase, finally reaching the stationary phase. It is in this latter phase that the main stressor events are more active. In the present work, we aim to understand whether the responses evoked by the sudden onset of a stressor, like what happens to cells going through the stationary phase, would be different or similar to those induced by a gradual increase in the same stimulus. To this aim, we studied the expression of the *HSP12* gene of the HSP family of proteins, typically induced by stress conditions, with a focus on the role of chromatin in this regulation. Analyses of nucleosome occupancy and three-dimensional chromatin conformation suggest the activation of a different response pathway upon a sudden vs a gradual onset of a stress stimulus. Here we show that it is the three-dimensional chromatin structure of *HSP12*, rather than nucleosome remodeling, that becomes altered in *HSP12* transcription during the stationary phase.

1. Introduction

Oxidative and metabolic stress are two relevant conditions to which *S. cerevisiae* cells are exposed during the stationary phase (Gasch and Werner-Washburne, 2002). Due to the progressive exhaustion of nutrients, the accumulation of damaged mitochondria and proteins, and the abundant ROS production occurring at this phase, yeast cells need to activate adaptive responses to ensure maximal cell survival (Werner-Washburne et al., 1993; Kaeberlein, 2010; Longo and Fabrizio, 2012). Among several highly specific stress response pathways (Gasch et al., 2000), the Environmental Stress Response (ESR) appears to be particularly effective against the major alterations that occur during the stationary phase (Werner-Washburne et al., 1993; Mager and De Kruijff 1995). Overall, during the stationary phase, there is a massive transcriptional shutdown (Choder, 1991) and a hundredfold decrease in protein synthesis rate compared to the exponential phase (Fuge et al., 1994). In addition, environmental stress can directly influence the expression of more than 900 genes, with an up-regulation of approximately 300 genes and a down-regulation of almost 600 genes (Gasch et al., 2000;

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Causton et al., 2001). In this complex scenario, many stress-related genes that directly drive the recovery from metabolic and oxidative stress are transcriptionally up-regulated (Werner-Washburne et al., 1996). Among the genes induced during the stationary phase, the HSP (heat shock proteins) family is one of the most relevant for the stress response (Verghese et al., 2012). Many heat shock proteins function as chaperones, preventing proteins from misfolding when subjected to stressful conditions such as heat, oxidative stress, and metabolic stress (Craig et al., 1993; Walter and Buchner, 2002). *S. cerevisiae* Hsp12p is a 109 amino acid amphipathic protein, encoded by a 330 bp long gene on chromosome VI, and it localizes to the plasma membrane (Sales et al., 2000) where it contributes to maintaining membrane fluidity (Welker et al., 2010; Herbert et al., 2012). The *HSP12* gene transcription is finely tuned to ensure a fast response to stress conditions. Its promoter contains two Heat Shock Elements (HSEs) characterized by nGAAn sequences, which are recognized and bound by Hsf1p, a specific factor activated upon phosphorylation (Ruis and Schüller, 1995). The *HSP12* cis and trans heat shock elements activation occurs following damaged or misfolded protein accumulation (Peffer, et al., 2019). In addition to HSEs, seven Stress Response Elements (STREs) are located upstream of the *HSP12* transcriptional start site (TSS; Kobayashi and McEntee, 1993; Ruis and Schüller, 1995). They control *HSP12* transcription by binding to Msn2/Msn4 (Moskvina et al., 1999) transcription factors, which redundantly control *S. cerevisiae* response to stress exposure, with their activity regulated by different partners (Sadeh et al., 2011). This allows maximizing the transcriptional stress response following different environmental stimuli (Boy-Marcotte et al., 1998).

During gene activation, the involvement of various regulatory strategies (Ruis and Schüller, 1995), as well as numerous transcription factors (Angel and Karin 1991; Hinnebusch and Natarajan 2002; Okazaki et al., 2007), is accompanied by chromatin changes (Erkina et al., 2008). It has been reported that many stress-related genes that undergo strong transcriptional induction display alterations in the three-dimensional (3D) organization of chromatin. This 3D arrangement of genomes occurs through complex chromatin folding. Different 3D interactions have been identified: (i) "chromatin loops" between gene elements consistently far from each other (e.g., enhancer and its promoter) and (ii) "gene loops" between elements occurring more closely on a gene, e.g., promoter and terminator sequences of a defined gene. Regarding *HSP12*, 3D alterations linked to gene transcription following heat shock have been described (Chowdhary et al., 2017). We therefore intended to study *HSP12* transcription during the stationary phase, with a particular focus on the associated chromatin changes.

2. Materials and Methods

2.1. Culture media and conditions

Yeast cultures were grown according to standard protocols (Sherman et al., 1983). YNB minimal medium (1% Yeast Nitrogen Base, 2% glucose, 1% Trp, 1% His, 1% Leu, 1% Ura) was used to grow all strains.

2.2. S. cerevisiae strains

WT: Matα; URA 3–52; TRP 1–289; HIS 3-Δ1; LEU 2–3112; gal2; gal10 *top1Δ*: Mat α; URA 3–52; TRP 1–289; HIS 3-Δ1; LEU 2–3112; gal2; gal10; TOP1::KAN

2.3. Primers

For gene expression analysis:

ACT1F: 5′ACGTTCCAGCCTTCTACGTTTCCA
ACT1R: 5′AGTCAGTCAAATCTCTACCGGCCA
*HSP12*F: 5′AGTCATACGCTGAACAAGGT
*HSP12*R: 5′CGTTATCCTTGCCTTTTTCG

For 3C loop analysis:

HSP12-47R: 5′-TTCAGAAGCTTTTTCACCGAATC
HSP12 + 279 F: 5′-AAAAGGCAAGGATAACGCTGAAG
HSP12 + 449 F: 5′-TTTTCTTTATGATGTGTGATGTTCC
ARS 504 F: 5′-GTCAGACCTGTTCCTTTAAGAGG
ARS 504 R 5′-CATACCCTCGGGTCAAACAC

For H3 occupancy-ChIP analysis:

NUC6 F: 5′-TCAGATGAAAGATGAATAGACATAGGA
NUC6 R: 5′-AAAGTAACATCCCAATGCGG
HSP12 –47F: 5′-ACGTATAAATAGGACGGTGAATTGC
HSP12 -47 R: 5′-TTCAGAAGCTTT

2.4. RT-qPCR analysis

Cells were grown in YNB minimal medium, starting from the exponential phase (0.5 O.D./ml), with a 24-h interval (24, 48, 72 h). A total of 7.5×10^7 cells were harvested at the exponential phase (d0), after 24 (d1), 48 (d2), and 72 (d3) hours. RNA was isolated using the method described by (Verdone et al., 1996). Briefly, 1.5 μg of RNA was treated with DNase I and then reverse transcribed using the iScriptTM cDNA synthesis kit following the manufacturer's instructions. One microliter of the resulting cDNA was amplified using a Mini Opticon Real-time PCR system (Bio-rad) with Sso Advanced SYBR Green supermix (Bio-rad) and specific primer pairs (*HSP12* F/R or ACT1 F/R) to detect the expression of *HSP12* or *ACT1*, respectively. The Ct values of *HSP12* RNA were normalized to those of *ACT1*.

2.5. Chromosome Conformation Capture

The Chromosome Conformation Capture (3C) assay was performed as described (Chowdhary et al., 2020) with minor modifications. After growth in YNB minimal medium, yeast cells (5.2×10^8 cells) were cross-linked at each timepoint (d0, d1, d2, d3) by adding 1% formaldehyde and incubating for 15 min at 30 °C with continuous shaking. Glycine (135 mM) was then added to stop the reaction for 5 min at 30 °C. Cells were centrifuged at 3500 rpm, washed with ice-cold TBS containing 1% Triton X-100, and resuspended in FA lysis buffer (Chowdhary et al., 2020). The cells were lysed with glass beads, and the chromatin mixture was divided into + L ("Ligated" samples), -L ("Digested only" samples), and Und ("Undigested" samples, not shown). For + L and -L samples, chromatin digestion was performed using 100 U of Taq I (approximately 1/10 of the total initial cells). The reaction mix was incubated at 60 °C for 7 h with continuous flipping. Taq I inactivation was performed with 10% SDS at 80 °C for 5 min. Chromatin was then diluted seven times to minimize inter-molecular contacts, and a final volume of 700 μl was adjusted with 10% Triton X-100 and H_2O as reported in (Chowdhary et al., 2020). After centrifugation, +L and -L samples were resuspended in 100 μl of 10 mM Tris-HCl (pH 7.5). The + L samples were subjected to intra-molecular ligation of cross-linked chromatin using T4 DNA Ligase (Thermoscientific) (10,000 units), and samples were incubated at 25 °C for 2 h. To all samples, H_2O was added to a final volume of 700 μl in the presence of 20 μg of DNase-free RNase A (30 min at 37 °C). The reaction was stopped by adding 3.5 μl of 20% SDS. Proteinase K (50 μg) was added to remove proteins (2.5 h at 56 °C). Samples were then incubated overnight at 65 °C to remove cross-linking. DNA was extracted twice with Phenol:Chloroform:Isoamyl alcohol (25:24:1) and once with Chloroform:Isoamyl alcohol (24:1). Finally, 95% ethanol precipitation was performed. Each sample was resuspended in 60 μl of RNase-free H_2O and subsequently amplified using Real-Time qPCR. The amplification was performed using sets of primers for the detection of two different intramolecular interactions at the *HSP12* gene: *HSP12*-47R/*HSP12* + 279F or *HSP12*-47R/*HSP12* + 449F. Pairs of primers were also used in divergent orientation for the detection of intramolecular interactions. Results were normalized to ARS504 (as in Chowdhary et al., 2017) signal amplified using convergent primers, and fold increases were plotted.

2.6. H3 chromatin immunoprecipitation

Cells were grown in YNB minimal medium and harvested at the exponential phase (0h-0.5 OD/mL) and after 24 h (d0), 48 h (d1), and 72 h (d2) of growth. The yeast cells at each time step were crosslinked by incubation with 1% formaldehyde at room temperature for 15 min. To stop the cross-linking reaction, 330 mM Glycine was added for 5 min. After centrifugation and removal of the supernatant, cells were lysed with HCl-treated glass beads in the presence of lysis buffer 140 (140 mM NaCl, 50 mM HEPES/KOH pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) and added with protease inhibitors (Complete EDTA-free Roche). Lysates were subjected to ultrasonic shearing to obtain DNA fragments with an average length of 500 bp. Chromatin was recovered in the supernatant after centrifugation at 13,000 rpm. Immunoprecipitation was then performed according to (Huang and Moazed, 2003). 350 μg of chromatin extracts were incubated with 3 μg of anti-H3 antibody (Millipore). Chromatin-antibody complexes were recovered using Dynabeads protein A (Invitrogen) according to the manufacturer's instructions. Subsequently, samples were extensively washed and eluted with 100 μl elution buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS). Two additional aliquots of chromatin extracts were treated: the negative IP-quality control BO ("beads only," without antibody incubation, not shown) and the reference control, whole-cell extract (W.C.E) (i.e., genomic sample, without incubation with either antibody or Dynabeads). Cross-linking was reversed by overnight incubation at 65 °C. After treatment with 70 μg of Proteinase K (56 °C, 2.5 h) and 35 μg of RNase A (37 °C, 30 min), DNA was purified using standard procedures. Samples were then amplified with a Mini Opticon Real-Time PCR system (BioRad) using *HSP12*-47 primer pair for promoter analysis. The average of the analyzed sequence after amplification reflects the amount of H3 protein present at the same region. *HSP12* promoter signals were normalized to NUC6 signal, the rDNA ARS-proximal region, which permanently hosts a nucleosome (Vogelauer et al., 1998).

2.7. Statistical analysis

All data are shown as mean and standard deviation calculated from at least three biological replicates. p-values were calculated using Student's t-test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

3. Results

Previous studies have reported the transcriptional induction of *HSP12* following heat shock, metabolic stress, and oxidative stress (Verghese et al., 2012; Peffer et al., 2019). The *HSP12* gene plays a crucial role in driving the stress response under these conditions.

Continuous exposure to stress conditions, such as those occurring during the stationary phase, may result in a prolonged transcriptional response that persists for several hours or days. It is conceivable that this response may employ different regulatory strategies compared to acute stress responses. Therefore, we investigated *HSP12* transcription during the stationary phase and the potential chromatin alterations occurring under these conditions.

3.1. HSP12 expression

We studied *HSP12* transcription during the stationary phase to determine the increase in its mRNA levels. RNA was prepared from yeast cells grown in YNB minimal medium for 24, 48, 72, and 96 h, corresponding to days 0, 1, 2, and 3, respectively. The mRNA levels of *HSP12* were measured by qRT-PCR during the exponential phase (day 0) and the stationary phases (days 1, 2, and 3), and the fold increase values were normalized to *ACT1*, which did not show significant changes under our experimental conditions. As shown in Fig. 1, we observed a gradual and continuous increase in *HSP12* mRNA levels along the stationary phase. In the exponential phase (day 0), *HSP12* mRNA levels were low. The transcript levels started to rise slowly at days 1 and 2, but the highest transcriptional response was observed at day 3, where they were 8-fold higher compared to day 0. These findings indicate that *HSP12* transcription is induced during the stationary phase and gradually increases over time.

3.2. H3 occupancy at HSP12 promoter

Since the stationary phase of *S. cerevisiae* and the oxidative stress response exhibit similar physiological responses (Werner--Washburne et al., 1993; Mager and De Kruijff 1995), we investigated whether there is a change in nucleosome positioning at the *HSP12* promoter during transcriptional induction (Fig. 1) in the stationary phase. Therefore, we conducted a ChIP analysis using an anti-H3 antibody to assess H3 binding at the *HSP12* promoter at the same time points as the transcriptional analysis (refer to Fig. 1 above). The samples were processed using the ChIP procedure (see Materials and Methods), and the immunoprecipitated DNA fragments were amplified via qPCR. To normalize the values to a region with a consistently bound nucleosome, we selected the NUC6 region at rDNA, which has been reported to be stably bound by nucleosome 6 in the mapped array (Vogelauer et al., 1998 and Materials and Methods).

Surprisingly, as depicted in Fig. 2, there is no significant alteration in H3 occupancy at the *HSP12* promoter during the stationary phase (d1-d3). Furthermore, H3 occupancy remains unchanged between the exponential phase (d0) and the stationary phase (d1-3).

3.3. HSP12 chromatin conformation during transcription activation

Previous studies have reported changes in histone acetylation and chromatin remodeling during gene activation (Erkina and Erkine, 2006; Erkina et al., 2008; Antonazzi et al., 2021). Additionally, alterations in the three-dimensional chromatin conformation of the HSP12 gene have been demonstrated following heat shock treatments (Chowdhary et al., 2017).

Given that H3 occupancy at HSP12 remains unchanged over time and does not contribute to substantial nucleosomal remodeling during the stationary phase (Fig. 2), we investigated whether there would be a change in the three-dimensional conformation of the

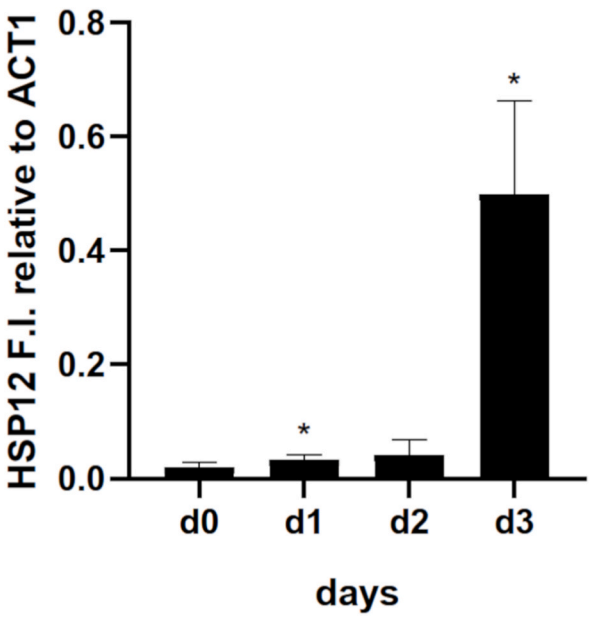

Fig. 1. *HSP12* expression in WT cells during exponential (d0) and stationary phase (d1-d3). Fold increase is relative to *ACT1* expression. Values, with standard deviation, are from three different biological replicates. *$p \leq 0.05$.

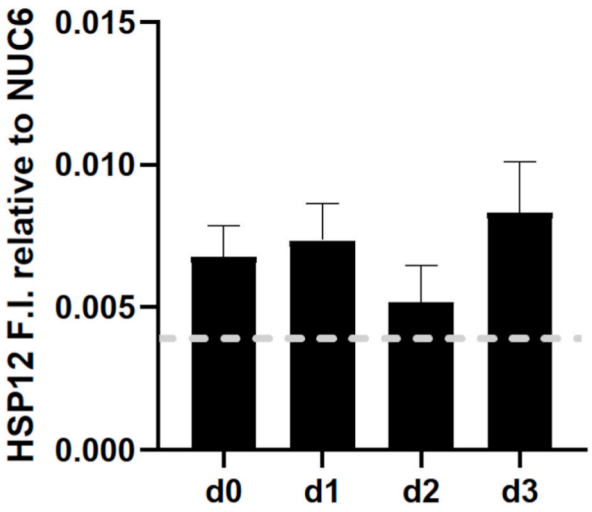

Fig. 2. ChIP analysis of H3 occupancy on the *HSP12* promoter at the exponential phase (d0) and during the stationary phase (d1-d3). Black histograms represent immunoprecipitated DNA, fold increase (F.I.) relative to NUC6. The grey dotted line corresponds to the average value of the whole cellular extract (W.C.E). DNA was amplified at the *HSP12* promoter and then normalized to the rDNA NUC6 signal. Values with standard deviation are from three biological replicates.

HSP12 gene as its expression gradually increases during chronological aging.

To examine this, we employed the Chromosome Conformation Capture (3C) technique to study the presence of intragenic interactions in *HSP12*. Yeast cells were subjected to formaldehyde cross-linking at specific time points (d0-d3). Subsequently, TaqI digestion, ligase treatment, and DNA recovery were performed (for detailed procedures, refer to Materials and Methods). As depicted in Fig. 3A, TaqI cuts the HSP12 DNA at three different positions (−47, +279, +449 from TSS, see map in Fig. 3, panel A). In the presence of three-dimensional proximities between linearly distant regions due to intramolecular interactions, amplification of otherwise divergent oligos becomes possible after the ligation step. The experiments were conducted following a protocol similar to

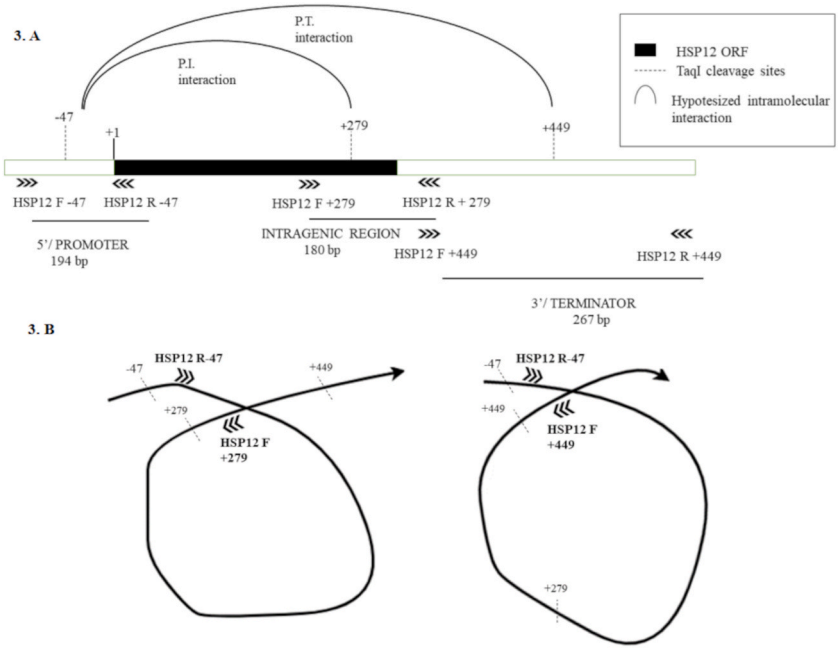

Fig. 3. (A) Schematic representation of primer positions in the *HSP12* gene and the hypothesis of 3D interactions. »» or «« indicate the primer positions and their direction. Dotted lines represent three cuts performed by the restriction enzyme TaqI: −47 corresponds to the promoter region; +279 is located within the intragenic sequence; +449 maps downstream of the *HSP12* termination. TSS = transcriptional start site. P–I represents promoter-intergenic region interaction; P-T represents promoter-termination region interaction. (B) Representation of hypothesized intramolecular interactions at *HSP12*. Primers in bold are those with a divergent orientation used for detecting P–I and P-T interactions.

(Chowdhary et al., 2020) with minor modifications (see Materials and Methods).

After extraction and purification, the samples were processed for qPCR using divergent primer pairs (HSP12 R-47/HSP12 F + 279 and HSP12 R-47/HSP12 F + 449, respectively, see map in Fig. 3, panel A). This enabled us to verify the presence of two possible interactions: a promoter-intragenic region interaction (P–I) and a promoter-terminator interaction (P-T) (illustrated in Fig. 3, panel B).

To confirm that amplification products are obtained only following restriction and ligation of the divergent oligos, we evaluated the qPCR products using agarose gel electrophoresis. Fig. 4, lane 1, shows the amplification obtained at day 0 using the divergent oligos located at the border between the promoter and intragenic region (refer to Fig. 3, panel B for map reference). Correct amplification was also observed when oligos located in the promoter and terminator regions were used (Fig. 4, lane 3). Both results likely indicate contiguity between the promoter and the intragenic and terminator regions, respectively. As a control, in the absence of ligation (-L samples; Fig. 4, lanes 2 and 4), no amplification was observed, suggesting that the contiguity was only detectable when ligation was performed.

The graphical representation of the qPCR analysis (Fig. 5) shows high levels of P–I interaction at day 0, which gradually decreased from day 1 to day 3. Simultaneously, we observed that P-T interaction was minimal and remained relatively stable during the stationary phase.

The values of the amplified fragments were normalized to ARS504, where no TaqI sites were present, using a pair of convergent primers (see Materials and Methods). The results presented in Fig. 5 demonstrate that during the stationary phase, the P–I loop, which was present in the exponential phase, gradually disappeared, while the P-T interaction, if present, remained stable.

3.4. *top1Δ mutant*

Previous studies have shown a global decrease in RNA levels during the stationary phase as a physiological mechanism during chronological aging (DeRisi et al., 1997; Gasch and Werner-Washburne, 2002). DNA topoisomerase I (*TOP1*) has been implicated in the global RNA shutdown observed in the stationary phase (Choder, 1991). Moreover, we studied additional genes exhibiting higher mRNA levels in *top1Δ* mutants during the stationary phase. This suggests that DNA topoisomerase I could function as a global transcriptional repressor at this phase (Table 1). DNA topoisomerase I is involved in the control of torsional stress (Capranico et al., 2017) and is essential for DNA replication (Cho and Jinks-Robertson, 2018) and transcription (Liu and Wang, 1987; Baranello et al., 2016) when regulation of supercoiling is required (Corless and Gilbert, 2017). It has also been found to have a noncanonical function as a scaffolding protein involved in the epigenetic regulation of rDNA stability (D'Alfonso et al., 2016; Di Felice and Camilloni, 2021).

Based on this evidence, we investigated *HSP12* transcription during the stationary phase in *top1Δ* mutant cells to assess whether TOP1 could contribute to the fine-tuning of *HSP12* mRNA levels observed in this growth phase.

Total mRNA was extracted from exponentially growing cells (day 0) and cells at different stationary phase time points (days 1–3). As shown in Fig. 6, *HSP12* mRNA levels increased rapidly in *top1Δ* cells during their growth, with a maximum observed at day 2 and a subsequent decrease at day 3. Compared to WT cells, *HSP12* gene expression was 13- to 17-fold higher in *top1Δ* cells during days 1 and 2 (compare WT expression shown in Fig. 1). Interestingly, despite the changes in mRNA levels, no nucleosome loss was observed at the *HSP12* promoter in the *top1Δ* mutant, similar to WT cells (not shown). To investigate the possible role of DNA topoisomerase I in the formation of tridimensional chromatin structures, we performed 3C analysis in the *top1Δ* mutant. The results showed that the P–I interaction was present at day 0 but decreased rapidly at day 1, while the P-T interaction was scarce and remained stable throughout the stationary phase (Fig. 7). These observations in the *top1Δ* mutant resembled those in WT cells, although the P–I interaction was 2-fold higher in *top1Δ* mutants compared to WT. Overall, both WT and *top1Δ* cells exhibited the disappearance of the P–I loop upon entering the stationary phase, albeit with more gradual dynamics in WT cells than in the *top1Δ* mutant. The second loop involving the

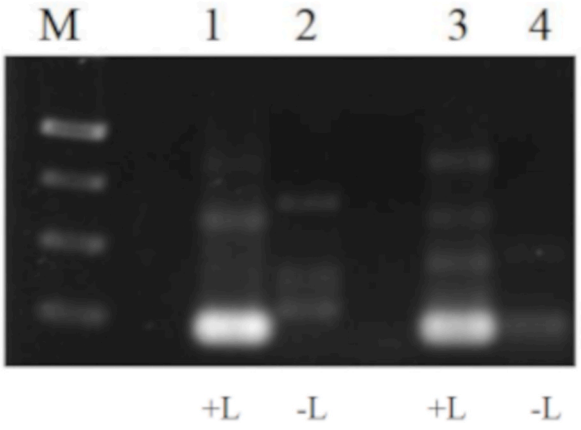

Fig. 4. Qualitative image of amplicons generated by RT-qPCR. Amplicons 1 and 3 correspond to P–I and P-T interactions, respectively, and were detected in WT cells at d0; both samples are treated with DNA Ligase after TaqI restriction. Amplicons 2 and 4 correspond to P–I and P-T interactions, respectively, in WT cells at d0; both samples are digested with TaqI only. M = marker; 1000, 750, 500, and 250 bp.

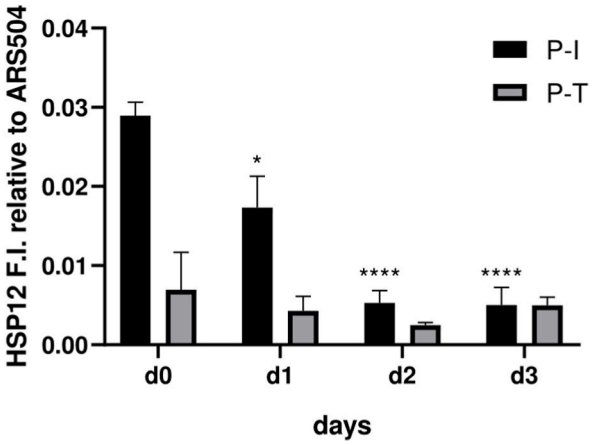

Fig. 5. *HSP12*P–I (promoter-intragenic region) and P-T (promoter and terminator) interactions during the stationary phase. After restriction and ligation, samples were amplified using divergent primers to detect intramolecular interactions. Results were normalized to ARS504, which was amplified using convergent primers. Values with standard deviations are from three biological replicates. *$p \leq 0.05$, ****$p \leq 0.0001$.

Table 1
Fold increase ratio between mRNA values measured in top1Δ and WT cells during the maximal expression in stationary phase (day 1,2,3). Fold increase for ATG8 and KGD1 relative to ACT. Values are from three biological replicates.

Genes upregulated during the exponential and stationary phase (by qRT-PCR)		Fold increase top1Δ/WT (max. expression in stationary phase)
Negative control	ACT	1058
Stress-induced genes	HSP12	17,658
	ATG8	7410
	KGD1	2178

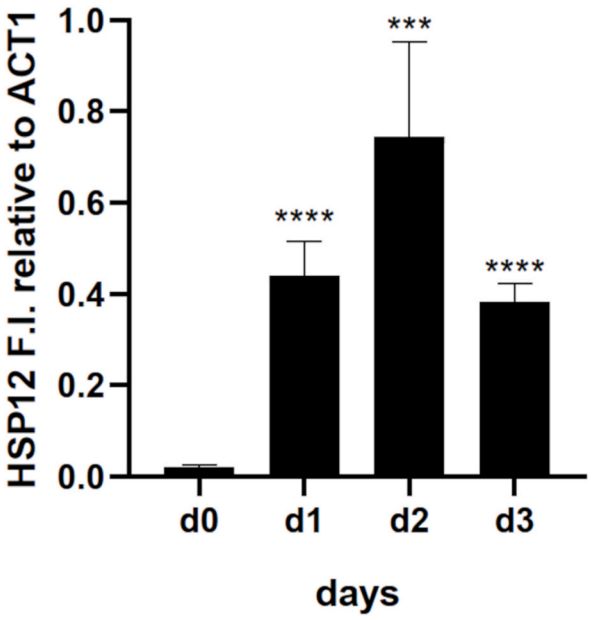

Fig. 6. *HSP12* expression in *top1Δ* mutants during the exponential phase (d0) and the stationary phase (d1-d3). Quantification is normalized to *ACT1* expression. Values with standard deviations are from three biological replicates. ***$p \leq 0.001$, ****$p \leq 0.0001$.

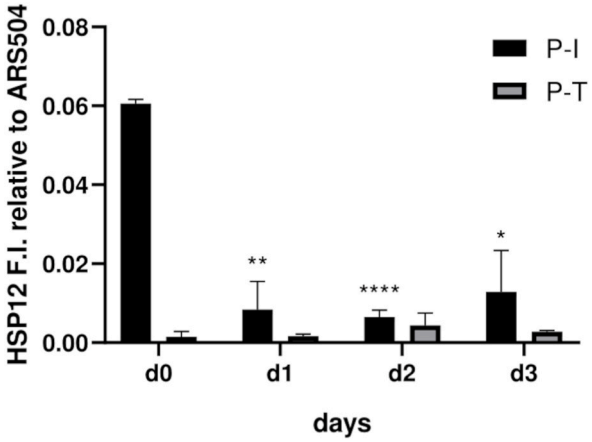

Fig. 7. *HSP12*P–I (promoter-intragenic region) and P-T (promoter and terminator) interactions during the stationary phase in top1Δ mutants. After restriction and ligation (when performed), samples were amplified using divergent primers to detect intramolecular interactions. Results were normalized to ARS504 as described in 38. Values with standard deviations are from three biological replicates. *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001.

promoter and terminator regions seemed to be almost absent, and its kinetics of disappearance could not be assessed.

4. Discussion

In yeast, various regulatory strategies are activated to respond to environmental stress, including the transcriptional induction of heat shock proteins such as *HSP12* (Verghese et al., 2012; Peffer et al., 2019). During the stationary phase of *S. cerevisiae*, a complex interplay occurs among nutrient exhaustion, compromised proteostasis, and ROS accumulation (Werner-Washburne et al., 1996), representing the burden of stress stimuli that cells experience during chronological aging. Due to the overlapping features between the responses to general environmental stress (heat shock, oxidative and metabolic stress) and those occurring during the stationary phase, we investigated common regulatory mechanisms could be involved. *HSP12* is a major regulator of the environmental stress response, and its activation is driven by Hsf1p and Msn2/4p, involving chromatin modifications (Erkina and Erkine, 2006; Sadeh et al., 2011). In this study, we examined whether *HSP12* undergoes similar activation during the stationary phase and observed a gradual increase in *HSP12* mRNA accumulation as growth progressed up to three days. The gradual nature of *HSP12* transcriptional activation distinguishes it from the response to single stimuli, such as heat shock, which typically triggers sudden transcriptional induction.

As previously mentioned, *HSP12* transcriptional activation following heat shock involves chromatin remodeling and three-dimensional arrangements. A previous study demonstrated that *HSP12* induction is highly dependent on SWI/SNF recruitment at the *HSP12* promoter (Erkina and Erkine, 2006), while a Gcn5-dependent mechanism was observed during oxidative stress exposure (Antonazzi et al., 2021). To investigate whether substantial chromatin rearrangements also occur during the stationary phase, we initially studied the occupancy of the +1 nucleosome at the *HSP12* promoter during the exponential and stationary phases. ChIP analysis of H3 presence revealed that nucleosome displacement is not directly involved in driving *HSP12* activation during the stationary phase, as there was no significant drop in histone content or occupancy at the promoter during the time points studied (Fig. 2). This observation suggests that the stationary phase-dependent transcription of *HSP12* does not rely on evident chromatin remodeling processes.

However, it was reported that Hsf1p drives the formation of tridimensional intramolecular and intermolecular interactions at HSP genes during heat shock (Chowdhary et al., 2019). Therefore, we wondered if similar chromatin conformations could be detected when *HSP12* becomes activated during the stationary phase. Through 3C experiments, we identified two types of intragenic interactions at *HSP12*: the promoter-intragenic region (P–I) and the promoter-termination region (P-T) interactions, which behave differently during the stationary phase. Specifically, the P–I interaction undergoes a progressive disappearance, while the P-T interaction does not show significant changes as the stationary phase progresses. Based on these data, we hypothesize that the P–I interaction plays a primary role in determining an open or closed chromatin state in relation to gene activation. Initially, the P–I interaction may be associated with a closed chromatin conformation that restricts optimal accessibility to the *HSP12* promoter by RNA polymerase II, as suggested by the low expression of the *HSP12* gene at day 0. As the P–I interaction decreases, *HSP12* mRNA levels increase. These findings suggest a repressive role for the P–I interaction and support a model in which enhanced transcription of *HSP12* during the stationary phase is caused by the gradual loss of this three-dimensional interaction.

Although many stress-related genes, including *HSP12*, are up-regulated during the *S. cerevisiae* stationary phase, the global mRNA levels are consistently lower compared to the exponential phase (Choder, 1991). DNA topoisomerase I appears to play a role in this process by driving global transcriptional repression during the stationary phase (Choder, 1991). This led us to investigate how *HSP12* expression is affected in *top1Δ* mutants. Our results demonstrate that the lack of topoisomerase I is associated with a more rapid and significant increase in *HSP12* mRNA during the stationary phase compared to WT cells. This finding prompted us to explore the

biological mechanism underlying the enhanced *HSP12* expression in *top1Δ* cells. Since +1 nucleosome occupancy does not appear to change during *HSP12* induction in both WT cells (Fig. 2) and *top1Δ* mutants (not shown), we focused on the same tridimensional intramolecular interactions examined previously. Similar to WT cells, we observed through 3C analysis that the P-T loop is hardly detected in *top1Δ* mutant cells at the analyzed time points. Conversely, the P–I interaction appears to be higher in the exponential phase compared to WT cells, and it undergoes an even faster decrease as the stationary phase progresses in *top1Δ* mutants. We hypothesize that the more rapid decrease of the P–I interaction in *top1Δ* cells could explain the sudden activation of *HSP12*, revealing a topoisomerase I-dependent mechanism of gene repression. In contrast, we observed that the P-T interaction does not change during *HSP12* induction, even in the absence of topoisomerase I, suggesting that this intramolecular interaction is not relevant for the regulation of *HSP12* activation.

In conclusion, we have demonstrated that in WT cells, *HSP12* mRNA increases during the stationary phase due to the emergence of various stress conditions, and this regulation occurs without significant nucleosomal rearrangements at the promoter. Furthermore, we have shown that under the same conditions, gene expression is increased in *top1Δ* mutants with different kinetics, indicating that DNA topoisomerase I plays an important role in controlling *HSP12* activation. Additionally, we have identified the occurrence of intramolecular interactions between the promoter and the intragenic region. The formation of this loop appears to play a crucial role in keeping *HSP12* transcription shut off during the exponential phase, while its disappearance during the stationary phase allows for gene induction. Thus, we suggest that a too-close proximity conformation may limit promoter accessibility. Moreover, we have shown that the contribution of topoisomerase I to regulating *HSP12* involves stress-induced chromatin rearrangements in three dimensions, emphasizing how this enzyme can influence many biological processes in response to stress conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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