Role of the stress-dependent MAP kinase Sty1 and the transcription factor Atf1 in transcription regulation in fission yeast

Miriam Sansó Martínez

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Trabajo dirigido por la Dra. Elena Hidalgo
Oxidative Stress and Cell Cycle Group
Departament de Ciències Experimentals i de la Salut
Programa de Doctorat en Ciències de la Salut i de la Vida
Universitat Pompeu Fabra

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Ha sido tan divertido habernos encontrado,
ha sido tan divertido que me hayas acogido,
ha sido tan divertido habernos disfrazado,
ha sido tan divertido reírnos de lo mismo.

Ha sido divertido hacer mil planes juntos,
Ha sido divertido que me hayas contagiado,
Ha sido divertido habernos insultado,
ha sido divertido, sí.

Ha sido divertido que me hayas enseñado
que tú tienes un camino que ya no cabe en el mío.
Ha sido en contra tuya que me hayas conocido,
ha sido muy extraño todo lo que nos ha sucedido...

NudoZurdo
Sintética, 2008

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ABSTRACT

In *Schizosaccharomyces pombe*, the MAPK pathway Sty1 is activated upon several stress situations, like osmotic and oxidative stress, stationary phase, UV radiation or heat shock. Since the modulation of gene expression is one of the main outputs of this response, we focused this Thesis work on the charactherization of the transcription regulation by the activation of the Sty1 pathway and through the transcription factors Atf1 and Pcr1. Moreover, we extend our field of interest investigating how stress–related chromatin remodelers are affecting the stress defence transcription of the cells.

En *Schizosaccharomyces pombe,* la vía de la MAPK Sty1 es activada ante diferentes situaciones de estrés, como son el estrés oxidativo u osmótico, fase estacionaria, radiación UV o choque de calor. Al ser la modulación de la expresión génica uno de los más importantes objetivos de esta respuesta, hemos focalizado el trabajo de esta Tesis doctoral en la caracterización de la regulación transcripcional mediada por la activación de la ruta de Sty1 y los factores de transcripción Atf1 y Pcr1. Además, hemos ampliado nuestra área de interés investigando el papel de remodeladores de cromatina relacionados con la respuesta a estrés y cómo a participan en la transcripción estrés-dependiente.

PROLOGUE

Single-cell organisms have to cope with a wide range of environmental fluctuations and exposition to a battery of toxic compounds. They have developed elaborate systems to sense variations in the intensity, concentrations or presence of such variables to adapt by applying the appropriate response, in order to survive and proliferate.

The definition of environmental stress uncovers any fluctuation of these variables, having an impact on the homeostasis and physiology of the organisms. The term environment includes both the external conditions where the organisms live and the internal environment of organisms where their metabolic reactions occur.

Schizosaccharomyces pombe is an ideal model to study stress responses due to the diversity of mechanisms it uses to respond to environmental stresses. In addition to a specific oxidative stress response (as found in bacteria), relying on the Pap1 transcription factor and the Tpx1 sensor, this yeast holds specific factors involved in the response to different kinds of stresses, like heat shock, heavy metals, etc. Moreover, like in mammals, the fission yeast possesses a general stress response which relies on the activity of the MAPK Sty1. Although Sty1 is not essential for the growth of *S. pombe*, the activation of this pathway is crucial for cell survival after stress, and a very important part of this response is a change in the transcriptional programme.

At the beginning of this project, it was kwon that the stress-dependent transcriptional response depends on Sty1 and the transcription factor Atf1, however the role of its partner Pcr1 was controversial. In this thesis we have demonstrated that kinase activity of Sty1 is essential to survive upon stress. We have reported the Sty1-dependent recruitment of RNA polymerase II to the stress genes upon stress. Analysis of the participation of Pcr1 and Atf1 in osmotic stress-dependent transcription has revealed their similar but not identical roles. We have shown the

binding of both proteins before and after stress to the CESR genes. We have demonstrated that Pcr1 is not a target of Sty1 kinase activity, and only its bZIP is enough for a proper stress response. Moreover, we have studied the role of chromatin remodelers in the stress response, conferring an important direct function to the SAGA histone acetyl transferase Gcn5, which counteracts with the deacetylase Clr3. Finally, we report some evidences that Gcn5 not only acts in the transcription initiation of stress genes, but also promotes transcription elongation.

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INTRODUCTION

1. ENVIRONMENTAL STRESS

Single-cell organisms have to cope with a wide range of fluctuations, such as variations in nutrient availability, pH, temperature, osmolarity, ultraviolet (UV) irradiation, but also exposition to a range of toxic compounds. They have developed elaborate systems to sense fluctuations in the intensity, concentrations or presence of such variables to adapt by applying the appropriate response, in order to survive and proliferate.

The definition of environmental stress uncovers any fluctuation of these variables, having an impact on the homeostasis and physiology of the organisms. The term environment includes both the external conditions where the organisms live and the internal environment of organisms where their metabolic reactions occur.

Like unicellular organisms, multicellular eukaryotes are exposed to environmental stresses and have developed mechanisms to handle them. In every organ of the human body, cells must adapt to stress situations and return to homeostasis. The list of stress-related pathologies would be long, among them neurodegenerative diseases such as Alzheimer and Parkinson, are known to be accelerated by the production of reactive oxygen species (ROS) causing DNA damage, emphasizing the importance of studying deeply the mechanisms by which cells fight against stress (reviewed by Kyriakis and Avruch, 2001)

2. GENERAL STRESS RESPONSE

Cells thrive in an extremely flexible way under non ideal conditions. To understand how they achieve this task, it is essential to study the way they sense unfavourable scenarios, which signalling

pathways transmit this information within the cell, and what are the resulting compensatory changes in gene expression. The modulation of gene expression is one of the main outputs of this response, occurring both at transcriptional and post-transcriptional levels.

2.1. CROSS-PROTECTION

It has been described that the exposure to low levels of a specific stress can trigger an adaptive response resulting in a transient resistance to higher levels of the same stress. This adaptive response is short-lived and requires new protein synthesis, and protein down-regulation, indicating that changes in gene expression are critical to achieve it. Interestingly, this adaptive response is not only limited to the same stress but also protects the cell against apparently unrelated negative stimuli. This phenomenon, known as “cross-protection”, suggests that common defence mechanisms are able to provide a basal level of protection, leading to the concept of a general stress response (Gasch *et al*., 2000).

2.2. CORE ENVIRONMENTAL STRESS RESPONSE

Global approaches have been decisive to elucidate the molecular responses of cells to diverse environmental stresses. Whole genome transcriptional profiles using microarrays have opened the door to understand how globally the cells stay in tune with the environmental conditions. Whole genome approaches revealed that the transcriptional profiles of the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) and fission yeast *Schizosaccharomyces pombe* (*S. pombe*) show a transcriptional response that is common in the majority of stress conditions (Causton *et al*., 2001; Gasch *et al*., 2000). This core response

is largely conserved between these two distantly related yeast species. The transcripts which are similarly up regulated in these different conditions involve genes related with carbohydrate metabolism, detoxification of ROS, protein folding and degradation, vacuolar and mitochondrial functions, autophagy, and metabolite transport. Conversely, the genes that are commonly repressed are related with energy consumption, growth-related processes, including RNA processing, transcription and translation, biosynthesis of ribosomes and nucleotides. This stereotypical response has been termed as "environmental stress response" (ESR) (Gasch *et al*., 2000; Causton *et al*., 2001) or as "common environmental stress response" (CESR) (Chen *et al*., 2003) in the budding yeast and fission yeast, respectively.

To regulate their core stress response, cells use different strategies, involving differential regulation of signal-transduction pathways and translating extracellular signals into specific intracellular responses. To implement this specific response, the stress-specific Mitogen Activated Protein Kinase (MAPK) pathways are the most important key regulators.

3. MAP KINASE PATHWAYS

Deciphering the mode of action of signal transduction pathways is a major area of interest in modern molecular biology. One of the most important signal transduction routes is represented by the MAPK pathway, which structure is highly conserved from yeast to humans. MAPK pathways are responsible of transducing a range of different external stimuli (environmental stresses, hormones, growth factors, and cytokines) in order to elicit an appropriate response, including a wide range of cellular functions, such as differentiation (cell growth and morphogenesis), proliferation, apoptosis and adaptation to environmental stress.

3.1. A CONSERVED MOLECULAR ARCHITECTURE: THE MAP KINASE MODULE

MAPKs represent a large family of proline-directed, serine/threonine kinases requiring the phosphorylation of tyrosine (sometimes serine) and threonine in the T/S-x-Y motif of their N-terminal activation loop by a dual-specificity MAPK kinase (MAPKK). MAPKK in turn, are phosphorylated on a conserved serine and threonine by a MAPKK kinase (MAPKKK). This sequential phosphorylation has a highly conserved molecular architecture and is often referred to as the "MAP kinase module" (see Fig. 1).

MAPK cascades are activated by sensors (frequently located in the plasmatic membrane) through phospho-relay systems, G-coupled proteins or receptor-tyrosine kinases (in mammals). Once the MAPK is active, it translocates from the cytoplasm to the nucleus, where it phosphorylates different substrates charactherized by a serine/threonine followed by a proline residue. Its usual targets are transcription factors (TFs), as well as other kinases, cytoskeletal proteins and phospholipases (Roux and Blenis, 2004).

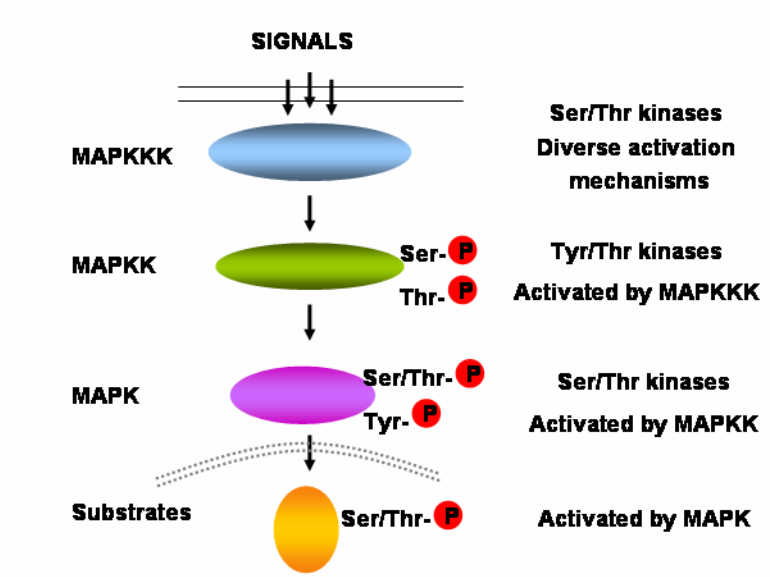


Figure 1. MAPK molecular architecture.

3.2. EFFICIENCY AND SPECIFICITY

MAPK modules are characterized by the presence of a single MAPK and various MAPKK and MAPKKK, ensuring on one side the crosstalk among different MAPK pathways (they share some module components), and on the other side allowing the activation of the same module by diverse stimuli trough different components. Moreover, to amplify the signal the single kinase protein levels are higher compared to the double and triple kinases in the cell.

The specificity and efficiency of MAPK are mainly due to the presence of scaffolding proteins binding to specific motifs. These scaffolds determine the sequence of physical interactions, insulating the components from inappropriate cross-talk or different localization, thereby helping a rapid signal transmission through the cascade. Not all the

modules have specific proteins exerting this function: some MAPK pathways use signalling components themselves as scaffolds.

In addition to scaffold proteins, other mechanisms ensure signal specificity, including sensing input systems and MAPK substrate specificity. Substrate selectivity is often conferred by specific MAPK docking sites present on physiological substrates, often at considerable distance from the phosphorylation site in the primary sequence. This mechanism allows for a strong interaction with selected MAPK subfamilies and exclusion of others (reviewed in Hohmann, 2002).

3.3. REGULATION BY PHOSPHATASES

Environmental stresses are variable in intensity and therefore cell response must fit with the particular levels of stress. The duration and magnitude of the activation is essential to determine the final outcome in the cell (Marshal, 1995), however, hyperactivation of a pathway can produce lethal effects. MAPK play a central role in preventing this situation and ensure an accurate intensity of signalling by modulating their phosphorylation status through different phosphatases: the same signal that activates the protein is the key point to switch off the cascade.

Three types of protein phosphatases can down regulate MAPKs: the Protein Tyrosine Phosphatases (PTPs), the Protein serine/threonine Phosphatases type 2C class (PP2C) and the Dual Specificity Phosphatases (DSPs), which remove the phosphate from Tyr, Ser or Thr residues. Some of them belong to the CESR response.

3.4. Stress MAP Kinases

MAPKs constitute a large and highly conserved protein family. However, not all members of this family are able to sense stress. For this reason, the members whose activity is triggered by stress are also known as Stress-Activated Protein Kinases or SAPKs. The end of this chapter intends to give a short overview on how lower and higher eukaryotes respond to stress through these class of kinases, and the specific substrates they regulate.

3.4.1. *Candida albicans*

A functional stress response machinery is crucial for the survival of this diploid yeast, which is the major systemic fungal pathogen affecting humans. Its success as pathogen is largely sustained by its resistance to adverse environments: inactivation of its stress response severely compromises its expansion capacity.

3.4.2.1. Hog1

C. albicans Hog1 is a MAPK playing a key role in response to multiple insults. It is the homologue of *S. cerevisiae* HOG1 and *S. pombe* Sty1. *C. albicans* Hog1 is activated by oxidative and osmotic stress, metals and the farnesol "quorum" molecule (San José *et al*., 1996; Alonso-Monge *et al*., 2003; Smith *et al*. 2004), promoting its phosphorylation and subsequent translocation into the nucleus, however only high doses of H_2O_2 are able to activate the kinase. A particularity of *C. albicans* is the way it responds to heat stress: instead of increasing its phosphorylation, a 23 to 37 ºC shift in temperature induces a decrease in Hog1 phosphorylation during the first hour post-stress (Smith *et al*., 2004). Hog1 activation by those stresses mounts a specialized core stress

response, what means that it is general and central (Smith *et al.,* 2004), similar to the one mediated by Sty1 in *S. pombe*. Although, recent studies have shown the role of alternative routes regulating this central response in *C. albicans* (Enjalbert *et al.,* 2006).

3.4.2. *Saccharomyces cerevisiae*

In the budding yeast *S. cerevisiae* different stresses control a core set of genes through different signalling pathways and TFs. *S. cerevisiae* has five different MAPK, two of which, SLT2 and HOG1, are implicated in stress responses.

3.4.1.1. SLT2

This kinase, also known as MPK1, is a component of the PKC pathway and belongs to the ERK family of protein kinases. It maintains cell wall integrity during heat shock, hypo-osmotic shock, cell wall damage and oxidative stress.

3.4.1.2. HOG1

The High Osmolarity Glycerol (HOG) pathway, is homonymous to its single kinase HOG1, a homologue of mammalian p38 MAPK. HOG1 is in charge of sensing osmotic shocks, and activation of the HOG pathway leads to a set of osmoadaptative responses, including metabolic regulation, cell cycle progression, translation and changes in gene expression.

The HOG1 MAPK module comprises three MAPKKK, SSK2, SSK22 (Maeda *et al*., 1995) and STE11 (Posas and Saito, 1997), which

activate the MAPKK PBS2. Once activated, PBS2 phosphorylates and activates HOG1, the MAPK of the pathway. The HOG pathway is principally activated by two independent mechanisms. The first one involves a two-component osmosensor composed by the SLN1-YPD1-SSK1 proteins, which activates SSK2 and SSK22. While the second mechanism acts through the transmembrane protein SHO1, the MAPKKK STE11, the STE11-binding protein STE50, the STE20 p21-activated kinase (PAK) and the small GTPase CDC42 (Posas *et al*., 1998; Maeda *et al*, 1995; reviewed in de Nadal *et al*, 2002).

3.4.3. Mammals

Five families of MAPK have been characterized in mammals: the Extracellular-Regulated Kinases (ERKs) ERK1 and ERK2, the Jun N-terminal Kinases (JNKs), the p38 MAPKs, ERK3 and ERK4, and ERK5. The more extensively studied MAPKs are ERK1/2, bearing the activation motif TEY and preferentially activated by growth; and the JNKs and p38 kinases, which are able to respond to stress conditions and harbour the TPY and TGY motifs respectively (Parson *et al.,* 2001; Roux and Blenis, 2004). We will briefly describe these last two.

3.4.3.1. JNK

The JNK pathway is strongly activated in response of ionizing radiation, oxidant stress, heat shock inflammatory cytokines, growth factors and reperfusion injury (Kyriakis and Avruch, 2001). Once the kinase is activated it translocates from the citosol to the nucleus. JNK is phosphorylated/activated by two different MAPKKs, MEK4 and MEK7, which activation patterns do not fully overlap, but cooperate. MEK4 and

MEK7 are phosphorylated by the MAPKKK MEKK1-4, MLK2 and 3, TPL-2, DLK, TAO1 and 2, TAK1 and ASK1 and 2. The repertoire of Ser/Thr kinases acting as MAPKKKs upstream of the stress-activated MAPKs is vast and diverse. This heterogeneity is consistent with the variety of different stimuli, which are able to trigger this MAPK pathway. The main JNK substrates are the TF c-Jun and ATF2 (Review by Johnson and Nakamura, 2007)

3.4.1.2. p38

Like JNKs, the p38 isoforms are strongly activated *in vivo* by environmental stresses and inflammatory cytokines and weakly by insulin and growth factors. In almost all instances, the same stimuli recruiting JNKs also recruit p38s (Kyriakis and Avruch, 1996). One exception is ischemia-reperfusion: while p38 is triggered during ischemia and maintained active during reperfusion, JNKs are activated only during reperfusion (Ginet, 2009).

The MAPKKs of this pathway are MEK3 and 6, while the MAPKKKs are MEKK3-4, TAO1, TPL-2 TAK1 and ASK1. Different isoforms of p38 have been identified, four isoforms are known exactly (p38α, β, γ, δ). One of the p38 substrates is the ATF2 transcription factor. The regulation of ATF2 by JNK or p38 is exerted at different levels. In basal conditions, ATF2 is transcriptionally inactive due to an intramolecular interaction between its DNA binding domain and the N-terminal region. This intramolecular inhibition is disrupted when ATF2 interacts with other proteins, such as E1A or c-Jun, or when it is phosphorylated by MAPKs, enhancing its transcriptional activity (Li and Green, 1996). Phosphorylation has also been shown to increase the activity of the isolated transactivation domain of ATF2, although the

mechanism involved remains unclear (Livingstone *et al*., 1995; Gupta *et al.,* 1995). Yet another mode of regulation involves ubiquitin-mediated degradation by the 26S proteasome: in that case, the phosphorylation of ATF2 by MAPKs protects it from ubiquitination and subsequent degradation (Moreno *et al.,* 1991, Firestein *et al*., 1998; Fuchs *et al*., 2000).

4. *Schizosaccharomyces pombe*, A MODEL SYSTEM FOR THE STUDY OF CELL CYCLE AND STRESS RESPONSE REGULATION

The fission yeast *S. pombe* is an excellent model to study stress responses and other multiple cellular processes (such as cell cycle regulation, splicing, etc.) at the molecular level. Some reasons for that are its facility of growing, maintenance and manipulation in the laboratory and its molecular similarity to higher eukaryotic cells. Moreover, the genome of *S. pombe* has been fully sequenced (Wood *et al*., 2002). Its genetic material is organised in just three chromosomes, containing less than 5000 genes scattered on approximately 14 Mb of total DNA.

These characteristics designate *S. pombe* as the smallest free-living eukaryote. As a result of the compactness of its genome the fission yeast contains few duplicated genes, making functional genetics easily amenable. Surprisingly though, *S. pombe* differs phylogenetically from *S. cerevisiae* as much as from humans. Finally, because *S. pombe* divides by bipartition, it is possible to assess the phase of the cycle in which the cell is or in which moment of the cell cycle the cell is arrested by simple nuclear staining followed by microscope examination.

4.1. *S. pombe* CELL CYCLE

4.1.1. Vegetative growth

In normal growth conditions *S. pombe* adopts a haploid mitotic cell cycle referred as vegetative growth. In these conditions *S. pombe* exhibits a standard cell cycle, in which DNA replicates during the S phase and in the course of the M phase chromosomes segregate and settle into two haploid nuclei. Both the S and M phases alternate separated by growth periods, known as G1 and G2. A peculiarity of *S. pombe* is that it undergoes cytokinesis just before next cycle's S phase. As a consequence, daughter cells remain together during nearly all the G1 phase, and G2 represents the longest phase of its cell cycle.

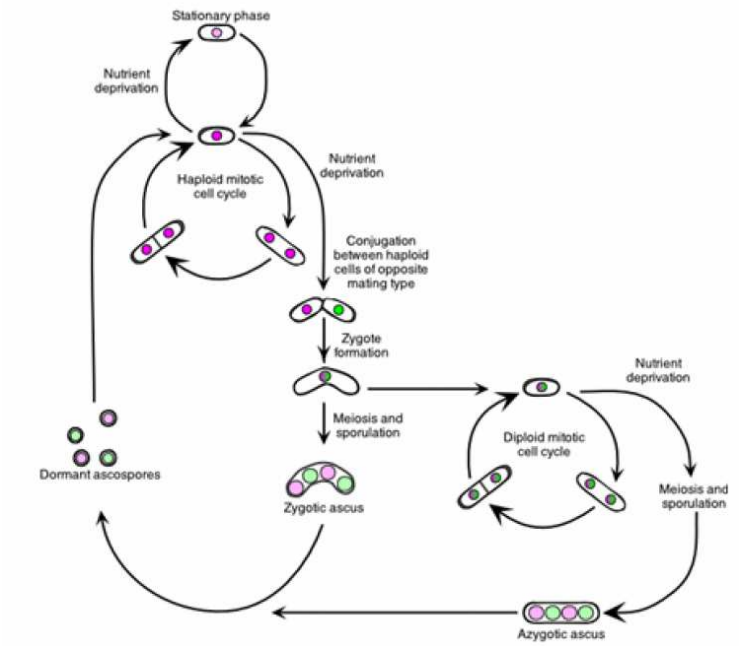


Figure 2. Complete cell cycle of *S. pombe*.

However, nutrient availability can modify this strategy of proliferation. When glucose is exhausted, fission yeast enters a quiescent stationary phase, while nitrogen starvation induces *S. pombe* to conjugate with a cell of the opposite mating type (M or h- and P or h+). Upon conjugation and if culture conditions turn out to favourable again, the cells can divide using a mitotic diploid phase, which represents however a very unstable state. Otherwise, if the environment continues to be unfavourable, diploid cells exit from mitosis to meiosis and form spores (Fig. 2).

4.1.2. Sexual reproduction

The exit from mitosis and the entrance in meiosis can be divided in two parts: the initiation of meiosis and the pheromone pathway.

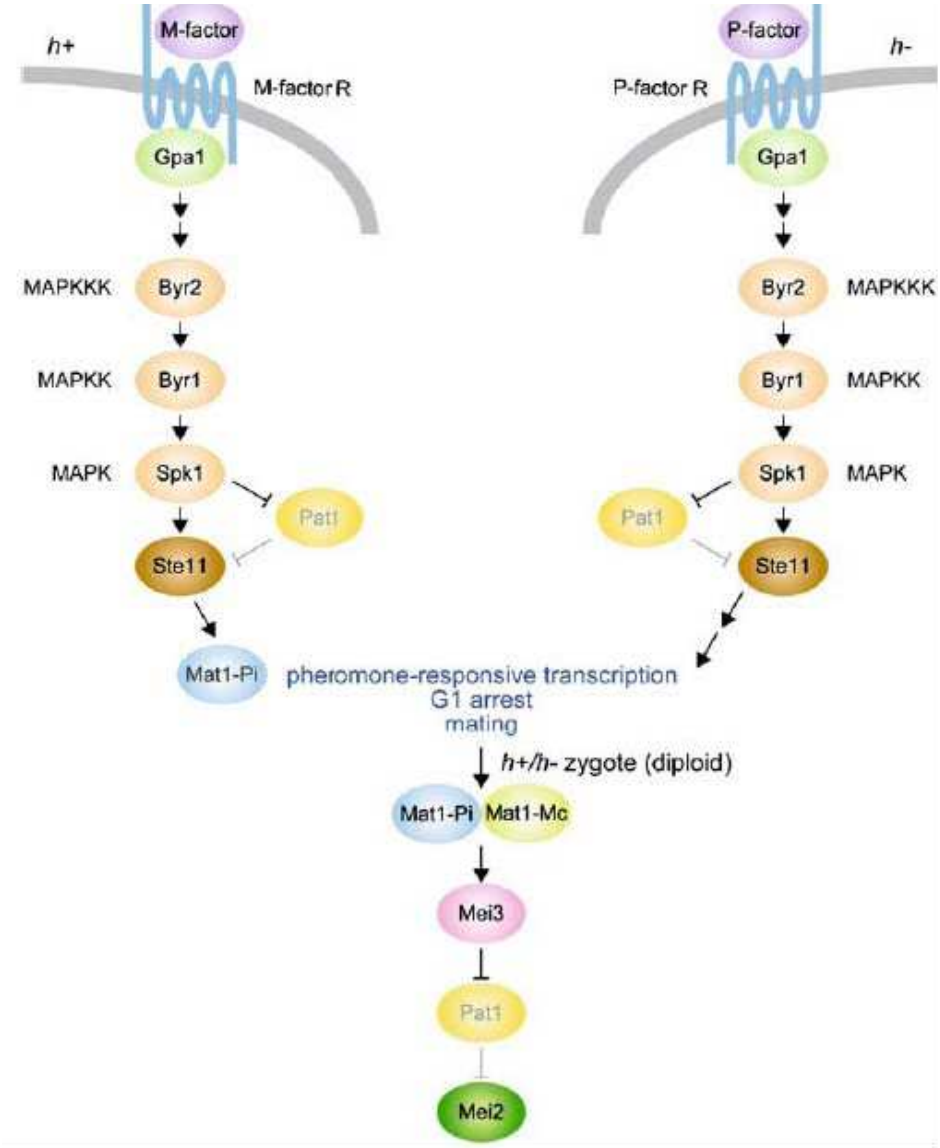


Figure 3. The pheromone pathway of *S. pombe*, initiation of meiosis. From Harigaya and Yamamoto, 2007

Initiation of meiosis. Nitrogen starvation results in G1 arrest and transcriptional activation of Ste11, a key regulator of the mitosis to meiosis switch (Maeda *et al*., 1990; Mochizuki and Yamamoto, 1992; Sugimoto *et al*., 1991). Both, cAMP-dependent Protein Kinase (Pka1) and the stress pathways are responsible for the induction of *ste11* expression. When Pka1 activity is reduced, its target, the TF Rst2 is dephosphorylated and becomes active allowing *ste11* up regulation (Higuchi *et al*., 2002). Alternatively, the Sty1-regulated TFs Atf1 and Pcr1 heterodimerize and activate *ste11* transcription. Furthermore, down-regulation of Pka1 and activation of Sty1 promote degradation of Cig2 B-type cyclin, resulting in delayed G1-S transition.

Pheromone pathway. This pathway is active when two opposite mating type cells, h+ and h-, conjugate. Binding of the opposite pheromone (P and M) to the receptor triggers a MAPK cascade (Byr2-Byr1-Spk1) resulting in *ste11* transcription activation and inhibition of the kinase Pat1 (Beach *et al*., 1985). After mating and conjugation, the sexual factors heterodimerize and promote *mei3* expression. Mei3 binds and inhibits the Pat1 kinase (McLeod and Beach, 1988) thereby preventing Mei2 from the inhibitory phosphorylation and triggering the initiation of meiosis (see Fig. 3).

The Ste11 TF activates numerous genes required for mating and meiosis, including *mei2*, which encodes an RNA-binding protein triggering meiosis. During vegetative growth, s*te11* transcripts are kept low, and the Pat1 kinase phosphorylates the Ste11 protein on Thr-173 and Ser-218, inhibiting its nuclear accumulation (Li and Mcleod, 1996). As Ste11 also regulates its own transcription (Kunitomo *et al*., 2000; Sugimoto *et al*., 1991), its nuclear exclusion mediated by Pat1 also contributes to keep low levels of the Ste11 protein. Under nitrogen starving conditions and

pheromone signalling *ste11* is up regulated, Pat1 inhibited and the Ste11 protein is dephosphorylated (reviewed in Harigaya and Yamamoto, 2007)

Meiosis consists in a specialized cell cycle composed by one S phase followed by two successive M phases. This strategy allows the formation of spores, a specialized cell type, which purpose is to resist in unfavourable environmental conditions. When the conditions improve, spores germinate and re-enter into a mitotic haploid cell cycle.

4.2. FISSION YEAST SPECIFIC STRESS RESPONSES

S. pombe is an ideal model to study stress responses due to the diversity of mechanisms it uses to respond to environmental stresses. In addition to a specific oxidative stress response (as found in bacteria), relying on the Pap1 TF and the Tpx1 sensor, this yeast holds specific factors involved in the response to different kind of stresses, like heat shock, heavy metals, etc. Moreover, like in mammals, the fission yeast possess a general stress response which relies on the activity of the MAPK Sty1; this type of response is presented in section 4. 3.

4.2.1. Pap1

S. *pombe* AP-1 TF, Pap1, is the homologue of yeast YAP1, *C. albicans* CAP1, and mammalian Jun, and is physiologically activated by low levels of ROS. The activation of Pap1 is regulated by nuclear localization: Pap1 is constantly imported to the nucleus and exported by interaction with the nuclear exportin Cmr1 via its C-terminal domain. In presence of low levels of H_2O_2, Pap1 suffers a conformational change due to the formation of a disulfide bond, preventing its interaction with Cmr1 (Vivancos *et al*., 2006). As a consequence, Pap1 accumulates into the

nucleus and its target genes are transcribed (Castillo *et al*., 2002). The targets of Pap1 include genes encoding for detoxifying proteins, like *ctt1* (catalase), *trx2* (thioredoxin), *trr1* (thioredoxin reductase), *pgr1* (glutathion reductase) and *tpx1* (thioredoxin peroxidase); and membrane transporters as ABC family proteins *hba2/bfr1* and *pmd1*. All these genes are part of the cellular adaptative response to oxidants. The ability of Pap1 to be activated by low levels of oxidants prepares the cell to survive to higher degrees of this stress (Quinn *et al.,* 2002).

4.2.2. Prr1

The *prr1* gene encodes the homologue of *S. cerevisiae* SKN7 TF. It is part of a two-component response system, which most likely acts as a direct transcriptional regulator of some oxidative stress response genes, independently of the Sty1 and Pap1 pathways (Omiya *et al*., 1999; Omiya *et al.,* 2000 and Buck *et al*., 2001). This signalling mechanism is common in bacteria but rarely found in eukaryotes. It has been identified in fungi (Santos & Shiozaki, 2001), plants (Urao *et al.,* 2001), but not in animals.

4.2.3. Zip1

Zip1 is a basic leucine zipper TF, playing a critical role in cadmium stress transcriptional response, by preventing the loss of viability in cells exposed to this heavy metal. *zip1* mutants are hypersensitive to cadmium. In wild type cells, cadmium exposure results in Zip1 up-regulation, leading to growth arrest and reduced cell size (Harrison *et al*., 2005).

4.2.4. Hsf1

The mechanism of heat shock response in *S. pombe* still remains elusive. The fission yeast possesses a highly conserved response to elevated temperature, resulting in the synthesis of a set of specific proteins called heat shock proteins (HSPs; Lindquist and Craig, 1988; Morimoto *et al*., 1994). In eukaryotic cells this response involves the activation of an essential TF named heat shock factor (HSF; Wu, 1995). The phosphorylation and oligomerization of HSF allows the stress-induced transcription of responsive genes by interaction with a highly conserved DNA sequence, the heat shock element (HSE; Amin *et al*., 1988).

In *S. pombe* Hsf1 is an essential TF (Gallo *et al.,* 1991). It is involved in heat shock response, but is also related to other kind of stresses. Its activation occurs posttranslationally in response to heat shock, but Hsf1 is also activated by cadmium (Gallo *et al.,* 1991; Saltsman *et al*., 1999). Interestingly, genes induced by other kind of stresses present putative HSEs in their promoters. This is the case for the CESR gene *ntp1*, even though this gene is primarily regulated by other factors such as the general stress response TFs Atf1 and Pcr1 (Paredes *et al*., 2004).

4.3. FISSION YEAST GENERAL STRESS RESPONSE: THE Sty1 PATHWAY

The MAPK Sty1 (also known as Phh1/Spc1) is closely related to mammalian p38 and like its homologue, responds to high osmolarity stress, heavy metals, heat shock, cold shock and nutrient limitation, as well as to a variety of agents inducing oxidative stress, such as H_2O_2,

paraquat, arsenite, alkylating agents and UV radiation (Millar *et al*., 1995; Shiozaki and Russell, 1995; Degols *et al*., 1996; Shiozaki and Russell, 1996; Degols and Russell, 1997). Although Sty1 is not essential for the growth of *S. pombe*, the activation of this pathway is crucial for cell survival after stress.

Mutants defective in *sty1*, or in other components of the pathway, are hypersensitive to ROS and the other stress agents listed above. The MAPK pathway is also related with cell cycle regulation. Accordingly, cells lacking *sty1* have problems in the G2-M transition, displaying a long cell size phenotype, which is even more severe under stress conditions (Shiozaki and Russell, 1995). Finally, *Δsty1* cells are almost sterile due to a markedly reduced induction of *ste11* (the master regulator of sexual differentiation in fission yeast, see section 4.1.2.) (Kato *et al.,* 1996).

4.3.1. Cascade components

A His-to-Asp phosphorelay system acts upstream of the Sty1 pathway. This relay is composed of three histidine kinases (Mak1/Phk3, Mak2/Phk1 and Mak3/Phk2) acting as sensors of the different types of insults; one phosphorelay protein (Spy1/Mpr1) (Oayama *et al*., 2000); and two response regulators (Mcs4 and Prr1) (Aoyama *et al*., 2001). It is still unclear how the sensor histidine kinases recognize and respond to different types of stress. However, several lines of evidence suggest that the phosphorelay protein Spy1 is activated by phosphorylation and negatively regulates the response regulator Mcs4 by binding and transferring its phosphate group. Finally, the anchor of the phosphorelay protein, Mcs4, binds to the MAPKKK and initiates the activation of the Sty1 cascade (see Fig. 4).

The MAPK module is composed of the MAPK Sty1, which is activated by the MAPKK Wis1 phosphorylating Sty1 at Thr-171 and Tyr-173 residues, and two MAPKKK Wak1 (also known as Wis4) and Win1, which are functionally redundant. Their only reported substrate is Wis1, which becomes phosphorylated in Ser-469 and Thr-437 (Shiozaki and Russell, 1996; Wilkinson *et al*., 1996; Samejima *et al.,* 1997; Shiozaki *et al*., 1997).

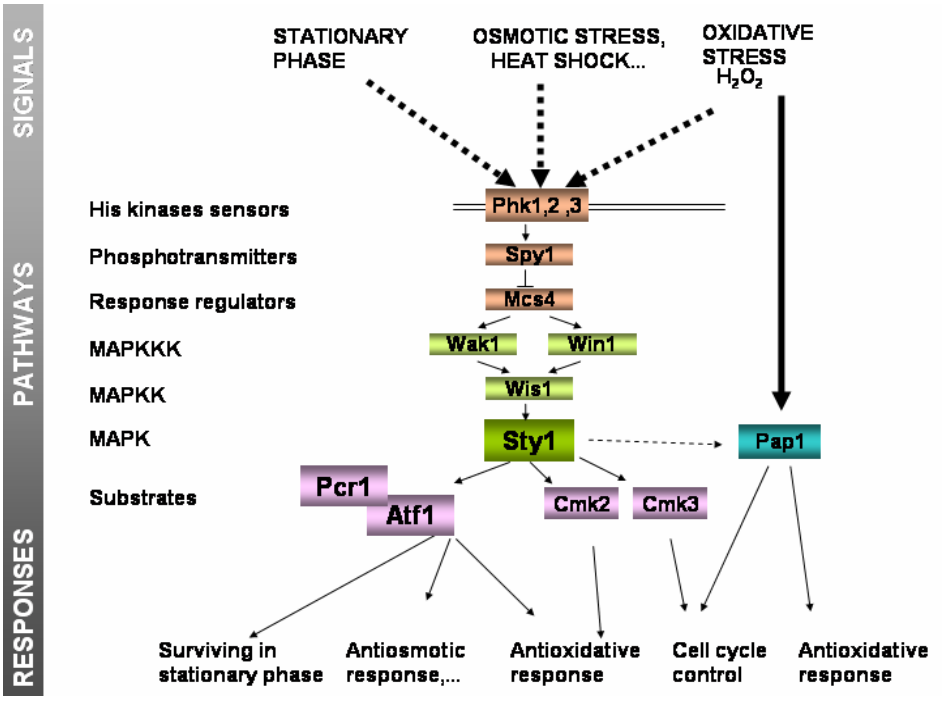


Figure 4. *S. pombe* stress response pathway.

4.3.2. Signals and physiological responses

4.3.2.1. Stress activates CESR, inhibits protein synthesis and alters cell cycle

The MAPK pathway regulates the global stress response, a stress-specific transcriptional program called CESR. This pathway enables the cell to sense multiple stressors in a dose-dependent manner (see section 4.2.3.). In response to those stressors, Sty1 triggers the CESR, but also down regulates the synthesis of proteins not directly involved in stress response and alters the cell cycle in order to prevent or repair the possible damages suffered by the cell.

4.3.2.2. Nitrogen depletion induces mating and meiosis

Nitrogen starvation results in Sty1 phosphorylation and entrance of the cell into the sexual pathway (see section 4.1.2.). The TF Ste11 is a master regulator of this pathway driving the cell into mating, meiosis and sporulation. *ste11* transcription is up regulated by Sty1 in an Atf1-dependent manner (Takeda *et al.,* 1995). In addition, Sty1 triggers the necessary G1 retention required for the proper induction of the sexual programme. This function is however not related to the transcriptional activation of Ste11. Accordingly, *Δste11* cells are sterile but have no defects in mitotic cell cycle.

The Pka1 pathway is also related with nutrients withdrawal responses but is regulated in the opposite way than Sty1. Following nitrogen starvation Pka1 is inactivated and results in Ste11 transcriptional activation (Takeda *et al*., 1995; Kanoh *et al.,* 1996; Stettler *et al.,* 1996). In basal conditions Pka1 constitutively phosphorylates the Rst2 TF, preventing its migration to the nucleus. Upon nitrogen depletion, Pka1 is inactivated, and non-phosphorylated Rst2 is able to migrate and bind to the STRE promoter sequence to activate *ste11* transcription.

4.3.2.3. Glucose starvation leads to stationary phase and chronological life span

S. pombe consumes glucose when growing exponentially in liquid culture. When the percentage of sugar becomes limiting in the medium, Sty1 gets activated and migrates into the nucleus to prepare the cell to enter in stationary phase (Zuin *et al.,* 2005). The main biochemical changes are the down regulation of metabolism, stop protein synthesis and cell wall thicknessing. This process is fundamental to ensure the long viability at stationary phase, what has been called chorological life span. Accordingly, cells lacking *sty1* do not survive in stationary phase (Kato *et al.,* 1996).

Sty1 is not the only regulator of the stationary phase, as for nitrogen starvation, the Pka1 pathway is also involved in this specific adaptive response. Cells lacking *pka1* have an extended life span in stationary phase and are also more resistant to oxidative stress (Roux *et al.,* 2006). Conversely, cells lacking the Cgs1 or Cgs2 regulatory subunits inactivating Pka1, loose viability in stationary phase (DeVoti *et al.,* 1991).

4.3.2.4. Meiosis induces recombination

When *S. pombe* cells enter meiosis due, for instance, to nitrogen depletion, homologue chromosomes recombine at a higher frequency than during mitosis. These recombination events, however, do not occur randomly but are localized in recombinant hotspots. Almost 50% of the natural hotspots are characterized by the presence of DNA sequences that are specifically bound by TFs (Petes, 2001).

One of the best characterized recombination hotspot in *S. pombe* is *ade6-M26,* constituted by a point mutation in the *ade6* gene generating

a DNA binding motif for the Atf1-Pcr1 heterodimer (Wahls and Smith, 1994; Kon *et al*., 1997), converting this region into a hotspot. It has been proposed that the MAPK-activated CRE-Atf1-Pcr1 complex helps to create an open chromatin configuration around *M26*, facilitating the access to meiotic recombination initiating proteins, resulting in the creation of the hotspot.

Another effect of the (G→T) point mutation in the *ade6-M26* locus is the creation a new transcription start site. As a result, in response to an osmotic stress signal, but not in response to oxidative stresses or heat shock events, the binding of the Atf1-Pcr1 heterodimer activates the transcription of an *ade6* minigene (Hirota *et al*., 2004).

4.3.3. Substrates of the MAP Kinase Sty1

Atf1. A well described substrate of Sty1 is Atf1, a TF belonging to the ATF/CREB family. This family of proteins shares a basic ZIP (bZIP) domain which contains a basic region that mediates sequence specific DNA binding properties and a leucine zipper that is required for the dimerization of two DNA binding regions (Fig. 5). Atf1 is the homologue of mammalian ATF2, a substrate of p38 and other MAPKs as well. In section 5.1.1., we will enter in more detail on Atf1 function.

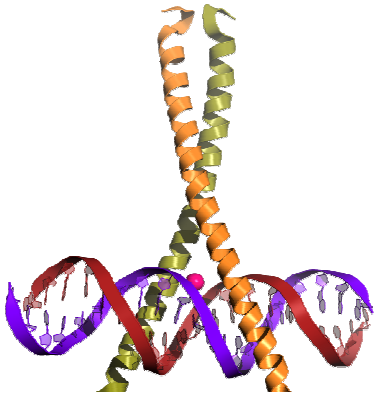


Figure 5. bZIP domain and CRE binding sequence of DNA.

Srk1. Another substrate of Sty1 is the Srk1 kinase, a serine/threonine kinase presenting

homology with mammalian calmodulin kinases. Srk1 has been found in complex with Sty1 and is directly phosphorylated by this MAPK (López-Avilés *et al*., 2008). Upon stress, Srk1 translocates from the cytoplasm to the nucleus in a Sty1-dependent manner. Srk1 regulates meiosis in fission yeast. Following nitrogen starvation, *srk1* deleted cells enter into meiosis significantly faster than wild-type cells (Smith *et al*., 2002).

Cmk2. The calmodulin-dependent kinase Cmk2 is another target of Sty1. This kinase is involved in G2 to M phase progression and is essential for the oxidative stress response. Sty1 phosphorylates cytoplasmic Cmk2 after oxidative stress, but not in response to other stresses. *cmk2* deletion or mutation of its Sty1-phosphorylable residue Thr-411 rescues the lethal phenotype of Wis1 overexpression (Sanchez-Piris *et al*., 2002).

4.3.4. Modulation and feedback

The activity of the Sty1 MAPK cascade has to be precisely regulated. *S. pombe* achieves this by three different means: kinases to transmit the environmental signals, changes in subcellular localization and phosphatases to turn off the pathway. Each mechanism is detailed below

4.3.4.1. Phosphorylation

The MAPKK Wis1 is physically associated with Sty1 until stress activates the pathway. Upon activation, Wis1 phosphorylates the TYG conserved motif of Sty1 (Thr-171 and Tyr-173), allowing the dissociation of the MAPK and its migration to the nucleus. The *Δwis1* phenotype is identical to mutations in each or both T and Y residues of the TYG motive of Sty1. In any of these configurations, Sty1 is not able anymore to

translocate into the nucleus resulting in stress sensitivity and elongated morphology of the cell (Gaits *et al*., 1998).

4.3.4.2. Nuclear migration

Once Sty1 is phosphorylated, so active, the kinase accumulates in the nucleus, where most of its stress roles are defined. In fact, in a *wis1* delta strain Sty1 doesn't accumulate in the nucleus (Gaits *et al*., 1998).

The mechanism by which Sty1 migrates into the nucleus is still unclear, since Sty1 lacks a typical nuclear localization signal. It has been proposed that Sty1 nuclear translocation could depend on its dimerization like for mammalian MAPK (Khokhlatchev *et al*., 1998). However, the size of a putative Sty1 dimmer would be above the limit allowed for passive diffusion between cytoplasm and the nucleus.

Cmr1 is a nuclear receptor involved in the export of proteins harbouring a nuclear export signal (NES). Sty1 has one functional NES between aminoacids (aa) 122 and 132. The export of Sty1 from the nucleus by Cmr1 has been well characterized (Gaits and Russell, 1999). Cmr1, in collaboration with the co-factor Hba1, constitutively exports Sty1. Lack of function of any of these two proteins results in Sty1 nuclear accumulation (Castillo *et al*., 2003).

Interestingly, when Sty1 is experimentally sequestered inside the nucleus and physically separated from the rest of the MAPK pathway, the protein is still accessible to activation by stress due to the ability of Wis1 to travel into the nucleus and phosphorylate its target in a regular way (Nguyen *et al*., 2002).

4.3.4.3. Phosphatases

The phosphorylation of Thr-171 and Tyr-173 of Sty1 by Wis1 is counteracted by different phosphatases. Thr-171 dephosphorylation is achieved by two specific Ser/Thr phosphatases of the 2C type class (PP2C), Ptc1 and Ptc3 (Nguyen and Shiozaki, 1999), whereas Tyr-173 is dephosphorylated by two tyrosine-specific phosphatases, Pyp1 and Pyp2 (Millar *et al*., 1995). Since the activation of Sty1 requires both phosphorylations of Thr-171 and Tyr-173 residues, the dephosphorylation of Tyr-171 alone is sufficient to inactivate Sty1. The combined deletion of *pyp1* and *pyp2* is lethal, as a result of the hyperactivity of Sty1 (Nguyen and Shiozaki, 1999), phenocopying *wis1* overexpression (Shiozaki and Russell, 1995). Pyp2 expression is enhanced as part of the CESR response, indicating a mechanism of negative feedback regulation: activation of Wik1-Wis1-Sty1 signal transduction pathway also promotes the transcription of *pyp2*, a gene coding for the phosphatase that switches off Sty1 activation (Shiozaki and Russell, 1996; Wilkinson *et al*., 1996). On the other side, *pyp1* expression is constitutive and poorly regulated by stress suggesting a role in the basal dephosphorylation of Sty1 (Degols *et al*., 1996).

The way PP2C proteins dephosphorylate Thr-171 remains still obscure. It has been reported that Ptc1 and Ptc3 dephosphorylate Thr-171 of Sty1 after heat shock (Nguyen and Shiozaki, 1999), what means a direct attenuation of the single kinase activity, as it happens in their homologues in *S. cerevisiae*, PTC1, 2 and 3 as phosphatases of HOG1 (Maeda *et al*., 1994). Conversely, it has also been reported that Ptc1, in front of an osmotic stress, does not dephosphorylate the single kinase, so it could act downstream Sty1 to negatively regulate the response (Gaits *et al*., 1997).

4.3.5. Sty1 mechanisms for stress defence

4.3.5.1. Transcription

Once Sty1 is phosphorylated and accumulates into the nucleus, it activates a complex transcriptional defence program against stress. The central aim of our Thesis work was to investigate Sty1 physiological role in stress-dependent transcription, this is why we develop further this aspect in section 5.

4.3.5.2 RNA stability

Global gene expression is not only regulated by TFs but also by modulation of RNA stability. Recent studies have shown that this type of regulation applies to the oxidative stress response. *S. pombe* counts with an RNA–binding protein, Csx1, that stabilizes *atf1* and *pcr1* mRNA (Rodriguez-Gabriel *et al*., 2003), and seems to work with the non sense mediated decay pathway, through the collaboration with the Upf1 protein (Rodriguez-Gabriel *et al*., 2006). *csx1* mRNA is slightly induced in an oxidant scenario and this increase is Sty1- and Atf1-dependent (Chen *et al*., 2003). Moreover, Csx1 also regulates global gene expression after oxidative stress and both processes are essential to survive in front of this insult, but they are dispensable for osmotic stress surveillance. Accordingly, Csx1 is phosphorylated directly or indirectly by Sty1 only after oxidative stress, but this phosphorylation is not essential for Csx1 function in the response (Rodriguez-Gabriel *et al*., 2003).

4.3.5.3. Translation

Stress response does not only affect the steady-state levels of mRNAs. Protein synthesis is down-regulated during this process, to protect cells from the generation of misfolded or toxic proteins (Dever, 2002; Kaufman *et al*., 2002). This phenomenon occurs in mammals and yeast through the reversible phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2a), resulting in protein synthesis inhibition (Dever, 2002). The same mechanism has been described in the fission yeast, but proved to be more evident in mutants disrupting the Sty1 pathway, in which phospho-eIF2a levels, which depend on Gcn2 and Hri2 kinases, are increased (Dunand-Sauthier *et al*., 2005).

Specific transcripts that are important for the stress response, including yeast GCN4 and human ATF4 transcription factors, escape from this general translation repression by relying on special regulatory sequences upstream of their coding regions (reviewed by Holcik and Sonenberg, 2005).

It has been recently shown that the Sty1 pathway participates to restore protein synthesis when environmental variables come back to normal values. To do so, Sty1 physically binds to translation initiation factors such as eIF3a, and translation elongation factors like eEF2 and eEF3. Supporting this data, the total amount and phosphorylation levels of eIF3a are dramatically reduced in *Δsty1* strain (Asp *et al*., 2008).

4.3.5.4. Cell cycle regulation

An important function of Sty1, once it is activated by UV, hyperosmosis or oxidative stress, is to increase the mitotic rate, known as “mitotic burst”, by shortening the G2 phase and allowing mitosis to occur

with a smaller size (Shiozaki and Russell, 1995; Kishimoto and Yamashita, 2000). This alteration in the cell cycle results from the Sty1-dependent accumulation of Cdc25, the phosphatase that dephosphorylates and activates the Cdc13/Cdc2 kinase complex. In *sty1* deleted cells, Cdc25 activity is reduced, generating a G2 delay and elongated cells, confirming that Sty1 activity affects cell cycle control (Millar *et al*., 1995, Shiozaki and Russell, 1995).

On the other hand, nitrogen starvation promotes sexual development in which cells arrest in G1, conjugate with partners of the opposite mating type, and then undergo meiosis. Nitrogen limitation induces activation of Sty1. Once the MAPK is active, and through Atf1 and Pcr1 (Watanabe and Yamamoto, 1996; Kon *et al*., 1998), it activates *ste11* transcription, which is critical for meiosis to proceed because is the main TF in sexual development (Shiozaki and Russell, 1996) (see section 4.1.2). In conclusion, cells lacking *sty1* are almost completely sterile.

The MAPK pathway has also a prominent role in a mitotic checkpoint, preventing sister chromatid separation, spindle elongation and entrance to cytokinesis in response to actin cytoskeleton damage (Gachet *et al*., 2001).

5. REGULATION OF STRESS-DEPENDENT TRANSCRIPTION BY Sty1

In *S. pombe*, genome-wide transcription studies revealed that there's a large number of genes (~700 out of 4970) whose expression levels changed two fold or greater after one stressor out of the five that had been analyzed, H_2O_2, sorbitol, cadmium, heat shock and MMS (Chen *et al*., 2003). The authors of this study found that in four out of five of those stresses 140 genes were commonly induced and 100 repressed.

This shared outcome was called the Common Environmental Stress Response (CESR). CESR is at the basis of the cross-protection phenomenon described in section 2.1. CESR up regulated genes are implicated in carbohydrate metabolism (*tps1, ntp1, fbp1*, etc.), signalling and transcriptional regulation (*pyp2, pcr1, atf1*, etc.), lipid or fatty acid metabolism, antioxidants (*cta1, gpx1, grx1*, etc.), DNA repair, transporters, protein folding, protein degradation and others. More than 70% of CESR genes are Sty1 dependent, and the majority of those are also Atf1 dependent. Nevertheless, the kinase controls the expression of more genes than Atf1, it is likely that Sty1 regulates additional TFs.

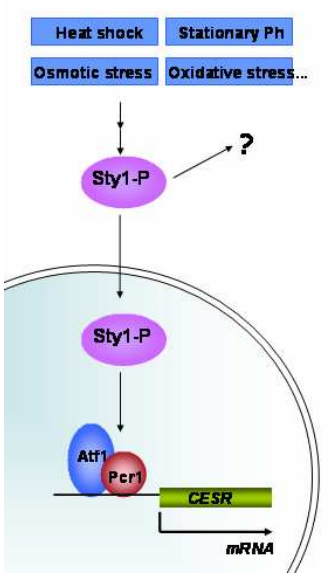


Figure 6. Sty1 regulates stress transcriptional response. Once Sty1 is active, translocates into the nuclei and actives CESR genes through Atf1-Pcr1 TFs

A large proportion of CESR downregulated genes are also derepressed in *Δsty1* cells. These genes are related to protein synthesis (ribosomal genes), cytoskeleton organization, transcription, etc. In general, the repressor role of Sty1 seems to be Atf1 independent, except in a very few cases.

Recent studies have shown that by varying stress conditions (timing of exposure, stressor concentration, etc.) the global patterns of gene expression are different in intensity and timing (Chen *et al*., 2008). These results highlight the richness, specialization, and plasticity of the regulatory mechanisms used by the cell to respond to stressors.

The TFs that are substrates of Sty1 are deeply implicated in this genome-wide regulation.

5.1. STRESS TRANSCRIPTION FACTORS

5.1.1. Atf1

As it has been introduced in section 4.3.3., Atf1 belongs to the ATF/CREB family of TFs and is the homologue of mammalian ATF2. This family of proteins binds to the same DNA sequence, the cAMP response element (CRE) (consensus: TKACGTCA) (Fig. 5). To be active as TFs, ATF/CREB proteins must dimerize.

In mammalian cells, the formation of bZIP TFs heterodimers has been proposed to be the basis of a large number of transcriptional regulatory interactions, increasing in this way the diversity of gene responses (reviewed in Wolberg, 1998). Atf1 conserved sequence is predominantly restricted to the C-terminus, containing the bZIP region (Takeda *et al*., 1995).

In vitro studies (Wahls and Smith, 1994) and co-immunoprecipitation (co-IP) experiments performed in *S. pombe* (Kanoh *et al*., 1996) suggest that Atf1 heterodimerizes with Pcr1, another bZIP TF. An Atf1 homodimer has been also reported *in vitro* but never *in vivo*. Cells lacking *atf1* partially phenocopy *sty1* mutations: they are sensitive to oxidative, osmotic, heat shock and heavy metals stress and their viability is reduced in stationary phase (Nguyen *et al*., 2000; Quinn *et al*., 2002; Zuin *et al.*, 2010). Atf1 was first identified by two genetic screens: one for sterile mutants showing a defect in G1 arrest following nitrogen starvation, the other for suppressors of the mating defect of *Δsty1* cells (Shiozaki and Russell, 1996; Takeda *et al*., 1995; Kanoh *et al*., 1996).

Several lines of evidence suggest a potential role of Atf1 in chromatin remodelling. As mentioned in section 4.3.2.4, the MAPK activated CRE-Atf1-Pcr1 complex is able to create an open chromatin configuration around the *ade6-M26* locus, facilitating the access to meiotic recombination initiating proteins and resulting in the creation of a recombination hotspot (Kon *et al*., 1997), which accompanies a CRE-like sequence due to a G to T transversion in the *ade6* coding region (Szankasi *et al*., 1988). In addition, components of the MAPK pathway are also needed, as Wis1 and Sty1 activated kinases (Mizuno *et al.,* 2001). Full induction of this chromatin alteration is facilitated by a histone acetyltransferase (HAT), Gcn5, and a Swi2/Snf2-like ATP-dependent chromatin remodelling factor (ADCR), Snf22 (Hirota *et al*., 2003), but it is repressed by the global corepressors Tup11 and Tup12 (Yamada *et al*., 2004). Recently the same authors reported other ADCRs (Hrp1 and Hrp3) and the Gcn5 coactivator Ada2, are also involved in this role (Hirota *et al.,* 2008).

The Atf1-Pcr1 heterodimer is also involved in the heterochromatinization of the *mat* locus, in a Swi6-HP1-dependent, RNAi-independent manner. *atf1* deletion or mutations in the ATF/CREB-binding sites of the *mat3-M* locus have subtle effects on the heterochromatin silencing (Thon *et al.,* 1999; Kim *et al.,* 2004). Atf1 and Pcr1 are required for proper histone deacetylation and H3 Lys-9 methylation in collaboration with the histone deacetylases (HDACs) Clr6 and the chromodomain silencing protein Swi6. Surprisingly, a similar activity has been reported on the stress dependent promoter of *ctt1* (Kim *et al.,* 2004).

Atf1 contains 11 potential MAPK sites (S/TP, a serine or threonine residue followed immediately by a proline) and Sty1 has been shown to phosphorylate Atf1 *in vitro* (Wilkinson *et al.,* 1996) and *in vivo* (Gaits *et al*., 1998). Since Atf1 is phosphorylated after stress and the kinase activity of

Sty1 is required for its transcriptional activity, it has been long believed that the phosphorylation of Atf1 was essential for its activation. However, by mutating the 11 MAPK phosphorylation sites of Atf1, Lawrence and colleagues have shown that the mutant protein has a similar activity than wild-type Atf1, if expressed at wild-type levels (Lawrence *et al.,* 2007). The observation that the levels of Atf1 phospho-mutant protein where extremely low lead the authors to conclude that the role of Atf1 phosphorylation by Sty1 is to stabilize the protein, suggesting that the phosphorylation of Atf1 by Sty1 is not required for the activation of the TF *per se* but rather for increasing its stability.

While the Sty1 kinase is accumulated inside the nucleus upon activation, Atf1 was shown to be nuclear resident protein by inmunofluorescence, even in basal conditions. The same authors reported that Sty1 needs Atf1 to be retained inside the nucleus upon stress (Gaits *et al.,* 1998).

5.1.2. Pcr1

Pcr1 is also an ATF/CREB TF containing a dimerizing bZIP domain. This small protein of 171 aa has a role in the early stage of sexual development, in association with the cAMP cascade (Watanabe and Yamamoto, 1996). Pcr1 is one of the TF controlling the transcription of the sexual regulator Ste11. Strain *Δpcr1* shares a number of phenotypes with strain *Δatf1* such as partially sterility (Kon *et al*., 1998), cold sensitivity, lack of transcriptional activation of nutrient starvation-dependent genes (*ste11* and *fbp1*) (Watanabe and Yamamoto, 1996) and deficient meiotic recombination. This last phenotype is explained by the fact that Atf1/Pcr1 heterodimers bind with higher affinity to recombination hotspots compared with individual TFs (Walhs and Smith, 1994; Kon *et*

al., 1997). As mentioned previously, the Pcr1-Atf1 heterodimer is also implicated in Swi6-HP1 dependent heterochromatin formation in the *mat* locus (Kim *et al*., 2004). For further information see section 5.1.1.

Pcr1 role in stress response is still obscure. By the time we started this project some authors had suggested that Atf1 but not Pcr1 was required for resistance to extracellular osmotic stress mediated by NaCl (Kon *et al*., 1997; 1998). However, Pcr1 is able to bind to stress promoters *in vitro*, but the binding affinity of the Atf1/Pcr1 heterodimer compared to each TF alone is not known. Pcr1 is a very small protein with the bZIP in its N-terminal domain (from 11 to 66 aa). It has been reported to be absent of MAPK phosphorylation sites (Kanoh et al., 1996). It is also always nuclear, and it has been reported to retain Atf1 in the nucleus by inmunofluorescence, that is, to be a nuclear anchor for Atf1 (Gaits *et al*., 1998). *atf1* and *pcr1* are CESR genes in *S. pombe*. This transcriptional activation suggests an important role in the regulation of stress-dependent genes.

5.1.3. Other transcription factors

S. pombe has 6 bZIP TFs: Atf1, Atf21, Atf31, Pcr1, Pap1 and Zip1. The role of Atf1, Pcr1 and Pap1 is well documented whereas the function Atf21, Atf31 and Zip1 in stress response is more specific and not so well described: their transcription is not regulated by the Sty1 pathway but, they are related with some stress responses.

Atf21 was cloned as a multicopy suppressor of *Δsty1* mating defects (Shiozaki and Russell, 1996). Its expression is only induced under osmotic stress with sorbitol 1.2 M (Chen *et al*., 2003) and meiosis (Mata, *et al*. 2002). Its main role is to regulate transcription during the late meiosis programme.

Atf31 works exclusively for late meiosis wave of transcription. Atf21 and Atf31 are required for the expression of overlapping sets of genes. Around 55% of the late meiosis genes are regulated by Atf21 and Atf31, including genes that are also transcribed in front of stress. In contrast, Atf21 and Atf31 are not required for the expression of stress genes induced as part of the nitrogen-starvation response (Mata *et al*., 2002).

Zip1 is a TF specific to cadmium toxicity (see section 4.2.3). *zip1* deletion causes severe cadmium sensitivity The regulation of *zip1* does not occur at the transcriptional level, but by protein degradation or stability through different phosphorylation degrees. The kinase in charge of Zip1 regulation has not been investigated but Sty1 could be related with. Genome-wide microarray demonstrates that many cadmium stress induced genes are Zip1-dependent (Harrison *et al*., 2005), most of these Zip1-dependent genes are over expressed in cells lacking *sty1*.

5.2. CHROMATIN REMODELERS.

Transcription, as recombination, repair and replication, is finely regulated by chromatin structure. This event preferentially occurs at accessible chromatin regions that are structurally modified using three mechanisms. The first involves the incorporation of histone variants which sequences differ from the canonical histones. The second mechanism uses the energy of ATP hydrolysis to remodel the nucleosome patterning along the chromosome fiber. Finally, chromatin-dependent factors can covalently modify histone N terminus. Several histone-tail modifications have been identified such as acetylation, methylation, phosphorylation and ubiquitinylation, which either regulate DNA to nucleosome association, or are used as hallmarks for other TFs

and chromatin modifiers. Only acetylation hes been related to the regulation of Sty1-dependent genes.

5.2.1. Histone variants

The nucleosome core particle consists of approximately 147 base pairs of DNA wrapped in 1,67 left-handed superhelical turns around a histone octamer, consisting of 2 copies each of the core histones H2A, H2B, H3, and H4. Although histones H4 and H2B are largely invariant, disparity is more evident within histone H3 and H2A variants (Malik and Henikoff, 2003). Histone variants further contribute to the intrinsic and extrinsic properties of the nucleosome core particle (NCP) by building specialized chromatin structures and affecting RNA polymerase II (RNAPII) activity.

One of the most studied variants is histone H2A.Z (highly conserved through most of eukaryotic evolution); when it substitutes H2A in the NCP it destabilizes interactions between H3 and H4, affecting nucleosome stability and chromatin folding (Suto *et al*., 2000). This destabilization is needed to improve accessibility of the nucleosomal DNA to the transcriptional apparatus; several studies strongly indicate that H2AZ variant is critical for maintaining a transcriptionally permissive open state (Stargell *et al*., 1996) and in protecting euchromatin from encroachment by silent heterochromatin (Meneghini and Madhani, 2003).

H2A.X, another H2A variant, is present in nearly all eukaryotes and is related with DNA double-strand breaks repair, in response to them H2A.X is rapidly phosphorylated and recruits repairing proteins as Rad51 (Celeste *et al*., 2003).

Other variants, like macroH2A or H2ABbd, are just present in vertebrates (Malik and Henikoff, 2003). .

5.2.2. ATP-dependent chromatin remodelers (ADCRs)

The restructuring of chromatin is often accomplished in an ATP-dependent manner, altering DNA-histone contacts to mediate nucleosomal structural alterations, removal and movement. There are currently four different families of ADCRs. All their members share five basic properties: an affinity for the nucleosome, beyond DNA itself; domains that recognize covalent histone modifications; a similar core catalytic ATPase subunit that belongs to the Snf2 ATPase superfamily; domains and/or proteins that regulate ATPase domains; and finally, domains and/or proteins for interaction with other chromatin or TFs.

Beside these common properties ADCRs are subdivided in four distinct well conserved families, SWI/SNF, ISWI, INO80 and CHD (Mi-2). Each of these families share unique domains residing within, or adjacent to, the ATPase domain conferring distinctive targeting properties and activities (reviewed in Clapier and Cairns, 2009).

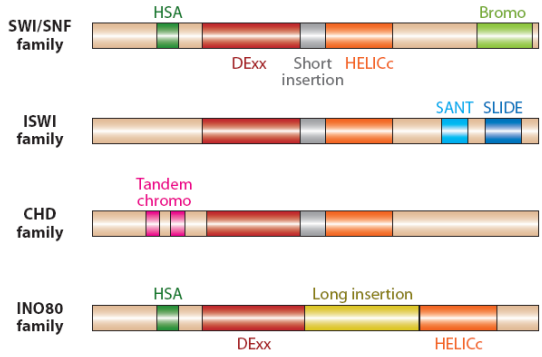


Figure 7. Remodeler Families, defined by their ATPase (Clapier and Cairns, 2009).

All remodeler families contain a SWI2/SNF2-family ATPase subunit split in two parts, DExx and HELICc (red and orange shaded boxes, Figure 7). While the SWI/SNF, ISWI, and CHD families share a distinctive

short insertion within the ATPase domain, INO80 family contains a long insertion. Each family is further defined by distinct combinations of flanking domains: a bromodomain and HSA (helicase-SANT) domain for the SWI/SNF family, a SANT-SLIDE module for the ISWI family, tandem chromodomains for the CHD family, and an HSA domain for the INO80 family. Although core components are conserved, SWI/SNIF family components from *S. cerevisiae* and *S. pombe* differ. As a consequence, their requirement for viability can differ dramatically between these two yeasts species.

In *S. pombe* SWI/SNF proteins can have opposite effects. For instance, while *snf22* represses iron-transport genes (Monahan *et al.*, 2008), the same protein decompacts the *ade6-M26* locus activating Atf1/Pcr1-dependent meiotic recombination and transcription of the *ade6* minigene under sorbitol induction (more detailed information in section 4.3.2.4) (Hirota et al., 2008). Hrp1 and Hrp3, other two *S. pombe* ADCRs from the CHD family, have similar effects on the *ade6-M26* locus, although the latter contributes more to decompaction (Hirota *et al*., 2008). As Snf22, Hrp3 has also an opposite activity silencing heterochromatin at the *mat3* locus (Jae Yoo et al., 2002).

5.2.3. Histone acetyl transferases

The most abundant post-translational modifications of chromatin is the reversible lysine acetylation of histones H3 and H4, which are related to transcription activation. Histone acetylation is carried out by a class of enzymes known as histone acetyltransferases (HATs), which catalyze the transfer of an acetyl group from acetyl-CoA to the lysine ε-amino groups on the N-terminal tails of histones. In the other hand, deacetylation of the same residues is an active process catalyzed by

enzymes called histone deacetylases (HDACs). This dynamic balance between HATs and HDACs activities not only regulates transcription, but also affects proper chromosome segregation during cell division, DNA replication and repair of double-strand breaks.

There are currently two hypothesis on how histone acetylation facilitates transcription. One proposes that by neutralizing histone charge, acetylation destabilizes histone–DNA and nucleosome-nucleosome contacts reducing chromatin compaction. The other, which is not exclusive, is commonly known as the "histone code" and claims that covalent modifications of histones provide epigenetic marks for gene expression, acetylation being usually considered as an activating mark.

Early indications that HATs were involved in transcription came from the observation that in actively transcribed chromatin, histones tend to be hyperacetylated, whereas in transcriptionally silent regions histones are hypoacetylated (Allfrey *et al.,* 1964).

HAT enzymes are divided into five groups: Gcn5-related acetyltransferases (GNAT), MYST, p300/CREB-binding protein (CBP), TATA binding protein-associated factors (TAFs) and hormone related HATs (reviewed in Carrozza et al., 2003).

HAT family	***S. pombe***	***S. cerevisiae***
GNAT	Gcn5	GCN5
	Sin3	ELP3
	Hat1	HAT1
MYST	Mst2	ESA1, SAS3
	Mst1(essential)	ESA1
TAF	Taf111	$TAF_{II}145$

Table 1. Classification of HATs from *S. pombe* and *S. cerevisiae*.

In *S. pombe* there is not HAT proteins from CBP and hormone related groups. Homologs to the rest of the families are detailed in table

1. Several HATs are found in fission yeast. For simplicity, we focus our description on the GNAT family, which is specifically related to the stress response.

5.2.3.1. Gcn5 and Spt-Ada-Gcn5 acetyltransferase complex (SAGA)

The S. cerevisae SAGA complex is related to: (i) deubiquitination of histone H2B by the trimeric complex formed by Ubp8 (Daniel *et al*, 2004; Henry *et al*, 2003), Sfg11 (Ingvarsdottir *et al*, 2005) and the small protein Sus1, that links SAGA-dependent transcription and nuclear mRNA export (Köhler *et al*., 2006). Deubiquitination of H2B regulates H3 methylation at some SAGA promoters (reviewed by Rodriguez-Navarro, 2009); (ii) acetylation of histone H3 by the Gcn5 subunit, as its going to be explained below.

The principal stress-related histone acetyltransferase is Gcn5, a GNAT-MYST superfamily protein and a component of the SAGA coactivator complex. SAGA was initially discovered in *S. cerevisiae* (Grant *et al.,* 1997). This multi-subunit complex contains several components displaying different enzymatic activities which are physically linked to other activators, histones and are involved in core promoter selectivity *in vivo*. It has been suggested that *S. cerevisiae* SAGA complex is important for the activation of highly induced stress-regulated genes (Lee *et al*., 2000). *In vitro*, *S. cerevisiae* GCN5 plays a key role in the preferential acetylation of H3 in Lys-14 (H3K14) on free histones (Kuo *et al*., 1996), but is also able to acetylate H3K9 when it is embedded in the SAGA complex (Grant *et al.,* 1999). In fact, native nucleosomal HAT proteins are generally found inside multi-subunit complexes (Grant and Berger, 1999). This feature enables several HATs to prefentially recognize specific histone residues in defined loci. Gcn5

Little is known in *S. pombe* about the relationship between stress and specific mechanisms of chromatin remodelling. The majority of studies establishing a link between these two processes have focused so far on the recombination hotspot *ade6-M26* and heterochromatin regions such as the *mat* locus.

In *S. pombe*, *gcn5* was identified as a gene encoding a putative homologue of *cerevisiae* GCN5, and the gene product was found in a SAGA-like complex, although it was unknown whether the protein had HAT activity (Mitsuzawa *et al.,* 2000). Later on, Gcn5 was described as one of the chromatin remodelers involved in the *ade6-M26* meiotic hot spot. When cells lack the *gcn5* gene, H3 acetylation levels around *M26* decrease and chromatin remodelling is slowed down. Moreover, H3 and H4 in the region are hyperacetylated in an Atf1- and Pcr1-dependent manner in early meiosis (Yamada *et al.,* 2004).

Another illustration of the role of HATs in stress response was revealed by microarray experiments showing that Gcn5 is a KCl-dependent gene regulator. Accordingly its deletion results in KCl and $CaCl_2$ sensitivity on plates. 67% of the genes downregulated in a *Δgcn5* strain in response to KCl contain an ATF/CREB site in their promoters, and half of them belong to CESR genes (Johnsson *et al*., 2006).

A recent publication has revealed a physical implication of Gcn5 in the fission yeast SAGA complex. The authors purified the whole complex and determined that its subunit composition is identical to its *S. cerevisiae* counterpart.

The same authors studied carefully different SAGA mutants and showed that it plays distinct roles in the proliferation-differentiation switch through opposing activities of Gcn5 and Spt8: Gcn5, in collaboration with its cofactors Ada2 and Ada3, represses *ste11* gene in basal conditions, while Spt8 is required for *ste11* induction under nitrogen limiting

conditions. Chip experiments revealed that al these components are bound to *ste11* gene promoter even in nutrient rich conditions (Helminger *et al*., 2008).

5.2.3.2. Sin3 and elongator complex

The elongator complex (ELP1-6) was originally identified as a component of hyperphosphorylated RNAPII holoenzyme isolated from budding yeast chromatin (Otero *et al.,* 1999). One of its subunits, ELP3, harbours motifs found in the GNAT family of HATs (Wittschieben *et al*., 1999). The human and yeast complexes are capable of acetylating histone H3 *in vitro* (Winkler et al., 2002; Hawkes et al., 2002; Kristjuhan *et al*., 2002). Deletion of yeast ELP3 results in decreased H3 acetylation *in vivo* (Winkler *et al*., 2002; Kristjuhan *et al*., 2002). Together this data led the hypothesis that elongator assists the transcription complex acetylating nucleosomes in the path of the elongating RNAPII.

However, other data collected on this complex have generated some controversy about its cellular role. First, it has been shown that most of the Elongator is actually localised in the cytoplasm (Pokholok *et al*., 2002). Second, attempts to detect different subunits (ELP1,2 and 3) of the yeast complex bound to chromatin, not only to active genes but also in genome, have failed (Pokholok *et al*., 2002). Third, it was reported that yeast Elongator is involved in exocytosis (Rahl *et al*., 2005) and finally, a recent picture has emerged where Elongator is involved in tRNA modification and affects translation (Esberg *et al*., 2006).

This new role in tRNA modification arised from fission yeast antisupressor screening, in which *sin3-193* mutant showed reduced levels of modified nucleoside mcm^5s^2U from a subset of tRNAs (Thuriaux *et al*., 1976). *The sin3* gene encodes a highly conserved protein showing 77 %

amino acid identity to *S. cerevisiae* ELP3. This paragraph summarizes most recent results obtained on *S. pombe* elongator complex, the rest of *elp* homologue genes are recognized by homology, but none of their functions has yet been studied.

Based on Sin3 data, the Byström group assumed that ELP3 could cause the same loss-of-suppression phenotype in *S. cerevisiae*. They characterized *elp* mutants resistance to zymocin (a toxin derived from *Kluyveromyces lactis*) and confirmed previous results showing that Elongator mediates tRNA modification. Zymocin acts as an RNAse degrading specifically modified tRNA. In the absence of ELP proteins, some tRNAs are no longer modified and therefore become refractory to zymocin degradation, allowing translation (Huang et al., 2005). The possibility of an indirect role of ELP in tRNA modification has still not been ruled out. However, evidences accumulate, supporting that the cause of the *elp* mutant phenotype is a consequence of altered translation. Consistent with this view, Esberg *et al.* found that two multi-copy supressors of *elp* mutants were tRNA genes (Esberg *et al*., 2006).

Back into its role in transcription elongation, the Elongator complex is more clearly associated with RNAPII in human cells. Different subunits of the complex are bound to a gene only after arrival of a hyperphosphorilated RNAPII (Metivier *et al*., 2003). Other labs have demonstrated that the complex primarily associates with coding regions, not only from genes in which the depletion of elongators is diminishing their transcription, but also in a number of unaffected genes (Kouskouti and Talianidis, 2005; Close *et al*., 2006). In transcription targets RNPAII was detected at normal levels at the promoter and the beginning of coding region, however decreasing amounts were detected getting closer to the 3'-end of the coding region. In paralel H3 acetylation levels (but not H4) were diminished (Close *et al*., 2006). These data provide significant

evidence that Elongator assists RNAPII during transcript elongation, but does not affect the initiation process.

5.2.4. Histone deacetylases

The levels of histone modification are the result of a continuous process of acetylation by histone acetyltransferases (HATs) and deacetylation by histone deacetylases (HDACs). Whereas HATs catalyze the transfer of an acetyl moiety from acetyl-coA to the ε-amino group of lysine residues, HDACs catalyze removal of the acetyl group, thereby reconstituting the net positive charge of the histone.

The first histone deacetylases were isolated by their ability to bind histone deacetylases inhibitors like trapoxin or trichostatin A (TSA). HDACs can be placed in three different phylogenic classes that predict their function and localization fairly well. *S. pombe* contains representative proteins from each subtype, as it is shown in Table 2.

HDAC	***S. pombe***	***S. cerevisiae***
Class I or Rpd3-like	Clr6 (essential) Hda1	RPD3 HDAC 2, 3 HOS2, 3
Class II or Hda1-like	Clr3	HDA1
Class III or Sir2-like	Sir2 Hst4	HST1, SIR2 HST2, 3

Table 2. Classification of HADCs from *S. pombe* and *S. cerevisiae.*

Class I HDACs display some sequence homology to members of class II, but not to those of class III. In agreement with this, class I and II HDACs are zinc-dependent enzymes, whereas the deacetylase activity of class III members is NAD^+ dependent.

5.2.4.1 Class I HDACs

S. cerevisiae RPD3, the founding member of class I HDACs, and the other members of this class have a region of homology over about 300 amino acids that is very well conserved among them. Class I enzymes can be described as sensitive to TSA, trapoxin and suberoylanilide hydroxamic acid. They tend to be nuclear and are generally ubiquitous in multicellular organisms. Association with co-repressors, like *cerevisiae* SIN3, is necessary for both proper targeting to specific chromatin loci and for their enzymatic activity (reviewed in de Ruijter *et al*, 2003). Several post-translational modifications have been reported a mean to regulate its deacetylase activity; for example phosphorylation of mammalian HDAC1 and -2 promote their enzymatic activity (Pflum *et al*., 2001; Galasinsky et al., 2002).

In *S. pombe,* Hos2 and Clr6 are class I histone deacetylases. Clr6 is homologous to the budding yeast histone deacetylase RPD3. *clr6* is an essential gene, and is required for repression of RNAPII dependent genes placed within the centromere, the telomeres and the mating type region (Grewal *et al*., 1998). In addition, the temperature sensitive mutant *clr6-1* has an elevated rate of chromosome mis-segregation. Clr6 has been purified in a complex with co-repressors Pst1 and Pst2 (Nakayama *et al*. 2003). Microarray analysis of a *clr6-1* mutant strain showed a repressive role of this HDAC in euchromatin. Basal transcription of 256 genes was affected, among them, 72 genes showed also a hyperacetylation state once analyzed the histone marks. Remarkably, 71% of the 72 Clr6 repressed genes are induced in meiosis and 46% in environmental stress. All these changes in histone modifications where

observed in the intergenic regions (IGR) and promoters, but no significant alterations were found in ORFs (Wiren *et al*., 2005).

The second *S. pombe* class I HDAC is Hos2, also known as Hda1 or Phd1. *Δhos2* cells showed enhanced gene repression of reporter genes placed within centromeres, telomeres, and the mating type loci (Olsson *et al*., 1998). Unexpectedly, this class I HDAC has a role in promoting high expression of growth-related genes by deacetylating H4K16 in their ORFs. Those genes are involved in cellular processes such as protein biosynthesis and major metabolic pathways. Accordingly to this data, *Δhos2* cells have a slower growth rate (Wiren *et al*., 2005).

5.2.4.2. Class II HDACs

Class II histone deacetylases have a similar enzymatic pocket to the Class I HDACs, therefore, are also sensitive to TSA, trapoxin and suberoylanilide hydroxamic acid. However, they are usually larger, and tend to be localized to the cytoplasm in the absence of signals that enables them to enter the nucleus. In mammals, class II HDACs tend to have a tissue specific or developmentally determined expression pattern (Verdin *et al*., 2003).

S. pombe has a single class II histone deacetylase. Although Clr3 is also in the cytoplasm, it is primarily nuclear, being detected both at the chromatin and nucleolar portions of the nucleus, with a high concentration at the outer periphery of the nucleus near the nuclear membrane (Bjerling et al., 2002). Clr3 was originally isolated in a screen to identify mutants which could not maintain silencing within the K region of the mating type locus (Ekwall and Ruusala, 1994). Early characterizations of *clr3-E36* cells, and later *Δclr3* cells, indicated a role for Clr3 in centromeric, telomeric, and rDNA silencing (Bjerling *et al*., 2002, Ekwall *et al*., 1998).

Clr3-myc has been shown to immunoprecipitate mainly in ORFs, but also to some extent in IGRs. The binding regions correlate with enriched H3K14 marks in a *Δclr3* strain. Interestingly, these ChIP on Chip results showed a preferential role in subtelomeric regions, where stress response genes are clustered (Wiren *et al*., 2005).

5.2.4.3. Class III HDACs

Class III HDACs, often referred to as Sirtuins, consist of a more divergent group of nicotinamide adenine dinucleotide (NAD) dependent enzymes (reviewed in Blander and Guarente, 2004). The founding member of sirtuins, *S. cerevisiae* SIR2, was isolated in a genetic screen for silencing at mating type (Rine and Herskowitz, 1987). *S. pombe* Sir2 is also required for silencing of all heterochromatic regions (Freeman-Cook *et al*., 2005). Sir2-Myc shares with Clr3 its preference for ORF binding compared to IGRs. This observation correlates with increased H3K9 acetylation at nucleosomes of the same regions in a *Δsir2* strain. Both HDACs, Clr3 and Sir2, share 33% of the targeted ORFs that are deacetylated (Wiren *et al*., 2005).

Hst4 is another *S. pombe* sirtuin, and has been implicated in telomere position effects, radiation damage response and chromosome segregation (Freeman-Cook *et al*., 1999).

OBJECTIVES

MAJOR OBJECTIVES

To better understand the transcriptional response regulated by the single MAPK Sty1 upon stress imposition in the fission yeast *Schizossacharomyces pombe*.

SPECIFIC OBJECTIVES

1. Elucidate how Sty1 is activating the transcriptional response inside the nucleus.
2. Understand the needing of two different transcription factors, Atf1 and Pcr1, in the stress-dependent transcription regulation.
3. Describe the regulation of Atf1 and Pcr1 through the Kinase Sty1.
4. Investigate the role of chromatin remodelers, like the HAT Gcn5, working in the stress-dependent transcription regulation.
5. Look for new chromatin remodelers involved in the stress response.

RESULTS

1. THE MAPK Sty1 ACTIVATES TRANSCRIPTION TRHOUGH RECRUITMENT TO STRESS PROMOTERS AND ITS KINASE ACTIVITY

Many signalling kinases modulate gene expression through regulatory phosphorylation of their target transcription factors or other chromatin-bound proteins. In *S. pombe*, the MAPK Sty1, through Atf1, is required for many of the stress-induced transcriptional changes that occur upon stress. Once this project started it was known that upon activation of the stress pathway, Sty1 is phosphorylated and it accumulates in the nucleus (Gaits et al. 1998; Gaits and Russell 1999). Meanwhile, several items remained unclear; it was still unknown if Sty1 binds to promoters and activates gene transcription or whether it simply phosphorylates its own transcription factors, which are then able to trigger transcription of specific genes through, for instance, recruitment of RNAPII. Moreover, it had not been determined if the kinase activity of the protein was essential for a proper stress response or whether localization of Sty1 was the only necessary aspect to activate transcription.

Some of these questions have been solved in our laboratory and by other colleagues (Lawrence et al., 2007; Reiter et al., 2008), along this thesis. In this chapter we resume the role of Sty1 in stress defence and the new features of its role in the transcriptional response.

1.1. CELLS LACKING Sty1 ARE SENSITIVE TO SEVERAL STRESS RESPONSES

The single MAP kinase Sty1 is not essential for *S. pombe* survival. Nevertheless, it is crucial for the cell survival in front of environmental stress. Accordingly, *Δsty1* strain is not able to proliferate under different stress conditions, such as growth on oxidative, osmotic and heat shock stress (Fig. 1A).

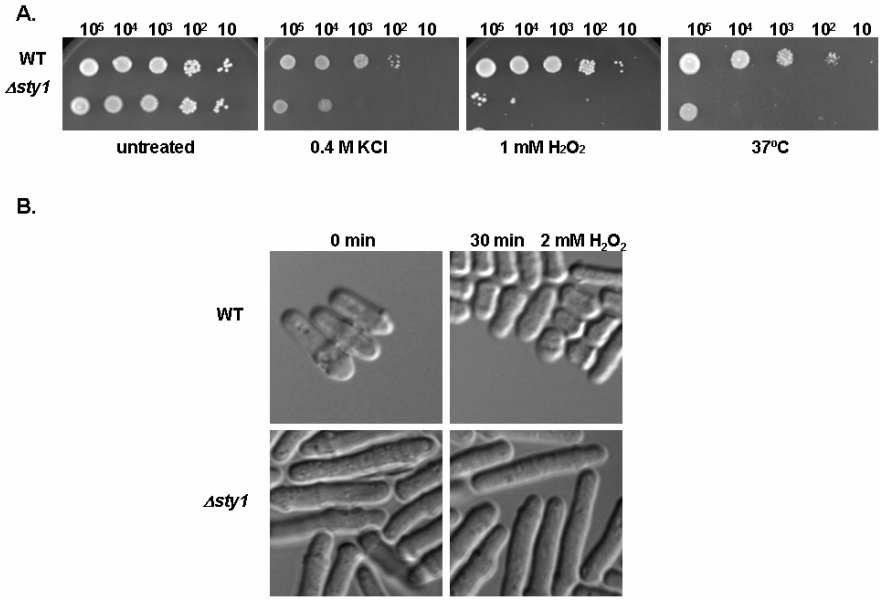


Figure 1. *Δsty1* phenotype in front of stress. A. *Δsty1* stress survival. Liquid cell cultures from strains 972 (WT) and AV18 *(Δsty1*) were grown in minimal medium (MM) and the indicated number of cells were spotted into MM with or without 0.4 M KCl, 1 mM H_2O_2, 37ºC. **B**. Morphological changes upon oxidative stress. Same strains as in A were grown in MM until OD_{600} of 0.5 and treated or not for 30 min with 2 mM H_2O_2. Pictures were captured with differential interference contrast (Nomarski) optics.

An important characteristic of cells lacking Sty1 is their elongated morphology compared to wild type cells, which is caused by a G2 cell

cycle delay (for more detailed information see section 4.3.4.4). In response to a mild oxidative stress, *S. pombe* reduces its size after cell division (Millar *et al.*, 1995). However, we observed that *Δsty1* cells subjected to the same stress do not undergo such size reduction (Fig. 1B). This morphological alteration represents one additional aspect of the Sty1-dependent stress response. Whether it only results from an altered cell cycle or if it is part of an active mechanism of defence to stress is still unclear.

1.2. PHOSPHORYLATED Sty1 TRANSLOCATES TO THE NUCLEUS AND BINDS TO STRESS-DEPENDENT PROMOTERS

Wis1 phosphorylates Sty1 in two residues: Thr-171 and Tyr-173 (Fig. 2A). This phosphorylation activates Sty1 in front of a stress situation, which results in Sty1 translocation to the nucleus. We are able to monitor these events using different strategies, such as Western blot, using a commercial anti phospho-p38 antibody (Fig. 2B) or by fluorescence microscopy using a GFP-tagged version of Sty1 (Fig. 2C). While Sty1 activation is immediate, the kinetics of its inactivation depends on the intensity of the stress.

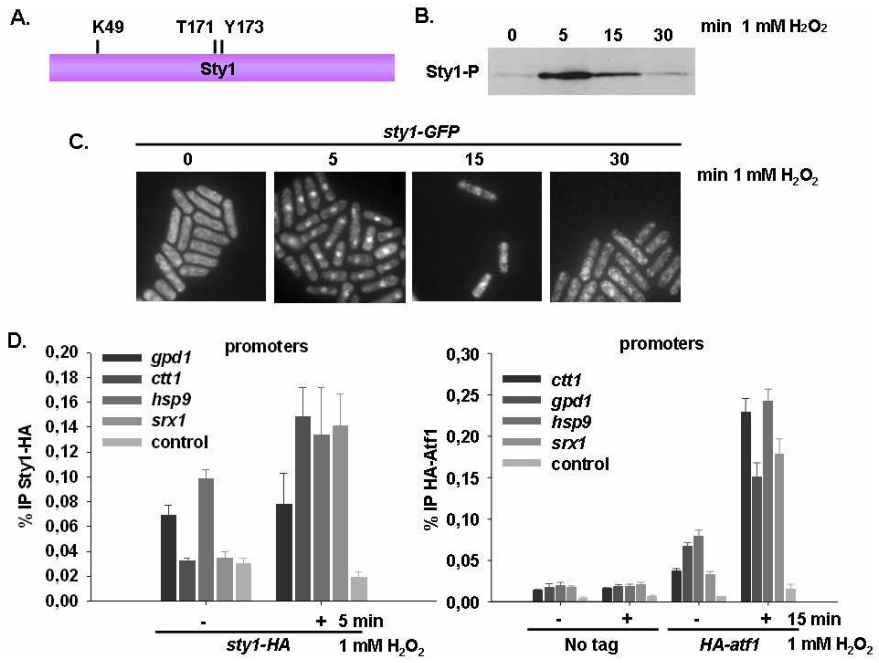


Figure 2. Sty1 is activated by stress. **A.** Schematic representation of Sty1 protein. **B.** Western blot analysis of Sty1 phosphorylation upon oxidative stress. WT cells (972) were grown in MM and cells were collected before and after treatment with 1 mM H_2O_2 at the times indicated. TCA extracts were prepared and protein was analysed by Western blot using anti-p38-P (Sty1-P). **C.** Nuclear Sty1-GFP accumulation upon H_2O_2 exposure. Cells from strain EHH5 (*sty1* gene tagged in C-terminal domain with GFP) were treated with 1 mM H_2O_2 in liquid MM. Cellular distribution of Sty1-GFP was determined by fluorescence microscopy at the times indicated. **D.** Sty1 and Atf1 binding to stress promoters. Strains AV8 (*sty1-HA*) and JM1066 (Δ*atf1*) harbouring p151.41x plasmid (containing the *nmt*-driven *HA-atf1* gene, p*HA-atf1*) were grown in YE5S and treated or not with 1 mM H_2O_2 during the time indicated, formaldehyde extracts were obtained and Sty1-HA or HA-Atf1 were precipitated using anti-HA monoclonal antibody. Recovered DNA was assayed by PCR amplification with primers encompassing the *gpd1*, *ctt1*, *srx1* and *hsp9* promoters or an intergenic region as a negative control. Data was obtained from three independent experiments and are expressed as mean+SEM.

Upon activation and migration to the nucleus, the kinase is recruited to the promoters of stress responsive genes like *gpd1, ctt1, srx1* or *hsp9*, and induces the transcriptional stress response, as shown in Fig. 2D by ChIP experiment. Surprisingly, we have found to kinds of stress

promoters in terms of Sty1 binding prior to stress. On one hand there are genes like *gpd1* and *hsp9* where Sty1 and its TF Atf1 are bound to their promoters even in the absence of stress, while genes like *srx1* and *ctt1* present recruitment of the kinase only after exposure to stress, correlating with lower basal binding of Atf1. This result does not correlate with nuclear migration of the kinase after stress exposure. It is worth pointing out that intracellular concentration of Sty1 is very high, and we speculate that it may be a small fraction of Sty1 inside the nucleus in basal conditions.

1.3. RNAPII RECRUITMENT TO STRESS GENES DEPENDS ON Sty1

Δsty1 strain is unable to proliferate in front of different stresses. In this background, it has been shown that the defence response is abolished at the transcriptional level (Chen *et al*., 2003). Accordingly, we have imposed different stresses of different strength to cell cultures, and observed that Sty1-dependent genes were totally switched off in the *Δsty1* strain (Fig. 3A).

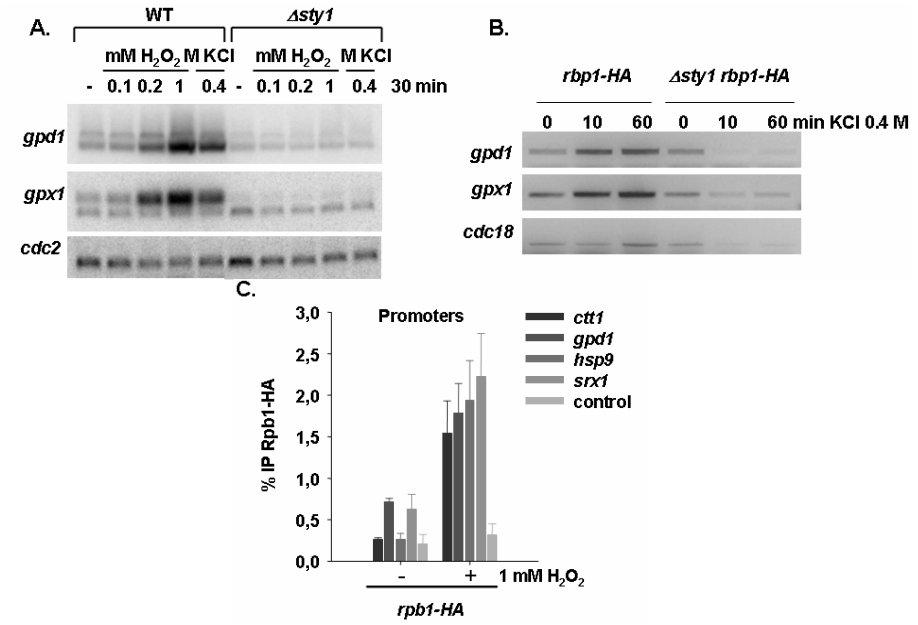


Figure 3. RNAPII is recruited to stress promoters in a Sty1-dependent manner. A. Impaired expression of stress genes in a *Δsty1* strain. RNA samples from strains 972 (WT) and AV18 (*Δsty1*) grown in MM till they reached an OD_{600} of 0.5 and treated or not (-) with H_2O_2 or KCl at the indicated concentrations for 30 min were analyzed by Northern blot. Radioactive probes of *gpd1* and *gpx1* were hybridized. As loading control *cdc2* probe was used. **B.** Recruitment of RNAPII to stress genes occurs in response to oxidative stress and depends on Sty1. Cell cultures from strains CN011 (*rpb1-HA*) and CN009 (*rpb1-HA Δsty1*) grown in MM as in A, were treated with 0.4 M KCl and samples were collected at the times indicated. ChIP experiment was performed using anti-HA monoclonal antibody to immunoprecipitate RNPAII through the large subunit Rpb1 tagged with HA. *gpd1* and *gpx1* oligos were used to amplify promoter regions. *cdc18* ORF oligos were used as control. **C.** RNAPII binds to stress promoters upon oxidative stress activation. Rpb1-HA cells (AV8) were grown in YE5S and treated or not with 1 mM H_2O_2 during 15 min, formaldehyde extracts were obtained and Rpb1 was precipitated using anti-HA monoclonal antibody. Recovered DNA was processed as in Fig. 2D.

In wild type cells, the recruitment of Sty1 to CESR gene promoters such as those of *gpd1* and *gpx1* correlates with RNAPII transcription, whereas in the absence of Sty1 RNAPII is not loaded on

promoters after stress impairment, demonstrating that RNAPII recruitment is Sty1-dependent (Fig. 3B).

The RNAPII binding upon stress is significantly greater compared to the Sty1 and Atf1 recruitment (Fig. 2D and 19A, respectively). This result suggests that the presence of the MAPK or the TF do not determine the onset of the transcription, and only the entrance of the RNAPII correlates with initiation of transcription.

1.4. NUCLEAR LOCALIZATION OF Sty1 IS NOT SUFFICIENT TO ACTIVATE TRANSCRIPTION

It is known that Sty1 and Pap1 travel to the nucleus to activate transcription (Gaits *et al*., 1998; Castillo *et al*., 2002). The localisation of Pap1 represents actually a major aspect of its regulation (Vivancos *et al*., 2006). When present into the nucleus, Pap1 is bound to promoters and activates the transcription of Pap1-dependent genes. To address if Sty1 behaves in a similar way (i.e. if Sty1 nuclear localisation is enough to activate transcription) we expressed a constitutive nuclear amino-tagged GFP-Sty1 protein from an episomal vector. This tagged version of Sty1 at its N-terminal domain unexpectedly displayed constitutively nuclear localization, probably as a consequence of a tag-induced structural change affecting Sty export (Fig. 4A). We analyzed the expression pattern of this strain in response to stress (Fig. 4B).

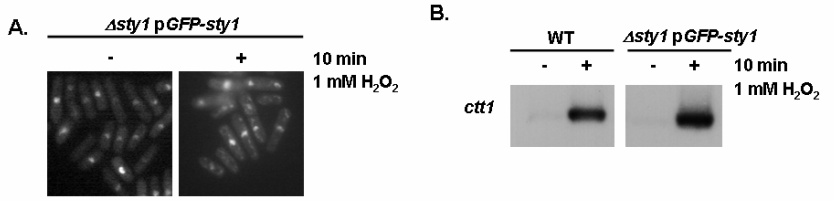


Figure 4. Constitutive nuclear accumulation of Sty1 is not sufficient to activate transcription. A. GFP-Sty1 is nuclear even in the absence of stress. Strain NT244 was transformed with the *nmt*-driven p179.41x (*Δsty1 pGFP-sty1*). Cellular distribution of the fusion protein in cells treated or not with 1 mM H_2O_2 for 10 min was determined by fluorescence microscopy. **B.** Nuclear accumulation of Sty1 does not activate transcription. Cell cultures from strains 972 (WT) and NT244 transformed with *nmt*-driven p179.41x (*Δsty1 pGFP-sty1*) were grown in MM until they reached an OD_{600} of 0.5 and treated with 1 mM H_2O_2. Samples were collected at the indicated time points, total RNA was analyzed by Northern blot and hybridized with *ctt1* radioactive probe as an example of classical CESR gene.

Our results show that strict Sty1 nuclear localization is not sufficient to transcribe Sty1-dependent genes but only happens when the protein is phosphorylated. It has been reported that the activation of Sty1 inside the nucleus is mediated by the MAPK kinase Wis1, which can travel into the nucleus and phosphorylate the single kinase (Gaits *et al*., 1998).

1.5. THE CATALYTIC ACTIVITY OF Sty1 IS ESSENTIAL FOR PROPER CELL CYCLE, SURVIVAL IN FRONT OF STRESS AND TRANSCRIPTION ACTIVATION

The kinase activity of Sty1 requires a functional ATP binding site. To address if the function of Sty1 only relies on its catalytic activity, we generated the p*HA-sty1·K49R* plasmid, expressing a HA-tagged version of Sty1 mutated in the predicted ATP binding site (Lys-49-Arg replacement) resulting in the so-called “kinase-dead” form of the enzyme (Fig. 5A). As control, we used a wild type HA-tagged Sty1 expression plasmid (p*HA-sty1*).

Both plasmids were transformed into a *Δsty1* strain. *Δsty1* cells transformed with p*HA-sty1·K49R*, had the same cell length defects as a *Δsty1* (Fig. 5B), were still sensitive to heavy metals in the media (Fig. 5C) and did not activate the transcription of CESR genes in front of oxidative

or osmotic stress (Fig. 5D). On the other hand, the *Δsty1* phenotype was rescued by p*HA-sty1* transformation: the cells behave as wild type with respect to cell length, stress resistance, and transcription suggesting that Sty1 is expressed at adequate levels and that the epitope does not inhibit Sty1 function. All together, our results suggest that the kinase activity of Sty1 is the only function of the protein required for transcription, cell cycle and stress surveillance and that no other part of the protein itself contributes to this activity.

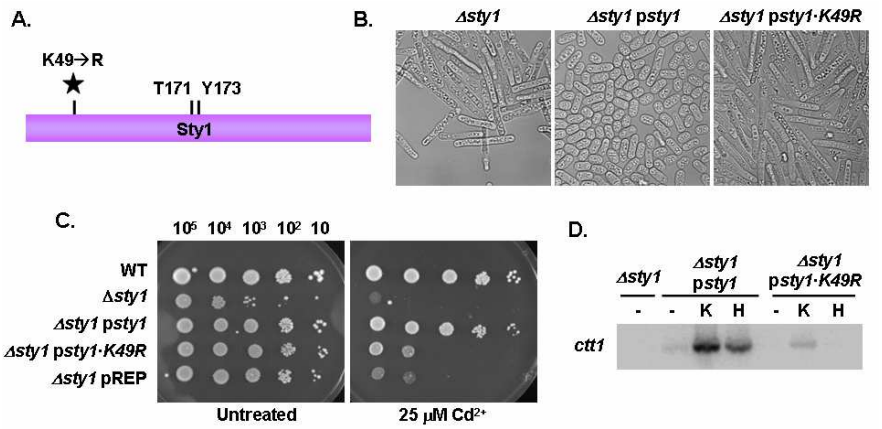


Figure 5. Kinase activity of Sty1 is essential for stress survival and proper cell cycle regulation. A. Schematic representation of Sty1. Phosphorylable Sty1 residues (T and Y) and point mutation to abolish kinase activity (K to R) are indicated. **B.** Sty1·K49R is not able to rescue morphology phenotype of *Δsty1* strain. Cells from strain NT224 were transformed with the *nmt*-driven p*sty1* (*psty1-HA-6His*) or p*sty1·K49R* (p*sty1·K49R-HA-6His*) were cultured in MM until OD_{600} of 0.5 without thymine for 18 hours. Cell morphology was analyzed by differential interference contrast microscopy. **C.** *sty1·K49R* mutant cells are sensitive to stress. Strains PN513 (WT), NT224 transformed with empty episomal *nmt*-driven plasmid pREP and the same strains as in B were grown in MM 10 to 10^5 cells were spotted into MM plates with or without 25 μM cadmium. **D.** Kinase activity of Sty1 is essential to activate stress genes. The same strains as in B were grown in MM and treated or not (-) with 0.4 M KCl (K), 1 mM of H_2O_2 (H). RNA samples were analyzed by Northern blot and hybridized with *ctt1* radioactive probe.

1.6. THE KINASE-DEAD Sty1·K49R CONFERS A BASAL ACTIVATION OF THE MAPK PATHWAY

One possible reason of the kinase-dead Sty1·K49R phenotype could be the inability of this modified protein to migrate to the nucleus. To address this hypothesis we mutated s*ty1* in its own locus and epitope-tagged the mutated gene with a GFP in its C-terminal domain. As control, we generated a strain targeted with the same tag but maintaining the wild type sequence of the gene.

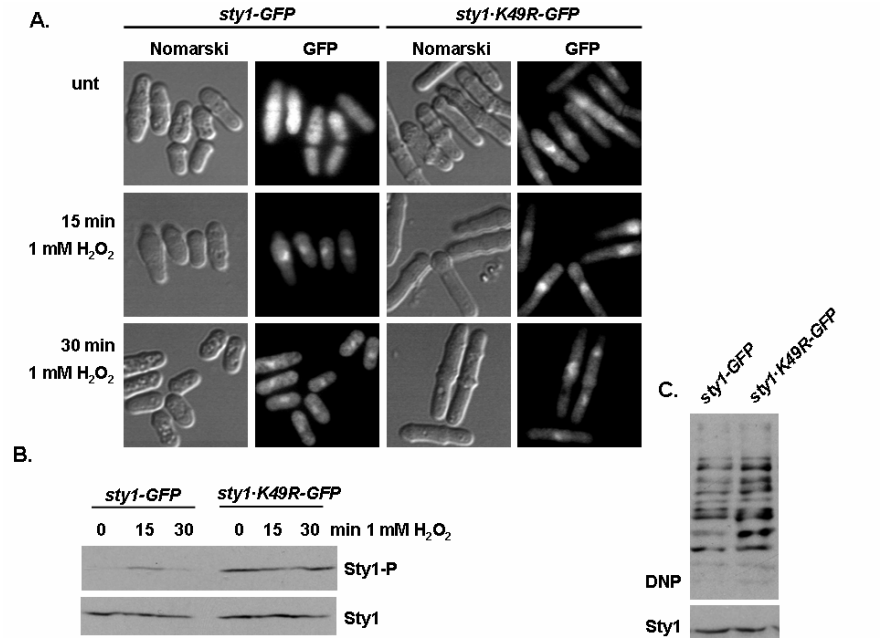


Figure 6. Sty1·K49R confers basal oxidative stress. A. Sty1·K49R-GFP localizes in the nuclei. Strains MS74 (*sty1-GFP)* and MS75 *(sty1·K49R-GFP)* tagged at the chromosomal loci were grown in MM and cellular distributions of the fusion proteins under non stressed conditions or treated with 1 mM of H_2O_2 at the times indicated were determined by fluorescence microscopy (GFP). The same cells under differential interference contrast (Nomarski) optics are shown. **B.** Sty1·K49R is basally phosphorylated. The same cells as in A were collected and TCA extracts were analyzed by Western blot using polyclonal antibodies to detect Sty1 with anti-Sty1, or with anti-p38-P for Sty1-phosphorylated form (Sty1-P). **C.**

Oxidative damage is exacerbated in cells expressing Sty1·K49R. Protein carbonylation from untreated samples as in A were analyzed by reaction of carbonyl groups with DNPH, followed by SDS-PAGE and Western blot by using anti-DNP (DNP, upper panel) and anti-Sty1 (Sty1, bottom panel) as loading control.

We then compared the localization of Sty1·K49R-GFP under normal and oxidative stress conditions with the localisation of the wild type tagged version of the protein. In normal conditions, we observed that Sty1·K49R-GFP accumulated in the nucleus, whereas the wild type-tagged counterpart was excluded from the nuclear compartment (Fig. 6A). Under stress conditions, both proteins were nuclearly localised (Fig. 6A).

After detecting the presence of Sty1·K49R-GFP into the nucleus, even in the absence of stress, we investigated the impact of this misregulation on the activation of the MAPK pathway, by monitoring the phosphorylation of Sty1 in the Sty1·K49R-GFP background compared to Sty1-GFP. Interestingly, we found that the mutant MAPK Sty1 was basally activated, correlating with its nuclear localisation under normal conditions (Fig. 6B). Finally, we measured the presence of elevated reactive oxygen species in the mutant strain, by analyzing protein damage due to oxidative stress. Protein carbonylation was detected using a 2.4-dinitrophenol antibody. As shown in Fig. 6C, Sty1·K49R-GFP confers basal carbonylation, which is the consequence of elevated ROS in the cell, even in the absence of stress.

2. THE TRANSCRIPTION FACTORS Pcr1 AND Atf1 HAVE DISTINCT ROLES IN STRESS- AND Sty1-DEPENDENT GENE REGULATION

The MAP kinase Sty1 regulates the transcriptional responses promoting cell survival triggered by different environmental stresses in *S. pombe*. Upon stress activation, Sty1 reversibly migrates in the nucleus, where it stimulates a transcriptonal response via the Atf1 transcription factor. Atf1 heterodimerizes with Pcr1, but the role of this association is still unclear. To clarify this, we have carried out a comparative analysis of *Δatf1* and *Δpcr1* cells individually, demonstrating that cells lacking Atf1 or Pcr1 do not share all the phenotypes reported for *Δatf1* cells. Transcriptome analysis reveals that the response to specific stresses of cells lacking either Pcr1 or Atf1 do not fully overlap; and even though most of the genes induced by osmotic stress are Atf1- and Pcr1-dependent, a subset of genes only require Atf1. Whereas most stress-dependent promoters requires the presence of Pcr1 to bind Atf1, we demonstrate here that some promoters are Pcr1-independent for the Atf1 binding *in vivo*. Moreover, Atf1 binding to promoters of stress genes is not dependent on the presence of the MAPK Sty1. Finally, genome wide analyses revealed a global repressive role on stress-dependent and -independent genes for both transcription factors.

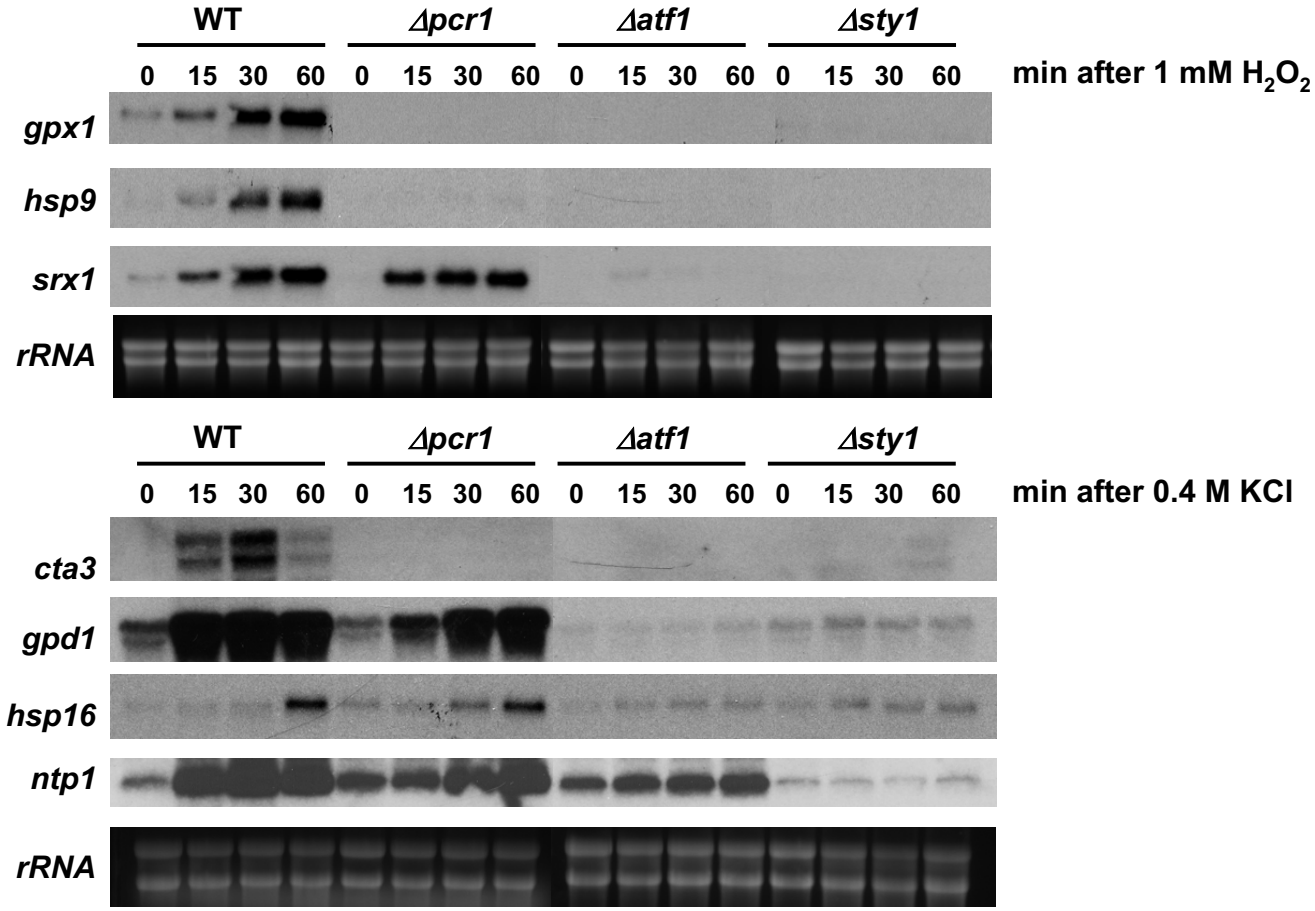
This Chapter includes:

Published results in the paper “The transcription factors Pcr1 and Atf1 have distinct roles in stress- and Sty1-dependent gene regulation” Miriam

Sansó, Madelaine Gogol, José Ayté, Chris Seidel and Elena Hidalgo. Eukaryiotic cell (2008) 7, 826-835.

Unpublished results that complement the previous manuscript.

Sansó M, Gogol M, Ayté J, Seidel C, Hidalgo E. Transcription factors Pcr1 and Atf1 have distinct roles in stress-and Sty1-dependent gene regulation. Eukaryot Cell. 2008; 7(5): 826-35.



Supplem. Fig. 1. Pcr1 is required for transcription of most, but not all, stress-response genes. Cultures of strains 972 (WT), MS5 (*Δpcr1*), AV15 (*Δatf1*) and AV18 (*Δsty1*), were left untreated (0) or were treated for 15, 30 or 60 min with 1 mM H_2O_2 or 0.4 M KCl, as indicated. Total RNA was extracted and analyzed by Northern blotting with the probes indicated. Ribosomal RNA (*rRNA*) was used as a loading control.

Supplementary Table I. Fold induction of A to G subsets of genes induced over two-fold in wild-type, *Δatf1* and/or *Δpcr1* cells

A= genes induced over 2-fold by KCl in wild-type, but not in *Δatf1* nor *Δpcr1,* cells (KCl induction is Atf1- and Pcr1-dependent)

NAME	WT- Unt.	*Δatf1*- Unt[a]	*Δpcr1* -Unt[a]	WT - KCl[a]	*Δatf1*- KCl[b]	*Δpcr1* –KCl[b]
SPAC23H3.15c	1.00	1.33	1.35	74.07	1.58	1.11
SPAC869.06c	1.00	1.12	0.93	26.81	1.15	1.48
SPACUNK4.17	1.00	0.81	0.87	16.93	1.15	1.48
SPAC513.02	1.00	1.44	1.75	16.41	1.69	1.45
SPBC24C6.09c	1.00	2.70	2.09	14.11	1.56	1.26
SPAPB1A11.03	1.00	1.43	2.32	12.48	1.18	1.37
SPAC637.03	1.00	1.28	1.74	12.11	1.18	1.47
SPAC869.09	1.00	1.21	1.27	11.86	1.33	0.96
SPCC757.03c	1.00	0.90	0.97	11.48	1.24	1.16
SPAC139.05	1.00	0.69	0.71	11.33	1.71	1.20
SPAC32A11.02c	1.00	1.81	1.72	10.69	0.97	1.14
SPAC3G6.07	1.00	3.11	1.69	10.38	1.82	1.63
rds1	1.00	0.31	0.42	9.22	1.54	0.93
eno102	1.00	0.55	0.75	9.14	1.37	1.27
gpx1	1.00	0.09	0.19	9.07	1.73	0.82
SPAC15E1.02c	1.00	0.37	0.48	8.60	1.22	0.84
mel1	1.00	0.56	1.75	8.49	1.15	1.08
SPBPB21E7.04c	1.00	2.84	10.04	8.14	1.59	0.47
SPAC4F10.17	1.00	1.15	0.94	8.05	1.23	1.04
ctt1	1.00	0.69	1.11	7.65	0.89	1.92
SPAC19G12.09	1.00	0.43	0.69	7.31	1.30	1.11
SPBC23G7.11	1.00	2.68	1.97	6.62	1.46	1.84
cta3	1.00	1.45	1.28	6.25	1.34	1.53
SPCC1393.12	1.00	0.73	0.75	6.15	1.12	0.97
gut2	1.00	0.61	0.59	5.94	1.02	1.20
SPBC725.10	1.00	1.31	1.56	5.54	0.94	1.88
SPAC11D3.01c	1.00	1.57	0.88	5.47	0.49	0.45
SPBC215.11c	1.00	1.15	1.34	5.45	1.14	1.81
SPAC4H3.08	1.00	1.14	1.08	5.45	1.24	1.17
SPCP31B10.06	1.00	2.12	3.02	5.40	1.52	1.28
gst3	1.00	0.97	1.41	4.96	1.51	1.41
SPBC660.06	1.00	2.29	3.17	4.72	1.18	1.44
SPAC5H10.02c	1.00	1.49	1.43	4.64	1.50	1.12
SPAC23C11.06c	1.00	0.96	1.18	4.55	1.06	1.09
SPAC869.08	1.00	1.25	1.58	4.39	1.37	1.35
SPBC1685.13	1.00	0.69	0.87	4.26	0.60	0.77
SPAC29A4.01c	1.00	0.99	1.39	4.25	1.63	1.48
SPAC3C7.13c	1.00	1.11	1.31	4.00	0.89	1.02
SPAC29A4.12c	1.00	1.25	4.73	3.54	1.05	1.60
SPCC736.15	1.00	0.92	1.63	3.45	1.20	1.45
SPAC6B12.03c	1.00	9.06	8.81	3.39	1.08	1.89
SPBC216.03	1.00	2.04	2.30	3.37	1.31	1.62
SPCC417.13	1.00	1.57	2.30	3.32	1.95	1.49
SPBC3H7.08c	1.00	0.89	1.56	3.31	1.40	1.37
SPAC23D3.11	1.00	0.66	0.82	3.21	1.04	1.16

SPAC3H8.09c	1.00	0.91	0.72	3.16	1.27	1.48
ish1	1.00	0.91	0.95	3.15	1.19	1.35
SPCC1322.04	1.00	0.55	1.06	3.15	1.22	1.11
SPAC750.08c	1.00	0.61	0.94	3.14	1.89	0.75
SPBC725.03	1.00	0.85	1.02	3.08	1.99	1.31
SPCC338.12	1.00	0.52	1.05	3.07	1.22	1.80
jmj1	1.00	1.44	1.06	3.05	1.11	1.46
SPAC2F3.05c	1.00	0.56	0.72	3.04	1.47	1.89
SPAC212.09c	1.00	0.59	0.96	2.98	1.76	0.69
SPBC30D10.14	1.00	1.22	1.45	2.92	1.57	1.68
glo1	1.00	2.04	1.99	2.89	1.44	1.57
SPBPB2B2.02	1.00	0.48	0.65	2.85	1.47	0.99
SPAC4D7.02c	1.00	1.00	1.25	2.83	1.33	1.72
SPBC713.11c	1.00	0.50	1.33	2.81	1.15	1.13
vip1	1.00	0.74	1.29	2.76	1.42	1.97
isp6	1.00	2.55	4.31	2.75	1.30	1.81
mae2	1.00	0.60	0.88	2.68	1.83	0.77
SPCP20C8.03	1.00	0.53	0.49	2.68	1.14	1.00
SPCC1322.07c	1.00	3.22	1.77	2.63	0.71	1.35
SPAC26H5.09c	1.00	0.96	1.10	2.63	0.81	1.10
SPAC22F8.03c	1.00	0.48	0.71	2.60	1.27	1.69
SPAC977.13c	1.00	0.92	1.19	2.55	0.87	0.96
atf1	1.00	0.13	4.53	2.54	1.46	1.70
SPBPB21E7.02c	1.00	1.18	1.14	2.54	1.46	1.15
SPBC1198.01	1.00	1.38	1.50	2.50	1.11	1.11
cmb1	1.00	1.29	1.45	2.48	1.84	1.40
SPCC736.13	1.00	1.05	1.52	2.47	1.29	1.31
SPBC557.06c	1.00	1.16	1.05	2.44	1.32	1.61
SPACUNK4.16c	1.00	1.48	2.61	2.41	1.43	1.43
SPCPB1C11.02	1.00	1.52	2.50	2.40	1.12	1.03
SPBP35G2.12	1.00	0.88	0.91	2.38	1.47	1.34
SPBC800.14c	1.00	1.03	1.94	2.37	1.93	1.50
exg3	1.00	1.17	1.56	2.36	0.96	1.04
SPBC216.04c	1.00	0.89	1.05	2.35	1.14	1.45
tms1	1.00	0.99	1.06	2.33	1.06	1.02
SPCC70.02c	1.00	1.01	1.61	2.31	1.52	1.37
SPAC13F5.07c	1.00	2.42	3.15	2.31	1.05	1.28
SPCC594.04c	1.00	1.53	1.58	2.30	1.17	1.20
SPAC3A11.10c	1.00	1.59	2.84	2.28	1.93	1.38
SPBC119.03	1.00	0.93	1.55	2.28	1.36	1.48
SPAC13F5.03c	1.00	0.75	2.03	2.27	1.00	1.14
SPBC1289.16c	1.00	0.74	1.51	2.25	1.24	1.39
SPCC569.03	1.00	0.78	0.77	2.24	0.98	0.92
SPAC29A4.17c	1.00	2.28	2.71	2.22	1.10	0.90
SPAC4G8.01c	1.00	1.07	1.03	2.22	0.94	1.18
SPAC8C9.16c	1.00	1.11	1.98	2.22	1.93	1.31
SPAC13D6.01	1.00	2.87	2.25	2.21	1.22	1.07
SPAC637.13c	1.00	1.37	1.29	2.21	1.00	1.24
btn1	1.00	2.04	1.96	2.21	1.04	1.84
SPCC4G3.03	1.00	2.02	2.13	2.19	1.46	1.19
sod2	1.00	1.30	1.18	2.18	0.62	1.00
fum1	1.00	1.07	0.97	2.18	1.09	0.66
SPBC19G7.18c	1.00	1.03	1.26	2.17	1.41	1.31
SPCC1281.04	1.00	0.79	0.76	2.15	1.09	1.28

SPAPB24D3.07c	1.00	0.47	1.78	2.15	1.24	0.84
SPBC83.19c	1.00	0.70	1.45	2.15	1.48	1.23
SPCC61.03	1.00	1.33	1.49	2.12	1.41	1.25
SPAC1F7.06	1.00	0.84	0.86	2.12	1.16	1.37
SPBC14F5.10c	1.00	1.78	1.67	2.12	1.29	1.54
SPAP8A3.03	1.00	1.04	1.13	2.11	1.32	1.58
SPAPB1E7.08c	1.00	0.99	1.08	2.11	1.33	1.88
isa2	1.00	1.47	1.74	2.07	1.49	1.42
SPAPB2B4.04c	1.00	0.93	1.08	2.07	0.96	1.19
SPBC2A9.02	1.00	0.74	0.95	2.07	0.80	1.14
fbp1	1.00	2.87	3.25	2.05	0.96	1.36
ppk15	1.00	1.76	1.88	2.05	1.29	1.58
SPCC1183.09c	1.00	1.45	2.02	2.03	1.05	0.87
mug66	1.00	2.70	2.41	2.02	1.03	1.12

B= genes induced over 2-fold by KCl in *Δatf1*, but not in wild-type nor *Δpcr1*, cells

NAME	WT- Unt.	*Δatf1*- Unt[a]	*Δpcr1* -Unt[a]	WT - KCl[a]	*Δatf1*- KCl[b]	*Δpcr1* –KCl[b]
SPBC32F12.10	1.00	1.26	1.69	1.93	2.02	1.15
psp3	1.00	1.19	1.83	1.88	2.25	1.47
oca2	1.00	0.64	1.65	1.77	2.52	0.76
SPBC713.09	1.00	0.99	1.48	1.75	2.86	1.62
SPAC3A12.08	1.00	1.10	1.51	1.66	2.13	1.66
SPAC24C9.06c	1.00	1.44	1.58	1.64	2.39	1.13
sod1	1.00	1.04	1.08	1.60	2.24	1.88
SPBC23G7.06c	1.00	1.17	1.64	1.58	2.03	1.39
SPBC8D2.11	1.00	1.03	1.16	1.57	2.05	1.70
SPBC1348.05	1.00	4.84	1.25	1.55	2.10	1.26
SPAC1952.09c	1.00	0.93	1.14	1.50	2.58	1.61
mag1	1.00	1.20	1.71	1.48	2.11	1.39
rhp14	1.00	1.11	1.06	1.48	2.02	1.60
cdc48	1.00	1.11	1.51	1.44	2.23	1.50
SPBC83.17	1.00	1.11	1.41	1.42	2.91	1.71
SPAC31G5.21	1.00	1.09	1.14	1.39	2.25	1.27
sti1	1.00	1.31	1.58	1.10	2.20	1.70
ssa1	1.00	0.75	2.59	1.04	2.13	1.30
SPAC4C5.05c	1.00	0.77	0.68	1.04	2.32	1.88
SPBC4F6.17c	1.00	0.89	1.74	0.92	2.14	1.68
wis2	1.00	1.03	1.47	0.81	2.03	1.43
SPBPB2B2.05	1.00	0.43	0.46	0.65	2.35	0.79

C= genes induced over 2-fold by KCl in *Δpcr1*, but not in wild-type nor *Δatf1*, cells

NAME	WT- Unt.	*Δatf1*- Unt[a]	*Δpcr1* -Unt[a]	WT - KCl[a]	*Δatf1*- KCl[b]	*Δpcr1* –KCl[b]
SPBC1685.05	1.00	1.25	1.26	1.99	1.20	2.44
SPBC1105.14	1.00	1.02	1.33	1.91	1.42	2.30
SPAC869.05c	1.00	1.16	1.66	1.87	1.87	2.36
SPBC12C2.03c	1.00	1.91	1.53	1.81	1.33	2.17
gst2	1.00	1.79	2.19	1.81	0.85	2.24
SPAC6B12.07c	1.00	1.09	1.86	1.75	0.97	2.65
srx1	1.00	1.36	3.53	1.71	0.97	2.88
SPAC24C9.14	1.00	1.11	1.39	1.57	1.60	2.02
SPCC569.05c	1.00	4.82	4.03	1.51	1.98	3.82

SPCC1183.11	1.00	1.12	1.67	1.49	1.37	2.82
pyp2	1.00	1.14	1.12	1.41	1.59	2.07
SPAC26F1.08c	1.00	0.81	1.49	1.36	0.99	2.24
SPAC1F12.10c	1.00	1.63	1.00	1.35	1.17	3.02
SPBC14F5.13c	1.00	1.34	1.22	1.35	0.80	2.32
sld3	1.00	1.34	1.28	1.31	1.38	2.67
cdc10	1.00	1.28	1.66	1.22	1.01	2.32
ppk31	1.00	0.86	0.65	1.22	1.05	2.20
atf21	1.00	0.90	0.94	1.18	1.27	2.15
SPAC3A11.09	1.00	0.89	0.83	1.14	1.19	3.00
SPAC29B12.10c	1.00	1.16	0.61	1.13	1.32	2.02
SPBC660.05	1.00	0.40	2.11	1.10	1.09	2.76
SPAC186.07c	1.00	0.68	2.14	1.07	1.05	2.13
SPCC1442.16c	1.00	1.01	1.25	1.05	1.66	2.64
SPBC839.16	1.00	0.93	1.17	0.79	0.88	3.08
SPAC4G8.03c	1.00	0.94	0.91	0.60	0.69	3.19
bfr1	1.00	1.09	1.47	0.40	0.57	2.19

D= genes induced over 2-fold by KCl in wild-type and *Δatf1*, but not in *Δpcr1*, cells (KCl induction is Pcr1-dependent and Atf1-independent?)

NAME	WT- Unt.	*Δatf1*- Unt[a]	*Δpcr1* -Unt[a]	WT - KCl[a]	*Δatf1*- KCl[b]	*Δpcr1* –KCl[b]
SPBC1289.14	1.00	0.64	0.46	39.72	2.36	0.96
SPAC13C5.04	1.00	0.84	0.82	6.00	2.79	1.09
SPCC794.04c	1.00	0.21	2.60	4.16	4.71	0.68
SPCC16A11.15c	1.00	1.33	1.51	3.45	2.34	1.44
SPAC57A7.02c	1.00	1.02	1.54	2.65	2.06	1.93
SPAC23G3.13c	1.00	1.44	2.39	2.23	2.12	1.74
SPAC22E12.03c	1.00	1.09	1.14	2.19	2.09	1.44
SPBC947.15c	1.00	1.02	1.35	2.19	2.05	1.47
SPBC20F10.03	1.00	1.43	1.26	2.18	2.06	1.98
SPBC1711.11	1.00	1.89	2.06	2.03	2.04	1.72

E= genes induced over 2-fold by KCl in *Δatf1* and *Δpcr1*, but not in wild-type cells

NAME	WT- Unt.	*Δatf1*- Unt[a]	*Δpcr1* -Unt[a]	WT - KCl[a]	*Δatf1*- KCl[b]	*Δpcr1* –KCl[b]
SPCC1739.06c	1.00	0.72	0.76	1.99	5.48	5.13
SPAC23A1.14c	1.00	1.03	1.36	1.80	2.37	2.06
SPBP16F5.08c	1.00	0.99	1.16	1.76	3.92	4.50
SPBC3H7.02	1.00	0.77	1.03	1.74	3.69	3.07
trx1	1.00	0.97	1.59	1.70	2.41	2.28
SPBC106.17c	1.00	1.00	0.82	1.54	2.64	3.24
zwf1	1.00	0.78	0.84	1.40	2.50	2.77
SPBC428.11	1.00	0.63	0.54	1.38	3.02	2.29
sua1	1.00	1.20	1.02	1.36	3.16	2.55
pof1	1.00	1.29	1.35	1.22	4.54	3.18
SPAC57A10.06	1.00	0.77	0.70	0.87	2.52	2.45

F= genes induced over 2-fold by KCl in wild-type and *Δpcr1*, but not in *Δatf1*, cells (KCl induction is Atf1- dependent and Pcr1-independent)

NAME	WT- Unt.	*Δatf1*- Unt[a]	*Δpcr1* -Unt[a]	WT - KCl[a]	*Δatf1*- KCl[b]	*Δpcr1* –KCl[b]

SPAC22A12.17c	1.00	3.38	2.62	19.57	1.28	2.16
SPAC22F8.05	1.00	1.13	1.78	11.28	1.33	2.50
SPBC56F2.06	1.00	2.86	6.38	8.17	1.10	3.56
SPAC4H3.03c	1.00	3.70	3.91	7.15	1.37	2.57
SPBC12C2.04	1.00	1.60	2.14	6.77	1.20	2.19
SPBC11C11.06c	1.00	0.38	1.16	6.15	1.22	3.34
SPBC23G7.10c	1.00	0.45	0.62	6.01	1.43	3.17
SPAC3C7.05c	1.00	1.75	1.89	5.71	1.66	2.58
SPBC428.10	1.00	0.87	1.01	5.18	1.56	2.23
SPBC16A3.02c	1.00	1.25	4.18	4.81	1.57	4.23
SPAC26F1.07	1.00	0.41	3.81	4.51	1.42	4.12
tps1	1.00	1.49	2.53	4.25	1.96	2.81
ntp1	1.00	2.56	4.07	4.07	1.92	2.74
SPBC21C3.19	1.00	0.19	0.63	3.60	1.33	5.72
gpd1	1.00	0.34	1.11	3.41	1.21	3.70
SPCC965.06	1.00	0.31	0.48	3.39	1.25	2.26
SPAPJ691.02	1.00	1.10	2.07	3.33	1.81	2.04
git5	1.00	0.82	1.32	3.17	1.21	2.19
SPCPB16A4.06c	1.00	1.15	1.10	3.17	1.21	2.07
SPBC23E6.03c	1.00	1.01	1.41	3.06	1.26	2.02
SPCC63.14	1.00	2.38	3.20	2.72	0.92	2.04
SPAC9E9.04	1.00	0.32	1.01	2.54	1.09	2.46
SPCC306.08c	1.00	2.22	2.93	2.41	1.84	2.19
SPACUNK4.15	1.00	2.17	4.07	2.30	0.88	2.69
ght2	1.00	1.21	1.22	2.30	1.76	2.90
SPAC26F1.04c	1.00	0.65	0.83	2.27	1.70	2.94
SPAC4H3.04c	1.00	1.23	1.89	2.25	1.76	2.00
SPAC3G9.11c	1.00	1.58	6.70	2.20	1.14	4.05
SPAC186.02c	1.00	0.80	0.94	2.16	1.59	2.21
SPAC16A10.01	1.00	1.27	1.47	2.05	1.14	2.74
SPBC21H7.06c	1.00	2.10	1.73	2.02	1.36	3.13
psi1	1.00	1.55	2.65	2.02	1.98	2.27

G= genes induced over 2-fold by KCl in wild-type, *Δatf1* and *Δpcr1* cells (KCl induction is Atf1- and Pcr1-independent)

NAME	WT- Unt.	*Δatf1*- Unt[a]	*Δpcr1* -Unt[a]	WT - KCl[a]	*Δatf1*- KCl[b]	*Δpcr1* –KCl[b]
zym1	1.00	0.52	0.87	33.15	2.08	2.61
SPAC27D7.10c	1.00	0.36	1.10	11.76	2.59	4.90
hsp9	1.00	0.03	1.08	10.65	4.08	5.12
SPAC27D7.09c	1.00	0.40	1.06	10.56	2.12	4.47
SPAC27D7.11c	1.00	1.50	3.53	9.86	3.25	3.16
hsp16	1.00	0.58	3.22	8.65	3.54	3.50
SPBPB2B2.08	1.00	0.08	0.12	3.63	43.61	28.56
SPBPB10D8.02c	1.00	0.07	0.15	3.35	6.31	3.93
SPAC15E1.10	1.00	0.98	1.43	3.08	2.20	2.02
SPBPB10D8.01	1.00	0.08	0.18	2.89	9.85	7.23
SPBP8B7.05c	1.00	0.98	0.89	2.75	2.35	3.53
SPCC330.06c	1.00	1.00	1.48	2.49	3.62	3.40
hmt2	1.00	1.18	1.16	2.34	3.98	3.63
SPBC106.03	1.00	1.25	1.52	2.16	2.37	2.42
SPAC343.06c	1.00	1.17	1.64	2.06	2.30	2.09

[a]Fold-inductions are indicated using the wild-type strain as a reference (with an assigned value of 1).
[b]The fold induction of these KCl treatments are refered to the respective untreated values of the same strain, and not to the wild-type untreated values.

Supplementary Table II. List of KCl-induced genes de-repressed or repressed over two-fold under untreated conditions in *Δatf1* and/or *Δpcr1* cells (unstressed and stressed values)

KCl-dependent genes de-repressed over 2-fold in *Δatf1* and *Δpcr1* cells

NAME	WT-Unt.	*Δatf1*-Unt[a]	*Δpcr1*-Unt[a]	WT-KCl[a]	*Δatf1*-KCl[b]	*Δpcr1*-KCl[b]
SPBPB21E7.04c	1.00	2.84	10.04	8.14	1.59	0.47
SPAC6B12.03c	1.00	9.06	8.81	3.39	1.08	1.89
SPBC56F2.06	1.00	2.86	6.38	8.17	1.10	3.56
isp6	1.00	2.55	4.31	2.75	1.30	1.81
SPACUNK4.15	1.00	2.17	4.07	2.30	0.88	2.69
ntp1	1.00	2.56	4.07	4.07	1.92	2.74
SPAC4H3.03c	1.00	3.70	3.91	7.15	1.37	2.57
fbp1	1.00	2.87	3.25	2.05	0.96	1.36
SPCC63.14	1.00	2.38	3.20	2.72	0.92	2.04
SPBC660.06	1.00	2.29	3.17	4.72	1.18	1.44
SPAC13F5.07c	1.00	2.42	3.15	2.31	1.05	1.28
SPCP31B10.06	1.00	2.12	3.02	5.40	1.52	1.28
SPCC306.08c	1.00	2.22	2.93	2.41	1.84	2.19
SPAC29A4.17c	1.00	2.28	2.71	2.22	1.10	0.90
SPAC22A12.17c	1.00	3.38	2.62	19.57	1.28	2.16
mug66	1.00	2.70	2.41	2.02	1.03	1.12
SPBC216.03	1.00	2.04	2.30	3.37	1.31	1.62
SPAC13D6.01	1.00	2.87	2.25	2.21	1.22	1.07
SPCC4G3.03	1.00	2.02	2.13	2.19	1.46	1.19
SPBC24C6.09c	1.00	2.70	2.09	14.11	1.56	1.26

KCl-dependent genes de-repressed over 2-fold only in *Δpcr1* cells

NAME	WT-Unt.	*Δatf1*-Unt[a]	*Δpcr1*-Unt[a]	WT-KCl[a]	*Δatf1*-KCl[b]	*Δpcr1*-KCl[b]
SPAC3G9.11c	1.00	1.58	6.70	2.20	1.14	4.05
SPAC29A4.12c	1.00	1.25	4.73	3.54	1.05	1.60
atf1	1.00	0.13	4.53	2.54	1.46	1.70
SPBC16A3.02c	1.00	1.25	4.18	4.81	1.57	4.23
SPAC26F1.07	1.00	0.41	3.81	4.51	1.42	4.12
SPAC27D7.11c	1.00	1.50	3.53	9.86	3.25	3.16
hsp16	1.00	0.58	3.22	8.65	3.54	3.50
psi1	1.00	1.55	2.65	2.02	1.98	2.27
SPACUNK4.16c	1.00	1.48	2.61	2.41	1.43	1.43
SPCC794.04c	1.00	0.21	2.60	4.16	4.71	0.68
tps1	1.00	1.49	2.53	4.25	1.96	2.81
SPCPB1C11.02	1.00	1.52	2.50	2.40	1.12	1.03
SPAC23G3.13c	1.00	1.44	2.39	2.23	2.12	1.74
SPAPB1A11.03	1.00	1.43	2.32	12.48	1.18	1.37
SPCC417.13	1.00	1.57	2.30	3.32	1.95	1.49
SPBC12C2.04	1.00	1.60	2.14	6.77	1.20	2.19
SPAPJ691.02	1.00	1.10	2.07	3.33	1.81	2.04
SPBC1711.11	1.00	1.89	2.06	2.03	2.04	1.72
SPAC13F5.03c	1.00	0.75	2.03	2.27	1.00	1.14
SPCC1183.09c	1.00	1.45	2.02	2.03	1.05	0.87

KCl-dependent genes repressed over 2-fold only in *Δatf1* cells

NAME	WT-Unt.	*Δatf1*-Unt[a]	*Δpcr1*-Unt[a]	WT-KCl[a]	*Δatf1*-KCl[b]	*Δpcr1*-KCl[b]
hsp9	1.00	0.03	1.08	10.65	4.08	5.12
SPBC21C3.19	1.00	0.19	0.63	3.60	1.33	5.72
SPCC794.04c	1.00	0.21	2.60	4.16	4.71	0.68
SPAC9E9.04	1.00	0.32	1.01	2.54	1.09	2.46
gpd1	1.00	0.34	1.11	3.41	1.21	3.70
SPAC27D7.10c	1.00	0.36	1.10	11.76	2.59	4.90
SPBC11C11.06c	1.00	0.38	1.16	6.15	1.22	3.34
SPAC27D7.09c	1.00	0.40	1.06	10.56	2.12	4.47
SPAC19G12.09	1.00	0.43	0.69	7.31	1.30	1.11
SPBC23G7.10c	1.00	0.45	0.62	6.01	1.43	3.17
SPAPB24D3.07c	1.00	0.47	1.78	2.15	1.24	0.84
SPAC22F8.03c	1.00	0.48	0.71	2.60	1.27	1.69
SPBPB2B2.02	1.00	0.48	0.65	2.85	1.47	0.99

KCl-dependent genes repressed over 2-fold in *Δatf1* and *Δpcr1* cells

NAME	WT-Unt.	*Δatf1*-Unt[a]	*Δpcr1*-Unt[a]	WT-KCl[a]	*Δatf1*-KCl[b]	*Δpcr1*-KCl[b]
SPBPB10D8.02c[c]	1.00	0.07	0.15	3.35	6.31	3.93
SPBPB2B2.08	1.00	0.08	0.12	3.63	43.61	28.56
SPBPB10D8.01	1.00	0.08	0.18	2.89	9.85	7.23
gpx1	1.00	0.09	0.19	9.07	1.73	0.82
rds1	1.00	0.31	0.42	9.22	1.54	0.93
SPCC965.06	1.00	0.31	0.48	3.39	1.25	2.26
SPAC15E1.02c	1.00	0.37	0.48	8.60	1.22	0.84

[a]Fold-inductions are indicated using the wild-type strain as a reference (with an assigned value of 1).
[b]The fold induction of these KCl treatments are refered to the respective untreated values of the same strain, and not to the wild-type untreated values.
[c]Those 5 genes repressed in *Δatf1* and *Δpcr1*strains and in bold belong to the cadmium response

2.1. Atf1 AND Pcr1 ARE NUCLEAR PHOSPHOPROTEINS

The bZIP TFs Atf1 and Pcr1 have been isolated in several screenings and they were soon reported to co-immunoprecipitate in extracts (Kanoh *et al*., 1996). We therefore decided to analyze whether they can form complexes *in vivo* before and after stress. We used formaldehyde to preserve possible interactions between Atf1 and Pcr1. As shown in Fig. 7A, two bands corresponding to Pcr1 could be detected immunoprecipitating HA-Atf1, before and after stress. Regarding the role of such interaction, early studies using inmunofluorescence have shown that the Atf1 and Pcr1 partners are nuclear, and that the nuclear localization of Atf1 was dependent on the presence of Pcr1 (Gaits *et al.,* 1998). However, we detected nuclear localization of both GFP-tagged proteins, expressed from an heterologous promoter, independently of the presence of the other bZIP partner (Fig. 7B).

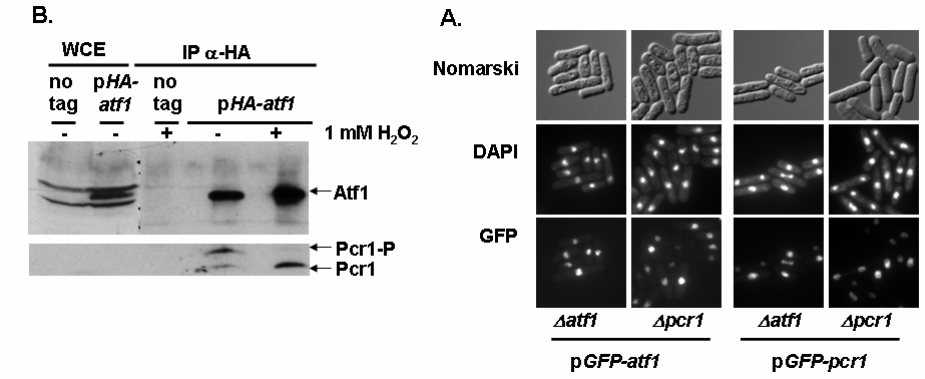


Figure 7. Atf1 and Pcr1 interact *in vivo* and are nuclear proteins. A. HA-Atf1 and Pcr1 interact in vivo before and after stress. Strains 972 (no tag) or MS9 (*Δatf1* with integrated, nmt-driven, p*HA-atf1*), were treated (+) or not (-) for 30 min with 1 mM H_2O_2, and formaldehyde extracts were obtained. 10 mg of total protein extracts were immunoprecipitated with anti-HA antibodies (IP α-HA), and the resulting immunoprecipitates were analyzed by SDS/PAGE and blotted with anti-HA or anti-Pcr1. As a loading control, 150 µg of whole cell extracts were loaded (WCE**). B.** Nuclear localization of GFP-Atf1 and GFP-Pcr1 proteins in *Δatf1* and *Δpcr1* strains. We used strains MS13 (*Δatf1* with integrated, nmt-driven, p*GFP-*

atf1), MS14 (*Δpcr1* with integrated, *nmt*-driven, p*GFP-atf1*), MS16 (*Δpcr1* with integrated, *nmt*-driven, p*GFP-pcr1*) and MS15 (*Δatf1* with integrated, *nmt*-driven, p*GFP-pcr1*). Cells were stained with DAPI to label DNA (center panels). The cellular distributions of the fusion proteins under non-stressed conditions were determined by fluorescence microscopy (GFP; lower panels). The same cells under differential interference contrast (Nomarski) optics are shown in the upper panels.

While analyzing the interaction between Atf1 and Pcr1, we noticed that endogenous Pcr1 appeared as a double band on Western blots. Treatment of cell extract with lambda phosphatase eliminated the slow migrating band, demonstrating that Pcr1 is a phosphoprotein (Fig. 8A). Furthermore, it is dephosphorylated in front of oxidative stress (Fig. 8B), but not osmotic stress (Fig. 2C of Sansó *et al.,* 2008), in a Sty1-dependent manner (Fig. 8C).

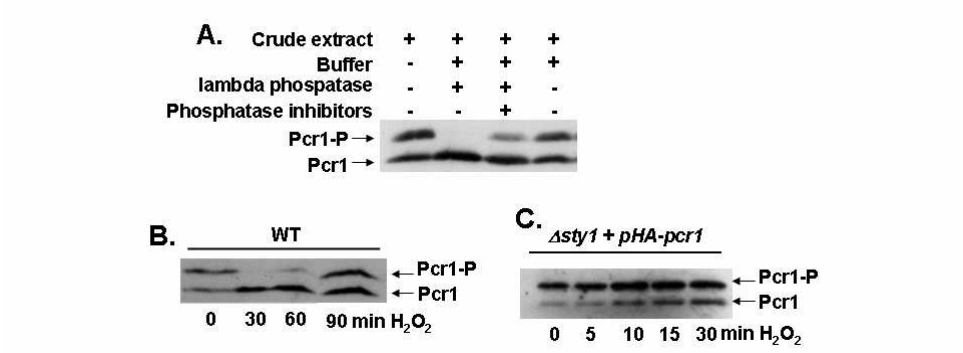


Figure 8. Pcr1 is a phosphoprotein. **A.** Pcr1 is phosphorylated under unstressed conditions. Native extracts from MS7 cells (Δpcr1) transformed with p138.41x, expressing HA-Pcr1, were prepared and incubated with (+) or without (-) lambda phosphatase in the presence or absence of phosphatase inhibitors, as indicated. Pcr1 was detected after SDS/PAGE followed by Western blot using polyclonal anti-Pcr1 antibodies. **B.** Pcr1 is dephosphorylated upon oxidative stress. Strain 972 (WT) was treated with 1 mM H_2O_2 stress for the times indicated in the figure. Boiled protein extracts were analyzed by Western blot analysis with anti-Pcr1 antibodies. Phosphorylated (Pcr1-P) and unphosphorylated (Prc1) protein forms are indicated with arrows. **C.** H_2O_2-dependent dephosphorylation of Pcr1 is Sty1-dependent. Strain NT224 transformed with p138.41x (Δsty1 + p*HA-pcr1*) was treated with 1 mM H_2O_2 stress for the times indicated in the figure. The phosphorylation status of HA-Pcr1 was analyzed by native extracts and Western blot was performed as in B .

2.2. Pcr1, A POSSIBLE TARGET OF THE Ptc1 PHOSPHATASE

Since the phosphorylation is not regulated by the main stress kinase, maybe the dephosphorylation is the step under regulation. It is has been reported that a PP2C phosphatase (see section 4.3.3.4), called Ptc1, impairs the stress inducible response in front of KCl when over-expressed. In that study, the authors postulated that PP2C negatively regulates a downstream element of the Sty1 pathway, and demonstrated that Atf1 phosphorylation is not the Ptc1 target (Gaits *et al*., 1997). Pcr1 was discarded at that time as possible target due to the false assertion about the absence of phosphorylable residues in the protein (Kanoh *et al*., 1996).

We checked if Ptc1 could be the phosphatase acting on Pcr1. Due to technical problems in order to detect Pcr1 protein by Western blot, we worked on its deletion phenotype and transcription profile in front of different stress stimuli. In Fig. 9A, we show that survival of *Δptc1* is slightly impaired in front of KCl or H_2O_2, representing an intermediate phenotype between *Δatf1* and *Δpcr*1. Looking at stress-dependent transcriptional regulation, we found that expression of Pcr1-dependent genes such as *gpx1* (see section 2.5) was also Ptc1-dependent. Moreover, those genes whose induction was partially dependent on Pcr1 (like *gpd1*) were completely Ptc1-dependent. Following the same trend, genes completely Pcr1-independent for stress induction, like *srx1*, were also Ptc1-independent (Fig. 9B).

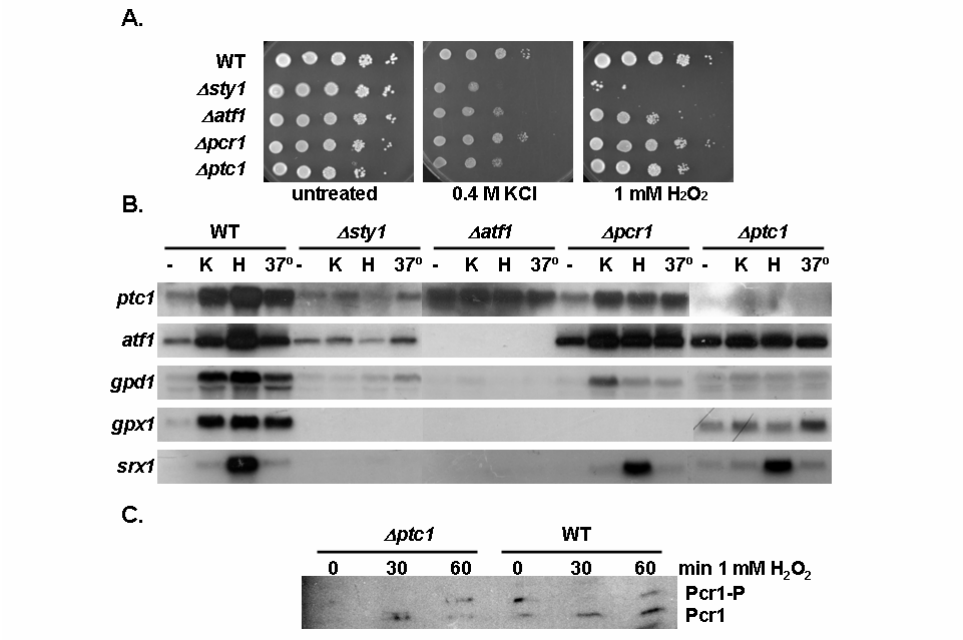


Figure 9. *Δptc1* phenotype upon different stresses. A. Liquid cell cultures from strains 972 (WT), AV18 (*Δsty1*), AV15 (*Δatf1*), MS5 (*Δpcr1*) and 834 (*Δptc1*) were grown in MM and 10 to 10^5 cells were spotted into MM plates with 0.4 M KCl, 1 mM H_2O_2 or nothing. **B.** The same strains as in A were exposed to 0.4 M KCl (K), 1 mM of H_2O_2 (H) during 30 min, left untreated (-) or cultured at 37ºC. Total RNA was extracted and analyzed by Northern blot using probes recognising common Sty1- dependent CESR genes such as *atf1*, *gpd1*, *gpx1*, *srx1* and a *ptc1*. **C.** Pcr1 dephosphorylation under oxidative stress in *Δptc1*. Strains 972 (WT) and 834 (*Δptc1*) were treated with 1 mM H_2O_2, samples were collected at the times indicated and TCA extracts were analyzed by Western blot with polyclonal anti-Pcr1

After solving protein detection problems using TCA extracts, we analyzed the dephosphorylation of Pcr1 upon oxidative stress. Unfortunately, we found it unchanged between wild type and *Δptc1* strains (Fig. 9C). In conclusion, although *Δptc1* cells have significant problems in stress dependent transcription, we could not demonstrate a link between Pcr1 phosphorylation and Ptc1.

2.3. MUTATIONS IN Pcr1 PHOSPHORYLABLE RESIDUE

To test the importance of Pcr1 phosphorylation status in oxidative stress response, we constructed mutant strains for Pcr1 SP MAPK site. As schematized in Fig. 11A, two variants were produced. One consists in a replacement of serine 122 by an alanine (S122A), mimicking non-phosphorylated Pcr1 protein. The other is a mutation of the same residue to aspartic (S122D), producing form equivalent to phosphorylated Pcr1. Both forms escape from any possible regulation. As background we created a novel *ura4* deleted *pcr1* (*pcr1::ura4*) in order to target the two engineered genes by homologous recombination at their original locus (Fig. 10A).

We first confirmed the successful mutation of the protein by sequence and electrophoretic mobility analysis of the mutants. We observed changes fitting with the two possible states of wild type Pcr1, phosphorylated or not (Fig. 10B). We also checked that the *pcr1::ura4* background strain showed no signal of Pcr1 protein (Fig. 10B).

Next we examined the transcriptional behaviour of the Pcr1 mutants we generated, comparing them to wild type and our new *pcr1::ura4* strain. Curiously, all of them showed an identical pattern as the wild type strain. Even more surprisingly, the *pcr1::ura4* deleted strain also showed a wild type pattern. In this context we suspected the presence of a short transcript, which could assume the whole function of the wild type protein. Using the same Northern blot we detected a lower migrating mRNA using a *pcr1* radioactive probe (Fig. 10C).

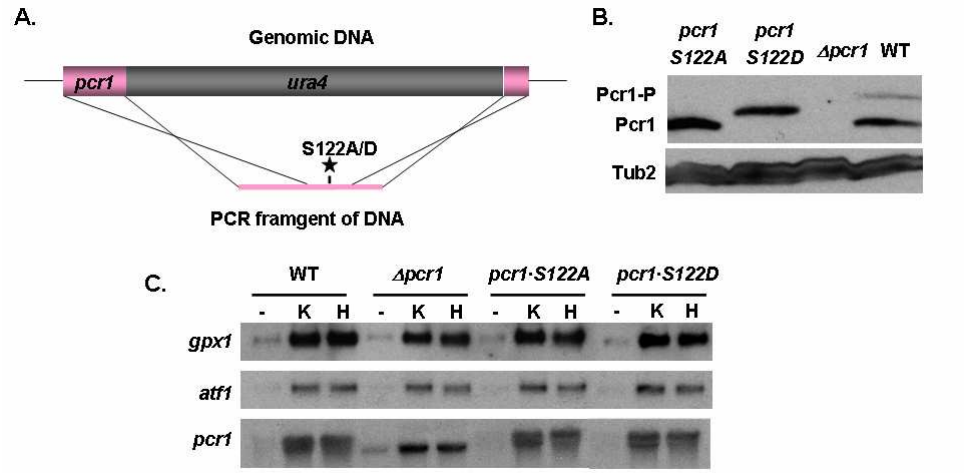


Figure 10. Punctual mutants of Pcr1. A. Schematic representation of *pcr1* gene and the *ura4* deleted version generated to produce the mutant Pcr1 forms in their own locus. *pcr1* was truncated at nucleotide +363 from its ATG to introduce *ura4* gene, leaving 150 nucleotides behind the disruptor. This new *Δpcr1* strain was called MS63 and transformed with DNA fragments containing the mutated S122D and S122A version of *pcr1*, obtaining MS73 and MS72 respectively. **B.** Electrophoretic mobility of Pcr1 mutants. TCA extracts from untreated strains 972 (WT), MS63 (Δ*pcr1*), MS72 (*pcr1·S122A*) and MS73 (*pcr1·S122D*) were analyzed by Western blot to check Pcr1 protein levels and mobility. **C.** Transcriptional behaviour of Pcr1 mutants in front of different insults**.** Total RNA from the strains as in B, treated or not (-) for 30 min with 0.4 M KCl (K) or 1 mM of H_2O_2 (H) were analyzed by Northern blot and hybridized with *gpx1*, *atf1* and *pcr1*.

Our results show that the SP site has no role in stress regulation; even more, only the first 356 nucleotides, which contain the bZIP domain of Pcr1, are enough to activate transcription.

2.4. THE Pcr1 TF IS REQUIRED FOR SURVIVAL OF SOME, BUT NOT ALL, STRESSORS

We tested the survival of *Δatf1* and/or *Δpcr1* strins under a variety of adverse stress conditions. In liquid cultures, both TFs are required for full survival upon oxidative stress or heat shock (Fig. 3A of Sansó *et al.*,

2008). In contrast, when the test is performed on solid plates containing NaCl, KCl, or sorbitol, the *Δatf1* cells survival is compromised while to *Δpcr1* cells behave as wild-type (Fig. 11). Similarly, Pcr1 is not sensitive to oxidative stress on solid plates (Fig. 11).

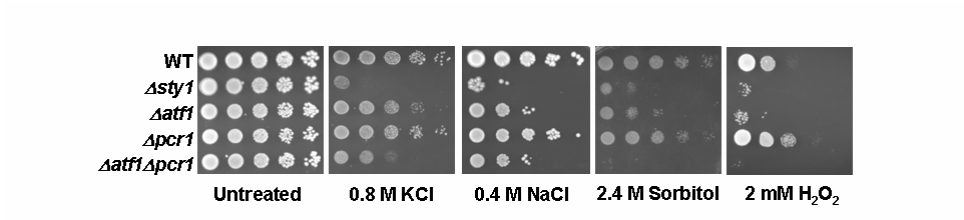


Figure 11. Atf1 but not Pcr1 is essential for survival in front of all major types of stress. Analysis of the stress resistance of WT, *Δsty1*, *Δatf1*, *Δpcr1*, and *Δatf1 Δpcr1* strains. Strains 972 (WT), AV18 (*Δsty1*), AV15 (*Δatf1*), MS5 (*Δpcr1*), and MS48 (*Δatf1Δpcr1*) at logarithmic MM growth were spotted from 10 to 10^5 cells in MM plates containing KCl, NaCl, sorbitol or 2 mM H_2O_2. at the indicated concentrations and incubated at 30ºC for 3 to 4 days. .

2.5. Pcr1 IS DISPENSABLE TO INDUCE SOME Atf1/Sty1-DEPENDENT GENES

Since Atf1 is necessary to survive in front of osmotic and oxidative stresses, but *Δpcr1* cells are not sensitive to these stressors on plates, we tested if this difference is also reflected in the induction of some Sty1-dependent CESR genes in both kind of stresses, oxidative (Fig. 12A) and osmotic (Fig. 12B). We did not observe any induction of *gpd1* and *hsp9* in response to oxidative stress in *Δpcr1*, nor for *cta3* in presence of KCl. Our results show that, while the Atf1/Pcr1 dimer is responsible for transcriptional activation of most CESR genes, not all these genes require Pcr1 function to respond to specific stresses, such as *gpd1*, *hsp16 and ntp1* upon KCl stress, or *srx1* upon oxidative stress

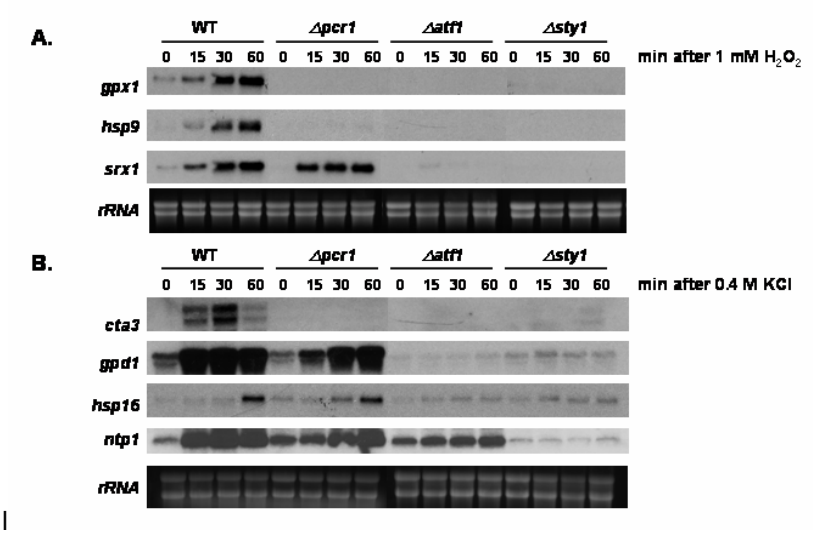


Figure 12. Transcriptional behaviour of Pcr1-dependent genes at long stress times. A. Long kinetics of oxidative stres transcription. Cultures of strains 972 (WT), AV18 (*Δsty1*), AV15 (*Δatf1*), and MS5 (*Δpcr1*) were treated with 1 mM H_2O_2 and samples collected at detailed times. Total RNA was hybridized with *gpx1*, *hsp9* and *srx1* probes, using as control mRNA stained with ethidium bromide. **B.** Long kinetics of osmotic stress transcription. The same strains and control as in A, but treated with 0.4 M of KCl, samples were collected at detailed times. Total RNA was hybridized with c*ta3*, *gpd1, hsp16* and *ntp1*.

2.6. Atf21 IS NOT THE Atf1 ALTERNATIVE PARTNER IN AN OSMOTIC STRESS

Atf21, another bZIP TF, is highly induced in front of osmotic stress induced by sorbitol 1.2 M (Chen *et al*., 2003) (see section 5.1.3). Based on this observation, we speculated that Atf21 could be the partner of Atf1 for the activation of Pcr1-independent genes in KCl stress conditions.

We constructed a *Δpcr1 Δatf21* double deleted strain. Survival experiments and Northern blot analysis revealed that *Δpcr1 Δatf21* cells had an identical phenotype to the *Δpcr1* strain (Fig. 13AB). These results

suggest that Atf21 is not likely to be the partner of Atf1 in KCl-dependent transcription, nor upon sorbitol and H_2O_2 exposure.

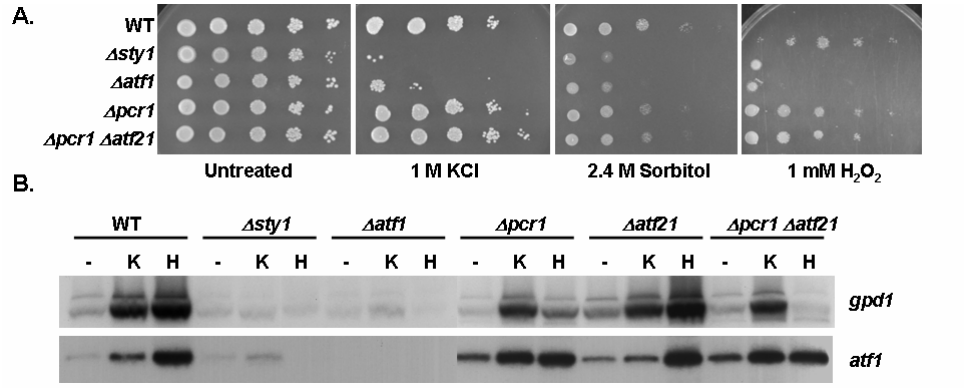


Figure 13. Phenotype and stress-dependent transcription in *Δpcr1 Δatf21*. **A.** Stress survival of cells lacking both Pcr1 and Atf21. Strains 972 (WT), AV18 (*Δsty1*), AV15 (*Δatf1*), MS5 (*Δpcr1*) and MS34 (*Δpcr1 Δatf21*) growing at logarithmic phase were spotted from 10 to 10^5 cells on MM plates containing different osmotic (1M KCl and 2.4 M sorbitol) and oxidative stressors (1 mM H_2O_2), let them grow at 30ºC during 3 or 4 days. **B.** Transcription of stress-inducible genes without Pcr1 and Atf21. The same strains as in A, as well as 368 (*Δatf21*) were treated with 0.4 KCl (K) or 1 mM H_2O_2 (H) for 30 min or left without treatment (-), total RNA was analyzed by Northern blot and hybridized with probes of *gpd1* and *atf1*.

2.7. Pcr1 OR Sty1 DO NOT DIRECTLY REGULATE Atf1 PROTEIN STABILITY

Levels of Atf1 protein are increased after stress, as previously described (Gaits *et al*., 1998). However, in Δ*pcr1* strain we observed low levels of Atf1, even after stress. Furthermore, Δ*sty1* strain has almost no detectable Atf1 protein (Fig. 5A of Sansó *et al*., 2008). Recently, it has been proposed that Atf1 stability is regulated by Pcr1, and by the Sty1 kinase (Lawrence *et al*., 2007). We hypothesized that the down regulation of some Atf1-dependent genes in Δ*pcr1* cells could result from lower concentrations of Atf1. To test this, similar levels of Atf1 protein were

obtained in *Δatf1*, *Δpcr1 Δatf1* and *Δsty1* cells by ectopically expressing wild type levels of Atf1 (Fig. 5B, upper panel of Sansó *et al*., 2008). This result indicates that Atf1 stability does not require Sty1 nor Pcr1. Moreover, Northern blot analysis of CESR genes revealed that levels of Atf1 have no impact in their induction once Sty1 or Pcr1 are deleted (Fig. 5B of Sansó *et al*., 2008).

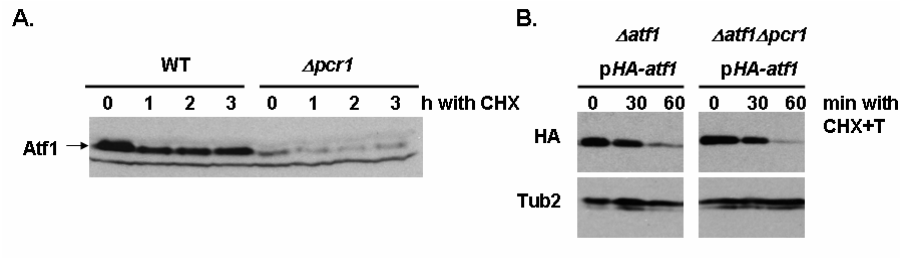


Figure 14. Pcr1 does not regulate Atf1 stability. A. Endogenous levels of Atf1 are not destabilized in the absence of Pcr1. Translation was inhibited by adding 200 μg/ml cycloheximide (CHX) into MM cultures at mid-log phase from strains 972 (WT) and MS7 (Δ*pcr1*). Samples were collected from 0 until 3 hours and total TCA extracts were analyzed by Western blot using anti-Atf1 to visualize Atf1 degradation ratio. **B.** Ectopic levels of HA-Atf1 are degraded in a Pcr1-independent way. Strains JM1066 (Δ*atf1*) and MS48 (Δ*pcr1* Δ*atf1*) harbouring p151.41x plasmid (containing the *nmt*-driven *HA-atf1* gene, p*HA-atf1*) were grown in MM without thiamine to mid-log phase. 10 μM Thiamine (T) and 200 μg/m of CHX were added to repress the expression of the *HA-atf1* gene and protein synthesis, respectively. TCA extracts from samples were collected at the times indicated to monitorize HA-Atf1 degradation. Monoclonal anti-Tub2 was used as loading control.

We further confirmed the Atf1 stability results by treating cells with a translation inhibitor (cycloheximide) and analyzing the stability of the endogenous Atf1 protein (Fig. 14A) or an HA-Atf1 fusion (Fig. 14B). In both experiments, the half-lifes of Atf1 or HA-Atf1 were comparable in presence or absence of Pcr1.

2.8. Atf1 IS ABLE TO BIND TO *CIS* ELEMENTS, EVEN IN THE ABSENCE OF Sty1

After addressing the interdependence of Atf1 and Pcr1 for the occupancy of target promoters (Fig. 6CE of Sansó *et al.*, 2008), we investigated the role of Sty1 in regulating the binding of Atf1 to promoters. We used an *nmt*-driven *HA*-tagged *atf1* plasmid to ensure detectable protein levels in the *Δsty1* background used in this experiment. We performed ChIPs under KCl stress for *cta3* (always Atf1-dependent / Pcr1-dependent), *hsp9* (Atf1-dependent / Pcr1-independent only in KCl conditions) and *srx1* (always Atf1-dependent / Pcr1-independent). Atf1 was bound to the promoters of these three genes before and after stress (Fig. 15), even without observing transcriptional activation (Fig. 5B of Sansó *et al.*, 2008).

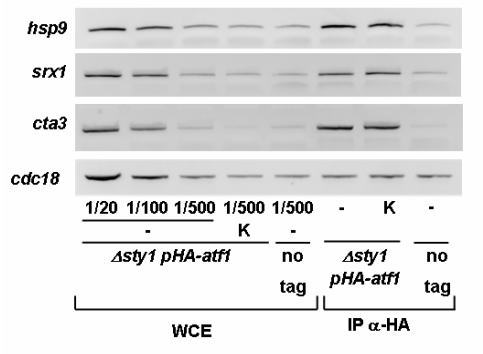


Figure 15. Atf1 binds to promoters even in the absence of Sty1. Strains 224 (*Δsty1*) transformed with episomal, *nmt*-driven p151.41x, expressing HA-Atf1 (*Δsty1* + p*HA-atf1*), and no transformed 972 (WT), were treated (K) or not (-) with 0.4 M of KCl. Cells were grown in MM to mid-log phase, formaldehyde extracts were obtained, and chromatin bound to Atf1-HA was isolated using anti-HA monoclonal antibody (IP α-HA). Recovered DNA was assayed by PCR amplification with primers encompassing the *hsp9*, *srx1* and *cta3* promoters or the *cdc18* ORF as a negative control. Three different concentrations of whole-cell extracts (WCE) were also analyzed to demonstrate that the quantity of amplified DNA is dependent on the amount of input (WCE) (1/20, 1/100 or 1/500).

3. Gcn5, A SPECIFIC HAT FOR THE STRESS RESPONSE

We are investigating the transcriptional response under the regulation of Sty1 and Atf1, thus this work embraces the participation of chromatin remodelers for proper transcription activation after stress imposition. Little is known in *S. pombe* about the relationship between stress and specific mechanisms of chromatin remodelling. The majority of studies establishing a link between these two processes have focused so far on the recombination hotspot *ade6-M26* and heterochromatin regions such as the *mat* locus.

It has been recently characterized the composition of the fission yeast SAGA coactivator complex (Helmlinger *et al.,* 2008). The authors found a high level of conservation of the complex, thus establishing *S. pombe* as a relevant system for the study of SAGA function *in vivo*.

We have focused this work in characterizing the role of Gcn5 in oxidative stress resistance through transcription activation and its regulation by the MAPK pathway.

3.1. Gcn5 AS PART OF Spt-Ada-Gcn5 ACETYLTRANSFERASE COMPLEX (SAGA) IS NECESSARY FOR THE OXIDATIVE STRESS TRANSCRIPTIONAL RESPONSE

We screened a collection of around 2700 haploid mutant strains for growth inhibition on H_2O_2-, caffeine-, menadione- or minimal media with glycerol-containing plates. One of the results that we obtained was the sensitivity to oxidative stress of strains lacking some chromatin remodelers, such as the HAT Gcn5, which was previously reported to be specifically required for adaptation to KCl- and $CaCl_2$-mediated stress in *S. pombe* (Johnsson *et al*., 2006). We decided to fully characterize the sensitivity of the *Δgcn5* strain to oxidative stress. As shown in Fig. 16A, the *Δgcn5* strain has a reduced survival on H_2O_2 plates compared to wild type. Similarly, other subunits from the recently described *S. pombe* SAGA complex (Helmlinger *et al*., 2009), like the cofactor Ada2 or the TATA binding protein (TBP)-interacting subunit Spt8, are also sensitive to the same stressor (Fig. 16B).

In order to confirm that Gcn5 has a HAT activity, we performed Western blot analysis over total extracts with polyclonal antibodies against acetylated histone H3 at residues K9 and K14. We examined levels of combined histone H3 Lys9 and 14 acetylation in the wild type and mutant *Δgcn5* strains. As expected, we found that H3K9/14 acetylation was dramatically reduced in the total extracts of cells lacking Gcn5 compared to wild type cells (Fig. 16C).

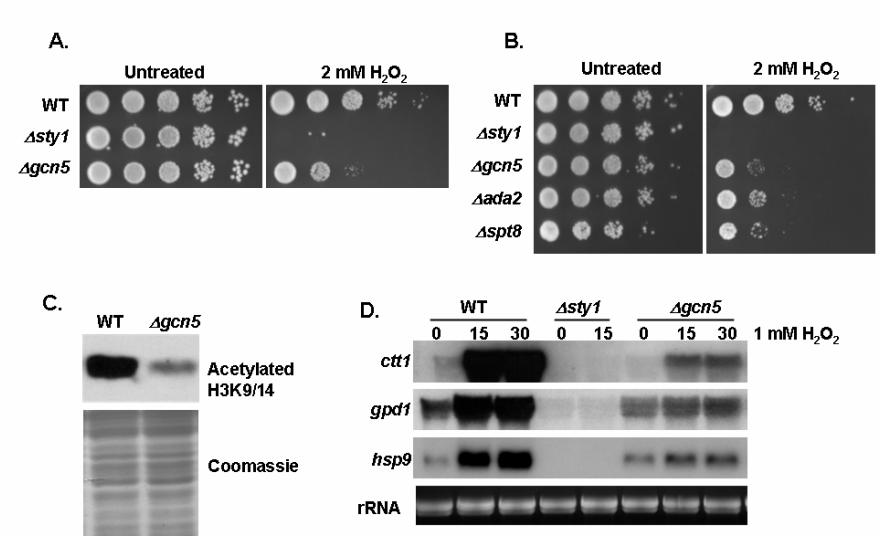


Figure 16. Gcn5, as the HAT of SAGA complex, is necessary for oxidative stress response. A. Gcn5 is required for growth under oxidative stress. Strains 972 (WT), AV18 (*Δsty1*) and MS112 (*Δgcn5*) were grown in liquid rich medium (YE5S) to a final OD_{600} of 0.5, and 10^5 to 10 cells were spotted onto YE5S plates containing 2 mM H_2O_2 or left untreated and incubated at 30℃ for 3 to 4 days. **B.** SAGA complex is required for growth under oxidative stress. The same strains as in A and MS183 (*Δada2*) and MS184 (*Δspt8*) were analyzed as in A. **C**. Histone H3 acetylation at lysines 9 and 14 is affected by Gcn5 mutant. Levels of H3 acetylation were determined by Western blot of TCA extracts from the strains 972 (WT) and MS112 (*Δgcn5*) with antibodies specific to H3K9/14 acetylation (Upstate). As loading control gel was stained with coomassie. **D.** Gcn5 is required for transcription of oxidative stress response genes. Cultures of the same strains as in A were treated with 1 mM H_2O_2 for the times indicated. Total RNA was extracted and analyzed by Northern blot with probes for *ctt1, gpd1* or *hsp9*. As control ethidium bromide stained rRNA was used.

Since Gcn5 is also required for survival upon oxidative stress and affects histone acetylation we tested whether induction of some Sty1-dependent CESR genes would be impaired in cells lacking this HAT. We used Northern blot analysis to measure gene expression in wild type, *Δsty1* (as control of no transcription) and *Δgcn5* strains. As shown in Fig. 16D the three Sty1-dependent genes tested, *hsp9, gpd1* and *ctt1*, had an impaired transcriptional response upon oxidative stress.

Our results show that Gcn5, as part of the SAGA complex, is necessary to the proper induction of Sty1-dependent genes upon H_2O_2, and so is required to survive in front of this stress condition.

3.2. Gcn5 BINDS TO PROMOTERS OF Sty1-DEPENDENT GENES AND IS FURTHER RECRUITED UPON H_2O_2 STRESS

To test whether Gcn5 directly regulates Sty1-dependet genes, we determined its physical association with some of those genes which transcription was impaired. For this analysis, we performed conventional PCR ChIP experiments using a strain in which Gcn5 was tagged with an HA epitope in the C-terminal domain of its own locus. We first confirmed that the tag did not affect CESR's inducibility nor H_2O_2 sensitivity in plates (data not shown). Our ChIP results showed a significant level of Gcn5 association to both *gpd1* and *hsp9* promoters before stress imposition (Fig. 17A). If Gcn5 acts as an activator of these stress genes and others, then one would expect that when those genes are induced upon H_2O_2 exposure, the level of Gcn5 association would increase. Indeed, we observed by Real Time PCR ChIP a recruitment of the HAT to several Sty1-dependent genes after 15 min of H_2O_2 stress (Fig. 17B).

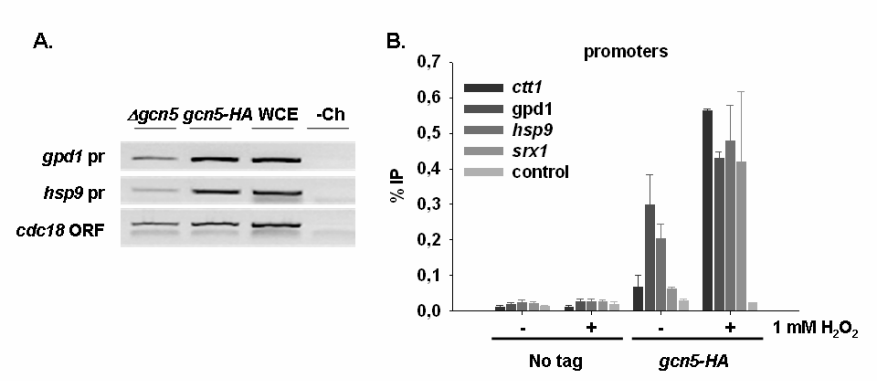


Figure 17. Gcn5 is physically associates to stress-dependent promoters. A. Representative ChIP data for Gcn5-HA occupancy at stress gene promoters. Cross-linked cell extracts from unstressed MS112 (Δ*gcn5*) and HMP47 (*gcn5-HA*) were immunoprecipitated with anti-HA antibodies, and assayed for the presence of the *gpd1* or *hsp9* promoter regions. As control, DNA was amplified from whole-cell extract (WCE) before immunoprecipitation. **B.** Gcn5 is targeted to stress promoters after, in some cases even before, H_2O_2 exposure. Strain HMP47 expressing chromosomic Gcn5-HA (*gcn5-HA*) and wild type 972 (No tag) were grown to mid log phase in YE5S and the treated (+) or not (-) with 1 mM of H_2O_2 for 15 min. Occupancy levels in promoter regions of *ctt1*, *gpd1*, *hsp9* and *srx1* were quantified by real-time PCR of anti-HA (12CA5) immunoprecipitation over the input DNA. Background levels are represented by amplification of an IGR region (control). Data was obtained from three independent experiments and are expressed as mean+SEM.

Both ChIP experiments showed a basal binding of Gcn5 to *gpd1* and *hsp9* promoters, which presence is almost not detectable in *srx1* and *ctt1*. Due to this fact, even though the total occupancy at all four promoters after stress is very similar (% of IP between 0.4-0.6), the fold-induction in binding before and after stress is very different: while the HAT is more than 8- and 6-fold recruited to *ctt1* and *srx1* respectively, in *gpd1* and *hsp9* the fold enrichments are 1.4 and 2.3 times.

Together, these results suggest that Gcn5 directly regulates stress-dependent gene expression, probably at the level of transcription initiation.

3.3. Gcn5 AND Atf1 DISPLAY VERY SIMILAR PROMOTER OCCUPANCY PATTERNS AT CESR PROMOTERS, WITH Gcn5 BINDING DEPENDING ON Atf1

We analyzed the *in vivo* binding of Atf1 to gene promoters by Real Time PCR ChIP. We used an HA-tagged version of the Atf1 protein, expressed from an episomal plasmid in *Δatf1* strain. We had already demonstrated that the concentration of Atf1 in this strain is similar to wild type levels (Fig. 5B of Sansó et al., 2008). Importantly enough, the pattern of Atf1 binding to promoters is very similar to that of Gcn5. On one hand, Atf1 was bound to all promoters prior to stress, especially to *hsp9* and *gpd1*. On another hand, Atf1 was further recruited to all four promoters after stress, reaching similar levels of occupancy but with different fold-inductions (Fig. 18A).

Next, we tested if Gcn5 was still bound to these genes in a *Δatf1* background. For this purpose we used the same chromosomic HA-tagged version of Gcn5. As expected, Gcn5 binding to CESR genes is abolished before stress when Atf1 is deleted, while after oxidative stress its binding is also abolished for *gpd1* and *hsp9*, and severely impaired for *ctt1* and *srx1* promoters (Fig. 18B). This slight increment in *ctt1* and *srx1* could be dependent on Pap1 binding, since both genes are also targets of the Pap1 TF, and by Northern blot it is possible to detect a slight increment of *ctt1* and *srx1* mRNA in a *Δatf1* background under high concentrations of H_2O_2, probably dependent on the TF Pap1 (see Fig. 12A and 13A). Thus, these results have demonstrated that Gcn5 basal binding and stress-dependent recruitment to Sty1-dependent promoters is dependent on the presence of Atf1.

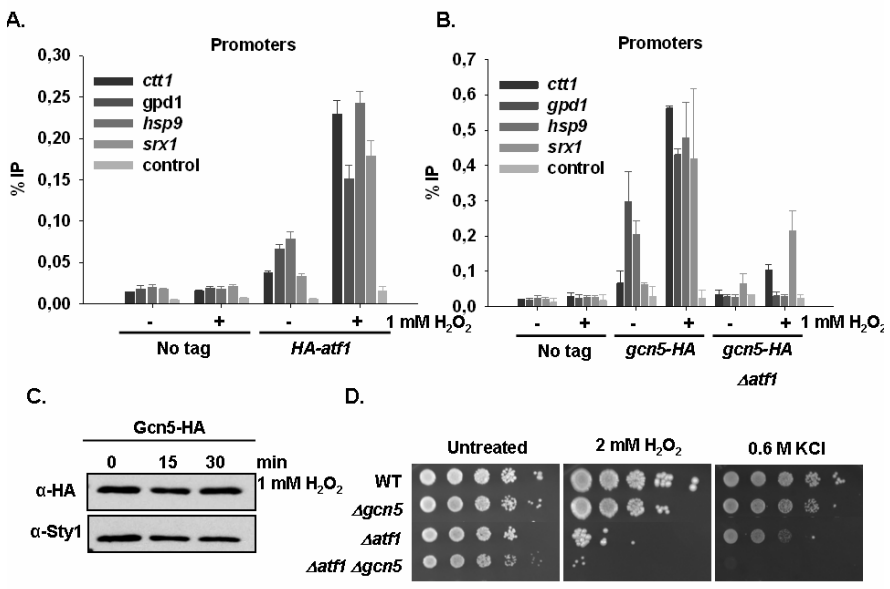


Figure 18. Gcn5 binding to stress promoters depends on Atf1. A. Atf1 association to stress-dependent gene promoters. Strain JM1066 transformed with p151.41x, expressing episomal HA-Atf1 (Δ*atf1*+ p*HA-atf1*) and wild type 972 (no tag) were processed as described in Fig. 18B. **B.** Gcn5 binds to stress promoters through Atf1. Strains HMP47 (*gcn5-HA*) and MS176 (*gcn5-HA* Δ*atf1*) expressing chromosomic Gcn5-HA (*gcn5-HA* and *gcn5-HA Δatf1*, respectively*)*, and wild type 972 (No tag) were processed as described in Fig. 18B. **C.** Oxidative stress does not modify Gcn5 electrophoretic mobility. Strain HMP47 (*gcn5-HA*) was treated with 1 mM H_2O_2 stress for the times indicated. TCA extracts were analyzed by Western blot with anti-HA (12CA5) to detect Gcn5-HA protein. As loading control Sty1 protein levels were measured with anti-Sty1 polyclonal antibodies. **D.** Genetic interaction between Gcn5 and the TF Atf1 upon stress. Strains 972 (WT), MS112 (Δ*gcn5*), AV15 (Δ*atf1*) and MS171 *(*Δ*atf1* Δ*gcn5*) were grown in YE5S to a final OD_{600} of 0.5, and 10^5 to 10 cells were spotted onto YE5S plates containing or not 2 mM H_2O_2 or 0.6 M KCl and incubated at 30ºC for 3 to 4 days .

Since Sty1 is a kinase that regulates stress transcription, and Gcn5 collaborates in this role, we speculate that Gcn5 could be a target of Sty1 and suffer a phosphorylation by the stress kinase. To test this we analyzed by Western blot the electrophoretic mobility of HA-tagged Gcn5 protein in basal conditions and after oxidative stress, but no changes in migration were observed (Fig. 18C). Furthermore, we looked for

phosphorylable residues in Gcn5 aminoacid sequence and there are no S/TP sites along the protein.

Finally, we tested whether the Gcn5 role upon stress is just restricted to Atf1 guidelines. We constructed a *Δatf1 Δgcn5* deletion mutant and compared its phenotype to the single deleted strains in front of H_2O_2 and osmotic stress on plates. As shown in Fig. 18D, deletion of *gcn5* impairs *Δatf1* phenotype for both conditions, suggesting that Gcn5 has other roles in addition to the Atf1-dependent transcription activation.

3.4. Gcn5 IS PREFERENTIALLY BOUND TO PROMOTERS BUT IS ALSO DETECTED IN CODING REGIONS OF STRESS GENES

Recently, it has been proposed that the *S. pombe* HAT Gcn5 is predominantly associated with the coding regions of highly transcribed genes (Johnsson *et al*., 2009). The same authors have later indicated that the presence of Gcn5 at stress promoters is also important for gene induction (Johnsson *et al.,* 2010). To establish under our experimental conditions whether Gcn5 also associates with the coding regions and terminal part of stress-induced genes, we performed ChIP over two stress genes: *gpd1* and *ctt1*.

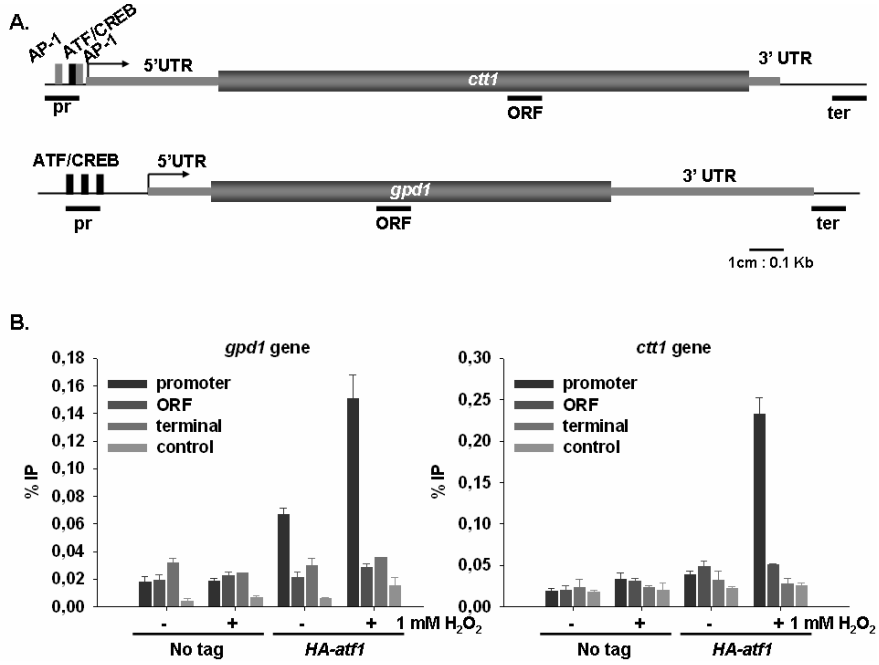


Figure 19. Atf1 exclusively binds to promoter regions. A. Schematic scaled representation of the *ctt1* and *gpd1* genes. For ChIP analysis, three primer pairs for each gene were designed to amplify PCR products (bars below the genes) spanning promoter region, coding sequence and terminal part of the gene. TF binding sequences in promoters are detailed as AP-1 and ATF/CREB for Pap1 and Atf1 binding sites, respectively. In the case of *ctt1* gene, binding site are described by Nakagawa *et al.,* 2000; about *gpd1* promoter, we based our primer design on ATF/CREB consensus sequence. 5' and 3' untranscribed regions were published by Lantermann *et al.,* 2010. **B.** Strain JM1066 transformed with p151.41x, expressing episomal HA-Atf1 (Δ*atf1*+ p*HA.atf1*) and wild type 972 (No tag) were grown to mid log phase in YE5S and the treated (+) or not (-) with 1 mM of H_2O_2 for 15 min. Occupancy levels in *gpd1* (left panel) and *gpd1* (right panel) genes were quantified by real-time PCR of anti-HA (12CA5) immunoprecipitation over the input DNA. Background levels are represented by amplification of an IGR region (control). The ChiP was assayed 3 times and the mean and the S.E.M. were plotted.

As the average size of DNA produced during the ChIP analysis was 500 bp we chose to examine association of Gcn5 with *gpd1* and *ctt1* as these genes are 1158 and 1539 bp in length, respectively. Primer pairs could therefore be designed sufficiently far apart so as not to amplify

promoter, ORF and terminal region from the same piece of DNA (Fig. 19A). On the other hand, *hsp9* and *srx1* genes are so short that were discarded for gene walking experiments. In order to ensure the proper primer design we first analyzed the Atf1 binding to the different regions of *gpd1* and *ctt1* genes. As shown in Fig. 19B, we can discriminate each region of the gene; HA-Atf1 binds to promoter regions of both genes after stress, remaining unbound to ORF and terminal part of the two genes tested. Gcn5, however, was clearly detected at ORFs, as well, although not at the same levels as at promoters (around 3-fold lower levels, before and after stress) (Fig. 20). As at its promoter, Gcn5 was found at the ORF of *gpd1* prior to stress, whereas it was mainly recruited at the *ctt1* ORF after stress in an Atf1-dependent manner (Fig. 20).

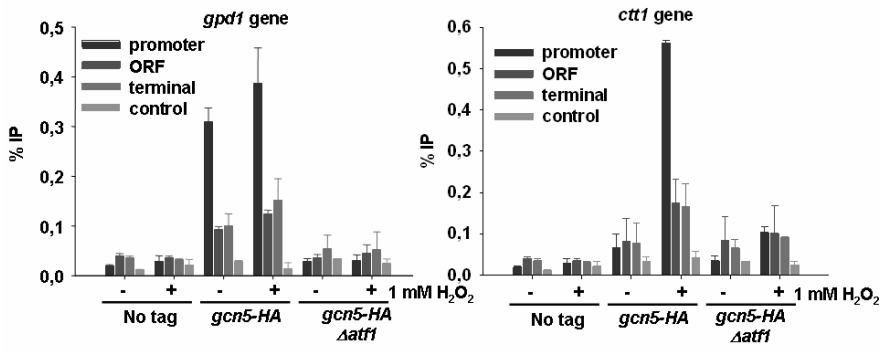


Figure 20. Gcn5 is recruited to promoters and coding regions of stress inducible genes. Strains HMP47 and MS176 expressing chromosomic Gcn5-HA (*gcn5-HA* and *gcn5-HA Δatf1*, respectively), and wild type 972 (No tag) were processed as described in Fig. 19B.

3.5. Gcn5 HAS A ROLE IN TRANSCRIPTION INITIATION BUT ALSO IN ELONGATION

Several laboratories working on *S. cerevisiae* and *S. pombe* have proposed a new role for Gcn5 in transcriptional elongation of highly

transcribed genes (Kristjuhan *et al*., 2002; Kristjuhan and Svejstrup 2004; Govind et al., 2007; Johnsson *et al*., 2009).

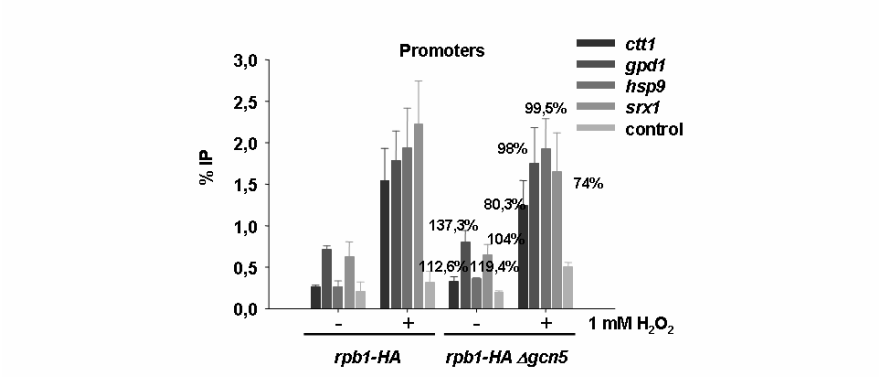


Figure 21. RNAPII recruitment to stress-dependent gene promoters is slightly impaired in Δ*gcn5* cells. Strains CN011 and MS47, expressing chromosomic Rpb1-HA (*rpb1-HA* and *rpb1-HA* Δ*gcn5*) were cultured and processed as described in Fig 17B. Percentages of Rpb1-HA recruitment in each region and condition for Δ*gcn5* background, and referred to wild type strain, are specified over the bars.

To test this hypothesis on our stress inducible genes we performed ChIP experiments to analyze the binding of the large subunit Rpb1 from RNAPII, tagged with HA, before and after treatment during 15 min with 1 mM H_2O_2, in wild type and *Δgcn5* backgrounds along *ctt1* and *gpd1* genes and also in *srx1* and *hsp9* promoters. As shown in Fig. 21, RNAPII occupancy in all promoters tested is increased after oxidative stress for both strains, moreover, cells lacking Gcn5 present higher levels of Rbp1-HA in basal conditions compared to wild type, but after treatment the RNAPII occupancy is slightly impaired. Alternatively, when we analyzed the occupancy along *ctt1* and *gpd1* genes we observed a stronger impairment of RNAPII recruitment along the coding and terminal region of these stress genes (Fig. 22A). From the cultures where we collected the ChIP samples we also collected RNA for Northern blot analysis of the *gpd1* and *ctt1* transcription, in order to ensure that HA-

tagging of Rpb1 is not altering transcription of both backgrounds, wild type and Δ*gcn5* (Fig. 22B).

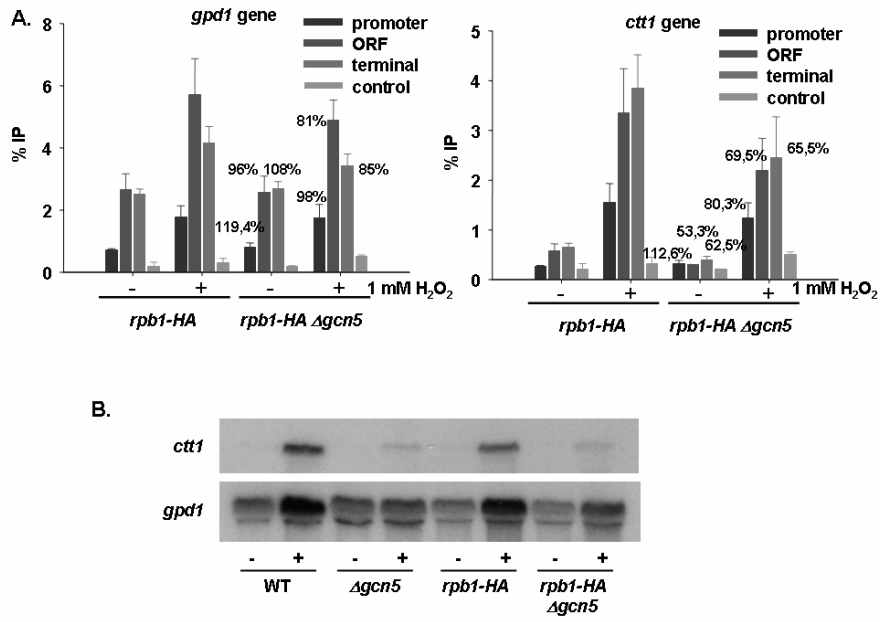


Figure 22. Density of RNAPII and transcription of stress-dependent genes in cells lacking Gcn5. A. Rpb1-HA recruitment to stress-dependent genes is impaired in Δ*gcn5* cells. Strains CN011 and MS47, expressing chromosomic Rpb1-HA (*rpb1-HA* and *rpb1-HA* Δ*gcn5*) were cultured and processed as described in Fig 19B. Percentages of Rpb1-HA recruitment in each region and condition for Δ*gcn5* background, and referred to wild type strain, are specified over the bars. **B.** Rpb1-HA tag does not affect transcriptional patterns. Cultures of strains 972 (WT), MS112 (Δ*gcn5*), CN011 (*rpb1-HA*) and MS47 (*rpb1-HA* Δ*gcn5*) were treated (+) or not (-) for 15 min with 1 mM H_2O_2 . Total RNA was extracted and analyzed by Northern blotting and hybridized with probes for *ctt1* and *gpd1*.

Cells displaying defects in RNAPII-dependent transcription elongation are sensitive to the drug 6-azauracil (6-AU). This drug inhibits transcription elongation by depleting the intracellular pools of GTP and UTP. Consistently, *Δgcn5* mutants showed enhanced sensitivity to 6-AU, even stronger than cells lacking the elongation factor TFSII (*Δtfs1*),

previously reported to be highly sensitive to this drug (Williams and Kane, 1996) (Fig. 23).

Taken together, these data suggest that Gcn5 not only affects transcription initiation of stress induced genes, but also affects the proper elongation of, at least, *ctt1* and *gpd1* genes.

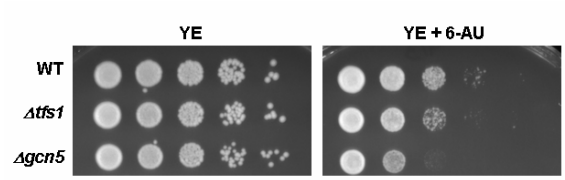


Figure 23. Cells lacking Gcn5 are sensitive to 6-AU. Strains 972 (WT), MS185 (*Δtfs1*) and MS112 (Δ*gcn5*) were grown in liquid rich media without uracil supplementation (YE) to a final OD_{600} of 0.5, and 10^5 to 10 cells were spotted onto YE plates containing or not 100 mg/ml of 6-AU and incubated at 30ºC for 3 to 4 days.

3.6. Gcn5 PROMOTES ACETYLATION AND NUCLEOSOME EVICTION FROM STRESS-DEPENDENT GENE PROMOTERS AND CODING REGIONS

H3 diacetylated on K9 and K14 in bulk chromatin is greatly reduced in Δ*gcn5* strain (Fig. 16C), and stress-dependent genes have an impaired transcription induction in this strain (Fig. 16D). Thus, we analyzed the H3K9/14 acetylation and total H3 levels of the stress-dependent promoters. After 15 min of oxidative stress we saw a marked depletion of total H3 (Fig. 24, right panel). After correcting for histone eviction, there was a net increase in H3K9/14 acetylation per nucleosome (H3K9-14Ac/H3 ratio) in all the promoters (Fig 24, left panel). Thus, transcriptional induction elicits increased acetylation of the H3 that remains in promoters. By contrast, deletion of *gcn5* substantially reduced the H3K9-14Ac/H3 ratio prior and after stress. Actually, Δ*gcn5* reduces

histone eviction from promoter to yield a net decrease in H3K9-14Ac/H3 ratio.

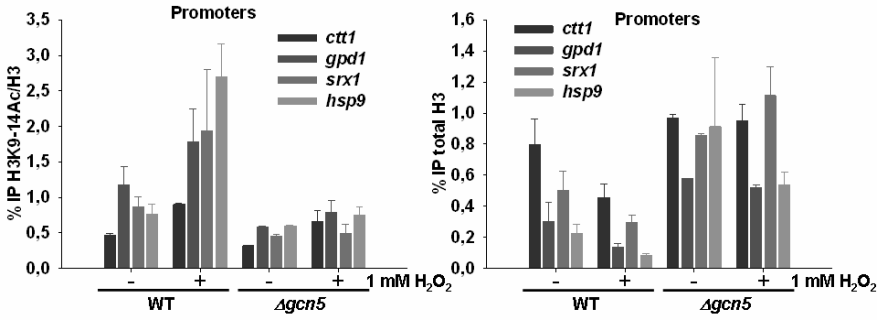


Figure 24. Deletion of Gcn5 affects H3K9-14 acetylation per nucleosome and H3 eviction in stress-dependent promoters. Strains 972 (WT) and MS112 (Δ*gcn5*) were grown to mid log phase in YE5S and the treated (+) or not (-) with 1 mM of H_2O_2 for 15 min. ChIPs were performed using antibodies specific for acetylated H3K9-K14 residues or against unmodified histone H3. Each column from left panel represents H3K9-K14 acetylation referred to total H3 values. Columns from right panel represent individual H3 percentage of immunoprecipitation. Occupancy levels in promoter regions of *ctt1*, *gpd1*, *hsp9* and *srx1* were quantified by real-time PCR of immunoprecipitation over the input DNA. The ChiP was assayed 2 times and the mean and the S.E.M: were plotted.

Having found that Gcn5 is targeted to the ORF, we extended our analysis to know whether Gcn5 also participates in transcription-coupled H3 acetylation and nucleosome eviction of coding regions. Analyzing *ctt1* and *gpd1* (Fig. 25) genes we observed a similar pattern for H3K9-14/H3 ratio and for H3 total levels to the one described for promoters: acetylation and nucleosome eviction at ORFs are both impaired in cells lacking Gcn5.

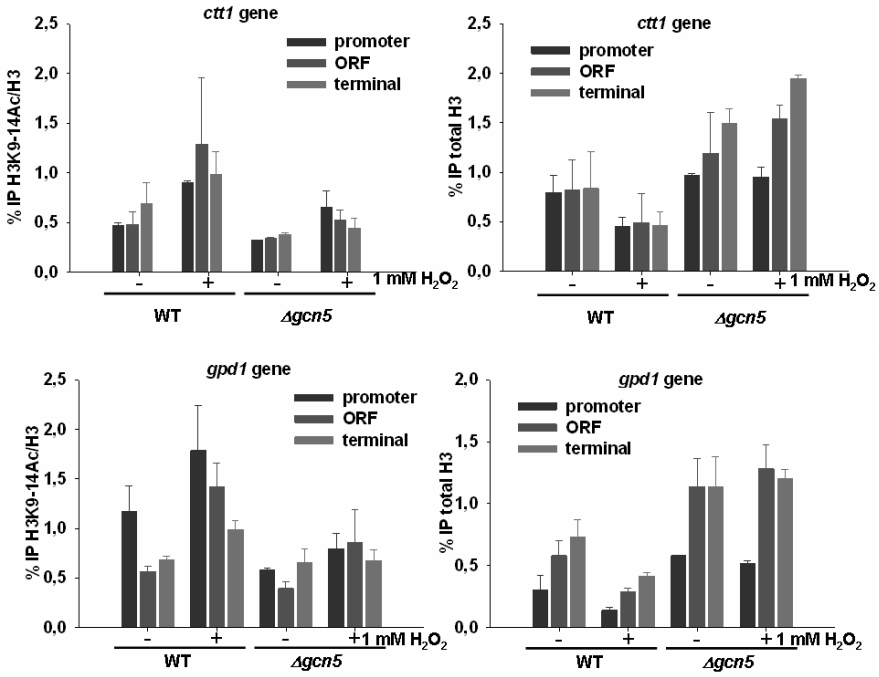


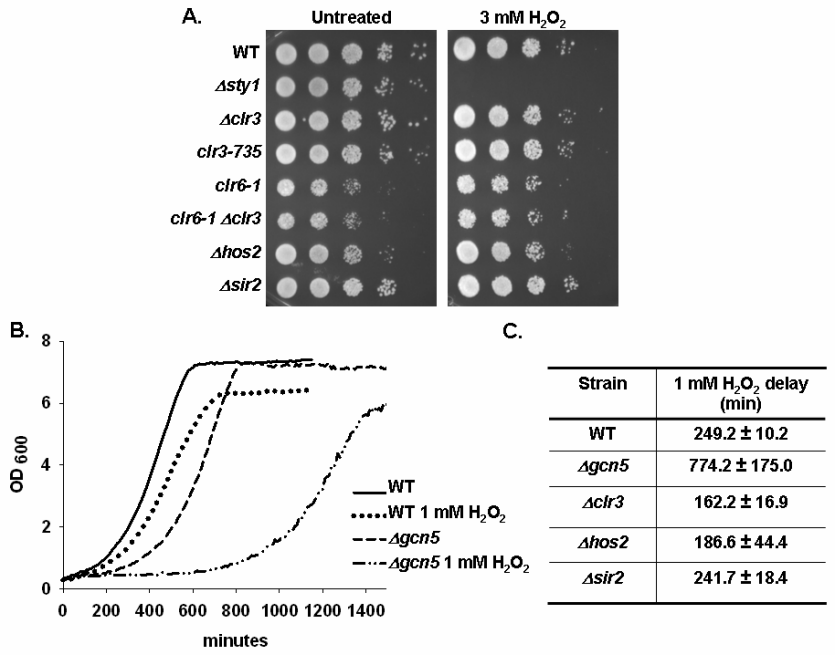
Figure 25. Deletion of Gcn5 affects H3K9-14 acetylation per nucleosome and H3 eviction in *ctt1* gene. The same experiment as in Fig. 24 was made with *ctt1* and *gpd1* gene specific oligos (see Fig. 19B).

Taken together, this data show that the direct activity of Gcn5 as HAT is affecting not only promoters, but also coding sequences of the stress inducible genes. Furthermore, its role is not limited to the histone acetylation, knowing that nucleosome eviction is also altered in the absence of Gcn5.

3.7. ROLE OF HDACS IN THE OXIDATIVE STRESS RESPONSE

In the genetic screening of the *S. pombe* deletion collection described before, we also isolated some chromatin remodeler mutants that were resistant to oxidative stress (5 mM H_2O_2), two of which were the

HDACs *Δhos2* and *Δclr3*. To identify HDACs that might have a role in stress transcription and might interact functionally with Gcn5, we analyzed the oxidative stress sensitivity of all the *S. pombe* HDACs available in the collection and some others kindly provided by different laboratories (see list of strains). First, we performed the test on solid plates with *Δclr3*, the temperature mutant *clr3-373*, the temperature sensitive (ts) *clr6-1*, the double mutant *Δclr3 clr6-1*, *Δhos2* and *Δsir2* strains (Fig. 26A). We could not observe consistent results, but slight resistance was appreciated for some strains. In order to test this possible resistant phenotype, we tested the same strains, except the ts mutant *clr6-1* due to its tendency to flocculate, by measuring every 10 min the OD_{600} of cultures treated or not with 1 mM of H_2O_2. As a control, we compared first the growth curves of wild type and *Δgcn5* strains. In order to standardize the effect of H_2O_2 exposure on the growth curves of different mutants, we exposed cultures at an OD600 of 0.1 to 1 mM H2O2 and measured the delay in minutes to reach an OD_{600} of 0.5 (Fig. 26B). Consistent with our previous results, this parameter was significantly reduced in the *Δclr3* and *Δhos2* strains, which indicates that these strains display a better adaptation to H_2O_2 than a wild type strain (Fig. 26C).



Strain	1 mM H_2O_2 delay (min)
WT	249.2 ± 10.2
Δgcn5	774.2 ± 175.0
Δclr3	162.2 ± 16.9
Δhos2	186.6 ± 44.4
Δsir2	241.7 ± 18.4

Figure 26. HDAC mutants survival in front of oxidative stress. A. Strains 972 (WT), AV18 (*Δsty1*), SPK19 (Δ*clr3*), SPK13 (*clr3-735*), SPK28 (*clr6-1*), SPK29 (*clr6-1* Δ*clr3*), MS186 (Δ*hos2*) and AZ95 (Δ*sir2*) were grown in YE5S to a final OD_{600} of 0.5, and 10^5 to 10 cells were spotted onto YE5S plates containing 3 mM H_2O_2 or left untreated and incubated at 30℃ for 3 to 4 days. **B.** Growth curves of wild type and Δ*gcn5* cells in the presence or absence of oxidative stress. Log-phase cultures at an OD_{600} of 0.1 of strains 972 (WT) and MS112 (Δ*gcn5*) were treated or not with the indicated concentrations of H_2O_2, and grown into microculture wells. Growth was monitored by measuring OD_{600} every 10 min at 30º for 24 h. **C.** Growth delay of several HDACs in front of oxidative stress. Strains 972 (WT), MS112 (Δ*gcn5*) SPK19 (Δ*clr3*), MS186 (Δ*hos2*) and AZ95 (Δ*sir2*) were cultured and treated as in B. Delay to reach OD_{600} of 0.5 between untreated and stressed cells from the same strain is represented in minutes.

We also examined the levels of H3K9/14 acetylation in the mutant strains to see whether they functionally might interact with the HAT Gcn5. As previously reported (Bjerling *et al.,* 2002), cells lacking Clr3, Clr6 and Hos2 showed increased levels of H3K9/14 acetylation compared to wild type strain (Fig. 27). Our data confirmed that class II HDACs (Clr3 and Hos2) are good candidates to counteract the role of Gcn5, while Sir2 was

discarded. About Clr6 (class I), more information is needed, like analyze the double mutant *Δgcn5 clr6-1* phenotype.

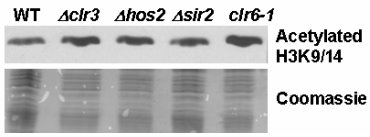


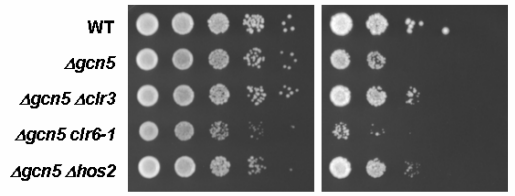
Figure 27. Histone H3 acetylation at lysines 9 and 14 in HDAC mutants. Levels of H3 acetylation were determined by Western blot of TCA extracts from the strains 972 (WT), SPK19 (Δ*clr3*), MS186 (Δ*hos2*), AZ95 (Δ*sir2*) and SPK28 (*clr6-1*) with antibodies specific to H3K9/14 acetylation (Upstate 06-599), and compared to total protein stained with coomassie.

3.8. GENETIC INTERACTIONS BETWEEN Gcn5 AND THE CLASS I AND II HDACS

To further investigate the significance of Clr3, Hos2 and Clr6 for Gcn5 function, we tested whether their deletion could suppress the sensitivity of *Δgcn5* cells in front of oxidative stress. Following the same experiments as in the previous section (3.6), we observed that deletion of *clr3* or *hos2* partially suppressed the sensitivity of *Δgcn5* to oxidative stress on plates. In contrast, the ts mutant *clr6-1* conferred a synthetic growth phenotype with *Δgcn5* (Fig. 28A). The growth curve profiles were consistent with the spot assays (Fig. 28B), but the improvement of Δ*gcn5* survival by deletion of Hos2 is not so significant to consider a compensation. In this last experiment we could include the *Δgcn5 clr6-1* mutant due to the lost of the flocculating phenotype observed in the single *clr6-1* mutant. Nevertheless, the double Δ*gcn5 clr6-1* was unable to resume growth at H_2O_2 imposition, while deletion of *clr3* clearly rescued Δ*gcn5* sensitivity phenotype. Clearly, the best suppressor of Δ*gcn5* (with a delay of 525 min over a wild type strain at H_2O_2 imposition; Fig. 28B) was Δ*clr3*, with a delay time very similar to that of a wild type strain.

Whereas Δ*hos2* could barely improve the delay of a single Δ*gcn5* mutant, the *clr6-1* ts mutant even impaired recovery upon stress.

A.



B.

Strain	1 mM H_2O_2 delay (min)
WT	249.2 ± 10.2
Δgcn5	774.2 ± 175.0
Δgcn5 Δclr3	362.2 ± 22.8
Δgcn5 clr6-1	------
Δgcn5 Δhos2	633.9 ± 38.7

Figure 28. Deletion of *clr3* specifically rescues Δ*gcn5* survival in front of oxidative stress. A. Strains 972 (WT), MS112 (Δ*gcn5*), MS168 (Δ*gcn5* Δ*clr3*) MS173 *(*Δ*gcn5 clr6-1*) and MS187 (Δ*gcn5* Δ*hos2*) were grown in YE5S to a final OD_{600} of 0.5, and 10^5 to 10 cells were spotted onto YE5S plates containing 3 mM H_2O_2 or left untreated and incubated at 30℃ for 3 to 4 days. **B.** Growth delay of HDAC mutants in Δ*gcn5* background under oxidative stress. The same strains as in A were analyzed as in Fig. 26C.

3.9. CELLS LACKING Clr3 HAVE AN ENHANCED STRESS-DEPENDENT TRANSCRIPTION AND RESCUES *Δgcn5* PHENOTYPE

Since Gcn5 is required to induce Sty1-depedendent genes upon oxidative stress while *Δclr3* cells are resistant to this insult, and both are acting no the same histone H3 residues, we tested whether the *Δgcn5* impairment for stress transcription could be rescued by deletion of *clr3*. Indeed, Northern blot analysis revealed that deletion of the HDAC Clr3 partially compensates the absence of the HAT Gcn5 (Fig. 29A). Even though the induction of those genes in the double deleted strain was not reaching wild type levels, it was significantly improved. Moreover, we tested whether the different stress transcription profiles correlate with antioxidant activities of the same strains. For this purpose, we analysed the H_2O_2 detoxification ability by measuring extracellular H_2O_2 using a ferrothiocyanate (FTC) assay. As shown in Fig. 29B, Δ*gcn5* has a poor

ability to detoxify the H_2O_2 added into the media, while Δ*clr3* cells can reduce oxidant levels faster than the wild type strain. Finally, mutation of *clr3* in Δ*gcn5* cells partially rescues their detoxification capacity. The experiment revealed a perfect correlation between transcription and antioxidant capacities.

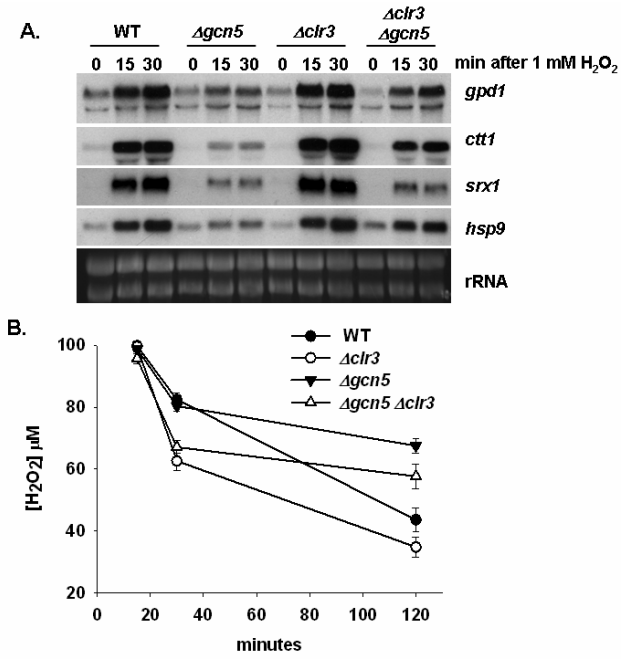


Figure 29. Clr3 deletion compensates Gcn5-dependent stress transcription. **A.** Stress-dependent transcriptional analysis of Δ*clr3* and Δ*gcn5* Δ*clr3* mutants. Cultures of the strains 972 (WT), MS112 (Δ*gcn5*), SPK19 (Δ*clr3*) and MS168 (Δ*gcn5* Δ*clr3*) were treated with 1 mM H_2O_2 for the indicated times. Total RNA was extracted and analyzed by Northern blotting with probes for *ctt1, gpd1*, *srx1* or *hsp9*. As control, ethidium bromide stained rRNA was used. **B.** Antioxidant activity measured by FTC assay. The same strains as in A were grown in YE5S to a final OD_{600} of 0.5 and treated with 1 mM H_2O_2. Samples were collected at 15, 30, 60 and 120 min and centrifuged, 1/10 dilution of the media without cells was subjected to FTC assay as described in Methods. Blank was measured by fresh YE5S, also 1/10 diluted.

3.10. Sin3, A NEW *S. POMBE* HAT INVOLVED IN STRESS-DEPENDENT TRANSCRIPTION ELONGATION

Gcn5 is not the only HAT of *S. pombe* (see chapter 5.2.3 from the Introduction), thus we have examined the stress sensitivity of other HAT proteins, like Sin3 and Hat1 from the GNAT family and Mst2 from the MYST family. First, we characterized the phenotype of the deleted strains upon oxidative stress. We realized that deletion of Mst2 or Hat1 does not compromise the survival of the cells in front of H_2O_2, while Δ*sin3* strain displays a strong sensitivity (Fig. 30A). This result correlates with our previous mutant collection screening, where Δ*sin3* cells were also sensitive to oxidative stress. Moreover, Sin3 is also necessary to survive in front of osmotic and heat stresses (Fig. 30B).

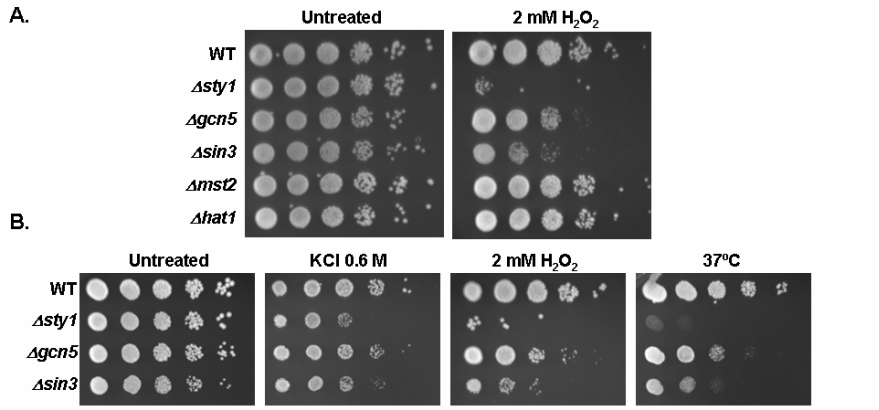


Figure 30. Sin3 mutant strain is sensitive to several stresses. A. HAT mutants survival in front of stress. Strains 972 (WT), AV18 (Δ*sty1*), MS112 (Δ*gcn5*), MS120 (Δ*sin3*), M199 (Δ*mst2*) and MS200 (Δ*hat1)* were grown in YE5S to a final OD_{600} of 0.5, and 10^5 to 10 cells were spotted onto YE5S plates containing or not 2 mM H_2O_2 and incubated at 30ºC for 3 to 4 days. **B.** Δ*sin3* sensitivity in front of different stresses. Strains 972 (WT), AV18 (Δ*sty1*), MS112 (Δ*gcn5*) and MS120 (Δ*sin3*) were analyzed as in A and spotted onto YE5S plates containing 0.6 M KCl, 2 mM H_2O_2 grown at 37ºC or left untreated and incubated at 30ºC for 3 to 4 days.

Nothing was known about Sin3 activity, except for its role in tRNA modification (see chapter 5.2.3.2 from Introduction) and its homology to *S. cerevisiae* ELP3. First, we confirmed the HAT activity of Sin3 acetylating histone H3K9 and K14 (Fig. 31A), like its homologue ELP3. Next, we examined the implication of Sin3 in the stress-dependent transcription. Consistent with our previous results, Δ*sin3* strain has also an impaired transcription activation of the tested Sty1-dependent genes (Fig. 31B). However, its defect is not as strong as for Δ*gcn5* cells, what correlates with the H3K9/14 acetylation levels for both backgrounds (Fig. 31A).

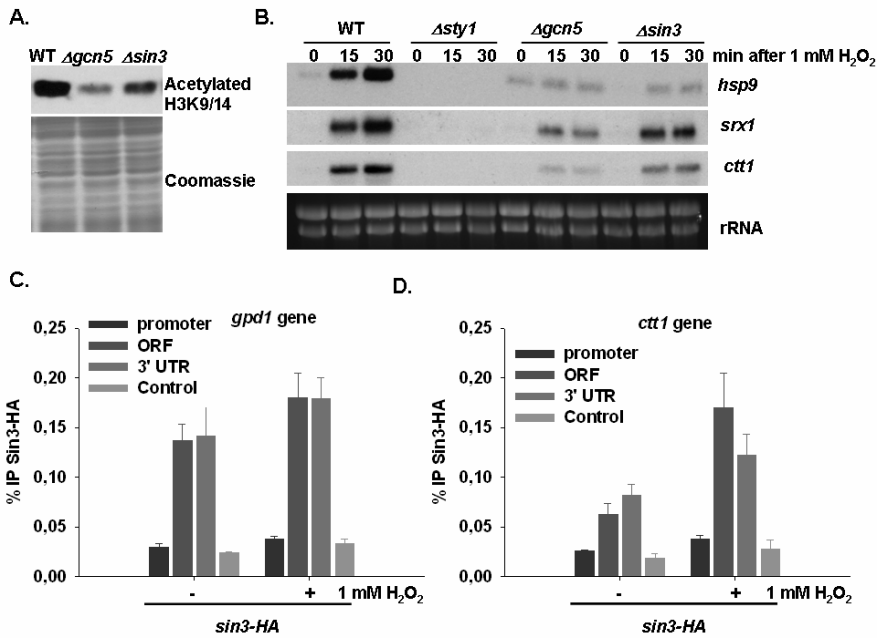


Figure 31. Characterization of Sin3 in stress-dependent transcription and HAT activity. A. Histone H3 acetylation at lysines 9 and 14 is affected by Sin3 mutant. Levels of H3 acetylation were determined by Western blot of TCA extracts from the strains 972 (WT) MS112 (Δ*gcn5*) and MS120 (Δ*sin3*) with antibodies specific to H3K9/14 acetylation (Upstate). As loading control gel was stained with coomassie. **B.** Sin3 is required for transcription of oxidative stress response genes. Cultures of the same strains as in A and AV18 (Δ*sty1*) were treated with 1 mM H_2O_2 for the times indicated. Total RNA was extracted and analyzed by Northern blot with probes for *ctt1, srx1* or *hsp9*. As control ethidium

bromide stained rRNA was used. **C.** Sin3-HA binds to coding regions of *ctt1* gene. Strain MS59 expressing chromosomic Sin3-HA was processed as described in Fig. 20B. **D.** Sin3-HA binds to coding regions of *gpd1* gene. The same experiment as in C made with specific oligos for *gpd1* gene (see Fig. 19B).

S. cerevisiae ELP3 is a cytoplasmic protein (known by fluorescent microscopy of a GFP-tagged version) (Pokholok *et al*., 2002), an it has been described as an elongator factor affecting stress-dependent transcription, but several laboratories have tried, without success, to immunoprecipitate it bound to chromatin (Pokholok *et al*., 2002). We constructed a strain with a genomic HA-tagged Sin3 protein in order to see whether the HAT is bound to stress-genes. Indeed, Sin3-HA is bound to coding regions and terminal part of *ctt1* and *gpd1* genes, but not to their promoters, as shown by ChIP experiments (Fig. 31CD).

This result suggests that Sin3, as its homologue ELP3, has a role in transcription elongation. In order to confirm this hypothesis, we tested the sensitivity to 6-AU of cells lacking Sin3. Besides this, we also analyzed the oxidative stress sensitivity on plates of other mutants lacking different proteins related with transcription elongation by homology, like Elp2, Elp4, Iki1, Iki3, and the protein codified by *SPAC30.02c* gene (homologue of KTI12 from *S. cerevisiae*), as well as the TFIIS factor Tfs1. This analysis revealed a severe oxidative stress phenotype for Δ*elp4*, Δ*iki3* and Δ*SPAC30.02c* strains, together with Δ*sin3*, as previously shown, and Δ*sty1* strain as positive control (Fig. 32A). The 6-AU sensitivity analysis was performed with all the strains related with elongation, those that were positive for oxidative stress sensitivity resulted also sensitive to the 6-AU drug (Fig. 32B). In fact, all these mutants showed a very strong sensitivity to 6-AU, when compared to Δ*gcn5* strain.

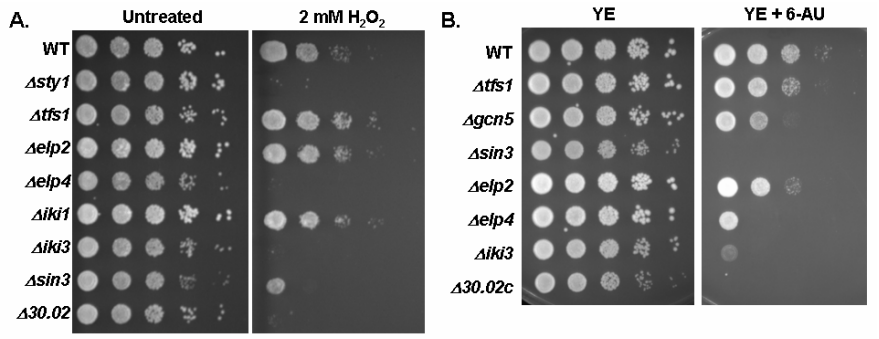


Figure 32. Transcription elongator factors and their role in oxidative stress survival. A. Putative elongator mutants stress sensitivity. Strains 972 (WT), AV18 (*Δsty1*), MS185 (Δ*tfs1*), MS112 (Δ*gcn5*), MS201 (Δ*elp2*), MS202 (Δ*elp4*), MS203 (Δ*iki1*), MS204 (Δ*iki3*), MS120 (Δ*sin3*) and MS205 (Δ*SPAC30.02c*) were grown in YE5S to a final OD_{600} of 0.5, and 10^5 to 10 cells were spotted onto YE5S plates containing 2 mM H_2O_2 or left untreated and incubated at 30ºC for 3 to 4 days. **B.** Putative elongator mutants 6-AU sensitivity. Strains 972 (WT), MS185 (Δ*tfs1*), MS112 (Δ*gcn5*), MS120 (Δ*sin3*), MS201 (Δ*elp2*), MS202 (Δ*elp4*), MS204 (Δ*iki3*) and MS205 (Δ*SPAC30.02c*) were grown in YE5S to a final OD_{600} of 0.5, and 10^5 to 10 cells were spotted onto YE plates containing 100 µg/ml of 6-AU or left untreated and incubated at 30ºC for 3 to 4 days.

In conclusion, these data indicate that Sin3 is a HAT protein acetylating H3K9 and K14 residues, affects stress-dependent transcription and probably, acting as a transcription elongation factor, since it is not bound to promoters and it is sensitive to 6-AU. Finally, the analysis of other possible elongator factors suggests that elongation is a crucial step for oxidative stress survival. Whether binding of Sin3 to these ORFs is Sty1- and/or Atf1-dependent is still to be determined.

DISCUSSION

1. Sty1 BINDS TO STRESS-INDUCED GENE PROMOTERS AND ACTIVATES TRANSCRIPTION THROUGH RNAPII RECRUITMENT

The MAP kinase Sty1 is largely known to be essential in several cellular programmes such as environmental stress defence, mating, stationary phase survival, meiotic recombination and normal cell cycle progression. Indeed, cells lacking Sty1 are sensitive to a wide range of stresses, are sterile, are deficient in hot-spot recombination and present an elongated morphology due to its G2 delay.

Modulation of gene expression has a central role in the Sty1 function, and it is mediated by the transcription factors Atf1 and Pcr1. Moreover, one of the firsts reported modifications of Sty1 in front of a stress situation is its phosphorylation and migration into the nucleus, where orchestrates the launch of alternative gene expression programmes to cope with new conditions. It still remained obscure if Sty1 was acting in a direct way necessitating its recruitment to chromatin for activating transcription or maybe phosphorylating the needed targets out from the genes. We have demonstrated how Sty1, in order of self-activating events, is phosphorylated by Wis1, transported to the nucleus and recruited to stress-dependent promoters and in an Atf1-depedent manner (Fig. 2BC). Moreover, the entering of RNAPII to this CESR genes is also concomitant with Sty1 activation, and is dependent on Sty1 recruitment to promoters (Fig. 2D).

One important conclusion arising from our work is that constitutive nuclear accumulation of Sty1 is not sufficient for transcriptional activation of the CESR genes. We have accidentally accomplished this by: (i) deleting the Crm1 cofactor Hba1 (Castillo *et al*., 2002); tagging Sty1 at its N-terminal domain with GFP (Fig. 4AB). In both cases, Sty1 is

constitutively nuclear but CESR gene expression is dependent on stress imposition; we suspect that upon stress, the MAPK kinase, Wis1, also travels to the nucleus, as previously reported (Nguyen *et al*., 2002), and is therefore able to trigger phosphorylation of constitutively nuclear Sty1. We can conclude that Sty1 has to be phosphorylated to activate transcription.

2. THE KINASE ACTIVITY OF Sty1 IS ESSENTIAL FOR PROPER CELL CYCLE, STRESS SURVIVAL AND TRANSCRIPTION ACTIVATION

Atf1, the main effector of the Sty1 pathway, is phosphorylated upon stress in a Sty1-dependent manner. However, it has been recently published that an Atf1 mutant with no phosphorylable sites, called Atf1-11M, is able to activate transcription (Lawrence *et al*., 2007). Besides this, Sty1 regulates a phosphorylation state of Pcr1, but it is not relevant for its role as a TF either (see below,section 6). In order to investigate the relevance of its kinase activity on Sty1 function, we analysed the mutant phenotype of a kinase dead Sty1 (Sty1·K49R). The phenotype of two strains lacking Sty1 or expressing Sty1·K49R were undistinguible (Fig. 5), even though Sty1·K49R can enter into the nucleus. Moreover, Sty1 is not only responsible of the induction of CESR genes upon stress, but also regulates steady-state levels of those genes (Chen et al., 2003), therefore disrupting the Sty1 pathway, through the kinase death version the protein, confers a basal activation of the stress response due to high ROS levels in the cell (Fig. 6) and also probably due to the low levels protein phosphatases, like Pyp2, that are transcriptionally Sty1-dependent and must dephosphorylate the kinase. Our results suggest that the kinase activity of Sty1 is the only property of the protein required for transcription,

cell cycle and stress survival and that no other part of the protein itself contributes to this activity. Fitting with our results, the catalytic activity of Sty1 has been reported to be necessary for Sty1 recruitment to stress-promoters (Reiter *et al*., 2008).

3. Atf1 AND Pcr1 PLAY SIMILAR BUT NOT IDENTICAL ROLES IN THE STRESS RESPONSE OF FISSION YEAST

Sty1-mediated transcriptional response is mainly dependent on the TF Atf1. About the importance of its partner Pcr1 for the stress defence there are different opinions; some authors have suggested that Atf1 and Pcr1 proteins, as heterodimer, play redundant roles in the CESR gene regulation, while Wahls and coworkers reported that Atf1, but not Pcr1, is required for resistance to extracellular NaCl and suggested that the heterodimer plays a key role in mating, meiosis, and hot spot-mediated recombination but not in global stress resistance (Kon *et al*., 1997 and 1998). All together suggests that these proteins are multifunctional and have distinct activities. Thus, in some contexts the proteins may have redundant roles while in other they may have distinct functions.

In this Thesis we provide new evidences of the functional differences between both TFs upon environmental stress. First of all, Pcr1 is not required for osmotic or oxidative stress on solid plates, a kind of adaptative version of suffering an environmental stress, while it is required for an acute stress imposition, like heat shock and oxidative stress in liquid cultures (Fig 3A, Sansó *et al*., 2008). Moreover, the combined deletion of both transcription factors confers a more severe phenotype compared to the single ones, what correlates with not fully overlapping roles in the stress defence (Fig 3B, Sansó *et al*., 2008).

In terms of transcription activation, we have reported that not all the Atf1-dependent genes are also Pcr1-dependent too, nor in oxidative neither in osmotic stress, as we have seen for *srx1* or *gpd1* and *hsp9*, respectively (Fig. 12). Genome-wide analysis of the Sty1- and Atf1-dependent transcription profiles have been published before, but no data about Pcr1-dependency was available. To globally identify new Atf1-dependent, Pcr1-independent stress genes we performed microarray experiments for one stress and in one time point, and it revealed that the overall KCl response is highly dependent on Pcr1, although a subset of 32 genes seems to be induced in *Δpcr1* cells as effectively as in wild-type cells (Table 1, Sanso *et al*., 2008). Among those Pcr1-independent genes we found some important transcripts for the osmotic stress resistance, for example *tps1* and *gpd1* (Soto *et al.,* 1999; Omiya *et al*., 1995), what correlates with the survival Δ*pcr1* cells in front of KCl compared to cells lacking Atf1.

Since it is not known whether Atf1 works as homodimer *in vivo*, and there are 32 genes whose expression was independent of Pcr1 upon KCl stress. We hypothesised that Atf21, a bZIP transcription factor that is strongly expressed under sorbitol stress conditions (Ohmiya *et al*. 1999; Chen et al., 2003), might substitute for Pcr1 by heterodimerizing with Atf1 at the promoters of these. However, a double knock-out strain *Δpcr1 Δatf21* is still resistant to osmotic stress conditions (Fig. 13A) and its stress-dependent transcription is identical to a Δ*pcr1* strain (Fig. 13B).

Some authors have hypothesised the existence of a Pcr1 homodimer, suggested by *in vitro* experiments (Wahls and Smith, 1994). Nevertheless, we found no one gene which transcription only relays on Pcr1 and not on Atf1, as expected by our phenotypic results.

4. Atf1 AND Pcr1 MODULATE THE BASAL EXPRESSION LEVEL OF A LARGE NUMBER OF STRESS-INDUCIBLE GENES

Our aim of interest is the stress-dependent transcription activation through Atf1 and Pcr1, even though it has been extensively characterized their roles in general repression. It was reported that under basal conditions Atf1 and Pcr1 are crucial for heterochromatin nucleation of the *mat* locus, via recruitment of the HDAC Clr6 for proper histone deacetylation of this chromosomic region (Kim *et al.*, 2004). It has also been reported that the Atf1/Pcr1 heterodimer functions both as an inducer and a repressor of chromatin remodelling at the *cgs2* promoter (Davidson *et al.*, 2004). According to our data, Atf1 and Pcr1 down-regulate the basal transcription of 4% and 6% of the whole *S. pombe* genome, respectively. Through their chromatin binding and remodelling activities, these proteins, acting alone or in combination with other factors, may facilitate the assembly of transcription, recombination or silencing machinery. The involvement of these stress transcription factors in general repression suggests that they might modify chromatin structure as a part of a programmed sequence of events that serves to cushion against the effects of environmental stresses. However, de-repression does not seem to be a factor in the roles of Pcr1 and Atf1 on stress activation, since in *atf1* or *pcr1* deletion mutant strains the basal expression the subset of genes highly induced by KCl is unaffected or only slightly elevated above wild-type levels (Supplementary Table I, Sansó *et al.,* 2008). In conclusion, the heterodimer Atf1-Pcr1 seems to bind to most CESR genes and is required for their activation upon stress. However, both proteins can probably work independent of each other, both regarding (i) stress-dependent gene activation (in cells lacking Pcr1, a subset of genes is still triggered by Atf1, which binds to their promoters

either alone or forming a heterodimer with a yet uncharacterized partner); and (ii) basal repression (in cells lacking Pcr1, 6% of the whole genome is de-repressed, whereas in the absence of Atf1 only 4% of the total mRNAs show enhanced basal levels).

5. Atf1 CAN BIND TO SOME PROMOTERS IN CELLS LACKING Pcr1 AND Sty1

It was published before that Pcr1 was acting as a nuclear anchor of Atf1, hence the low abundance of nuclear Atf1 in Δ*pcr1* cells (Gaits *et al.,* 1998). However, we know that cells lacking Pcr1 have very low levels of Atf1 protein. Therefore, we expressed GFP-Atf1 from a heterologous promoter in wild-type and *Δpcr1* cells, demonstrating that Atf1 is fully nuclear in cells lacking Pcr1 (Fig 1C of Sansó *et al*., 2008). At the same time we observed that both proteins are nuclear residents before and after stress, correlating with our ChIP experiments, in which both Atf1 and Pcr1 are bound to most CESR promoters (exemplified by *cta3*) before and after stress (Fig 6B, Sansó *et al*., 2008). Even more, we have confirmed that the Atf1 binding to stress-dependent genes is restricted to their promoter regions, where its TKACGT binding sequence is located (Fig. 19B). The binding of Atf1 to *cta3* promoter region requires the participation of Pcr1 (Fig 6C of Sansó *et al*., 2008). The *in vivo* protein-DNA binding experiments presented here also indicate that in wild-type cells both Atf1 and Pcr1 are bound to the *gpd1* and *hsp9* promoters, whereas Atf1, but not Pcr1, can be detected at the *srx1* promoter (Fig 6DE of Sansó *et al.,* 2008). We propose that the Atf1-Pcr1 heterodimer is normally bound to most promoters, with very few exceptions. The small bZIP factor Pcr1 probably stabilizes the interaction of Atf1 with all stress-gene promoters, but it is however dispensable in others such as *srx1*. The lack of Pcr1

would thus weaken Atf1 binding to most, but not all, of these promoters. All these ChIP results correlate with and confirm our previous transcriptional-dependency data.

Finally, we have demonstrated that the ability of Atf1 to bind to promoters is Sty1-independent (Fig. 15). This result suggests that basal binding of Atf1 to promoters is not dependent on its phosphorylated status.

6. Pcr1 AND Atf1 STRESS DEPENDENT POST-TRANSCRIPTIONAL REGULATION

Regarding posttranscriptional regulation of these transcription factors, we demonstrate here that Pcr1 is a phosphoprotein, and dephosphorylation occurs upon oxidative, but not osmotic, stress in a Sty1-dependent manner (Fig. 8). Surprisingly, analysis of strains expressing mutant Pcr1 forms with substitutions at its Ser 122, the only MAP kinase phosphorylation site in Pcr1, have demonstrate that the Pcr1 phosphorylation status is not relevant for its function, moreover, Pcr1 apparently does not harbour a non-bZIP activation domain involved in stress-response (Fig. 10). This suggests that the only role of Pcr1 in activating transcription is to provide DNA-binding site specificity to the Atf1-Pcr1 heterodimer. Actually, it has been published a similar result when demonstrating that only the bZIP fragment of Pcr1 is required to activate meiotic recombination hotspot *ade6–M26* (Gao *et al.,* 2008). It is noteworthy, however, that the Sty1-dependent phosphorylation of Atf1 upon stress does not seem to be essential for its role as a transcription factor (Lawrence *et al.*, 2007).

It has been recently suggested that stress induction of CESR genes is critically dependent on the concentration of Atf1 (Lawrence *et al.*,

2007). The authors claim that basal Sty1-dependent phosphorylation stabilizes the Atf1 protein, since the steady-state protein levels of a mutant Atf1 lacking all Sty1 phosphorylation sites are 10-fold lower than the wild-type protein. However, we have shown here that Atf1 protein steady-state levels, when expressed under the control of the *nmt* promoter, are not dependent on the presence of Sty1 (Fig. 5B, Sanso *et al*., 2008). The same study also proposed that Atf1 stability is regulated by its heterodimeric partner Pcr1, since the expression of Atf1 in a strain lacking Pcr1 is lower than in wild-type cells (Lawrence *et al.*, 2007). Nevertheless, we show here that the levels of *nmt*-driven Atf1 protein in *Δpcr1 Δatf1* cells are very similar to those in a single mutant *Δatf1.* Our results suggest that Atf1 protein levels are mainly regulated at the level of transcription and/or translation, rather than by protein stabilisation mediated by basal Sty1-dependent phosphorylation or dimerization with Pcr1.

7. BOTH Atf1 AND Sty1 ARE LOADED TO SOME, BUT NOT ALL, CESR GENES, PRIOR TO STRESS; RNAPII IS STRONGLY RECRUITED TO ALL PROMOTERS UPON STRESS

A careful examination of the Atf1 and Sty1 positioning on stress promoters before stress has revealed a similar pattern of loading. Genes like *gpd1* and *hsp9* have shown a basal binding of Atf1 and Sty1, which recruitment is induced upon stress. In the other hand, *srx1* and *ctt1* promoters present low bindings of the Atf1 and Sty1 proteins before stress imposition. In order to discard possible experimental interferences in those results we have confirmed that the Sty1-pathway is not activated in the presence of formaldehyde, a reagent necessary for ChIP cross-linking, since no phosphorylation of the kinase has been observed by

Western blot (data not shown). More over, growing cells in MM has resulted in the same Sty1 recruitment, like the attempt to change the Sty1-tag positioning from the CTD to the NTD (data not shown). These observations do not correlate with nuclear migration of the kinase after stress exposure, even though is not discarded the possibility of a small fraction of Sty1 acting inside the nucleus in basal conditions. At the same time, the basal Sty1 binding correlates with the previously reported levels of mRNA for those four genes (Schmidt *et al.,* 2007): *gpd1* and *hsp9* have high levels of transcription in basal conditions while *srx1* and *ctt1* mRNAs are modestly represented in untreated cells. We still do not know the phosphorylated status of the bound Sty1, and further experiments will be necessary to understand the role of the kinase in basal conditions.

8. Gcn5 IS REQUIRED FOR OXIDATIVE STRESS RESPONSE

We have shown that Gcn5 is essential for oxidative stress survival, with similar phenotypes on plates to other SAGA components, like the coactivator Ada2 and the Spt8 subunit (Fig. 16AB), what indicates that this Gcn5 acts as part of the SAGA complex and Spt8 does not have an opposite role to Gcn5, as it has been publish for the *ste11* gene regulation (Helmlinger *et al*., 2009). The oxidative-stress induction of transcription for CESR genes is strongly dependent on Gcn5 activity, while steady-state levels of the same genes are not affected (Fig. 16D and 29A).

We have demonstrated that Gcn5 directly regulates the CESR genes; the HAT is recruited to stress promoters in an Atf1-dependent fashion (Fig. 18B). Whether Gcn5 binding depends on Sty1 remains to be determined. The transcription factor and the HAT, together with the kinase Sty1, show the same binding pattern to CESR promoters: *gpd1*

and *hsp9* promoters before stress imposition already bind Atf1, Sty1 and Gcn5, and their binding is not strongly induced upon stress, while in *srx1* and *ctt1* promoters there are less amounts of the three proteins before treatment and a very high recruitment upon stress (Fig. 2D and 18AB). Interestingly, we have shown that Gcn5 role upon stress embraces more than Atf1-dependent transcription, as genetic analysis revealed (Fig. 18D); for instance, the transcription factor Pap1 could be another mark for Gcn5 recruitment to DNA, as we have seen for *ctt1* and *srx1* promoters, where Gcn5-HA is still immunoprecipitated in cells lacking Atf1 (Fig. 18B). Since the phosphorylable targets of Sty1 are still unknown, we hypothesized that Gcn5 could be post-translationally modified by the kinase. Actually, Gcn5 cannot be a target of Sty1 considering that its sequence has no putative sites to be phosphorylated by a MAPK and its electrophoretic mobility doesn't change upon stress (Fig. 18C).

9. Gcn5 PROMOTES TRANSCRIPTION IN PROMOTERS AND ALSO IN CODING REGIONS

We obtained evidences that relate Gcn5 not only with transcription initiation, but also with elongation. We report that Gcn5 occupies the *ctt1* and *gpd1* promoter regions, like Atf1, but also binds to coding sequences during transcriptional induction although a lesser extend (Fig. 19). We confirmed this result with the certainty that we are not detecting a binding signal in the neighbourhood; our chromatin fragmentation is correctly made to discriminate 500 bp between binding sites, as we demonstrate with the limited location of Atf1 to promoter regions (Fig. 19B). Moreover, the sensitivity to 6-AU of Δ*gcn5* strain suggests problems in transcription elongation (Fig. 23). Accordingly to our data, several laboratories have reported that *S. cerevisiae* Gcn5 has a

role in transcription elongation. Additionally, *S. pombe* Gcn5 is bound to coding regions of highly transcribed genes in basal conditions and affects the acetylation levels of H3K9/14 in the same class of highly transcribed genes (Johnsson *et al*., 2009). However, it is worth to point out that the elongation phenotype to 6-AU is much less sever that of cells lacking elongator subunits or the HAT Sin3 (Fig. 32B).

10. HOW DOES Gcn5 REGULATE RNAPII-DEPENDENT TRANSCRIPTION?

Our ChIP analysis upon stress imposition have shown a slight impair of RNAPII recruitment in promoters in Δ*gcn5* cells, and a more severe loss of recruitment of RNAPII in coding regions. As expected, the density of RNAPII in the ORF of *ctt1* and *gpd1* nicely reflected the same tendency in mRNA levels, as shown by Northern blot (Fig. 22). Analysis in H3 acetylation and total H3 levels in those genes reflects a strong impairment in histone H3 eviction from promoters and coding regions, what also affects the acetylation of residues K9 and K14 of H3 per nucleosome, a ratio that is clearly decreased upon stress in a Δ*gcn5* strain compared to wild type cells (Fig. 24 and 25). This means that opened state of the chromatin is not a crucial step for RNAPII recruitment. We have demonstrated that the entry of RNAPII in promoters is not dependent on Gcn5, and we have shown that the HAT is not only present at upstream activating sequences but also localizes in the coding regions. The main difference comparing Δ*gcn5* and wild type cells is the chromatin compactation of the stress genes due to the number of nucleosomes. Thus, we hypothesised that Gcn5 acetylates histones from the CESR genes, what promotes nucleosome eviction and maintains processivity of RNAPII facilitating its promoter clearance.

We still have to analyze whether Gcn5 entering in CESR genes depends on Sty1, but we already have shown that RNAPII loading totally depends on the presence of the kinase Sty1. We hypothesized that Sty1 could be marking something else, nor the TF neither the HAT, in order to to recruit the RNAPII.

11. DELETION OF Clr3 SPECIFICALLY SUPPRESSES Δ*gcn5* PHENOTYPE

Lack of the Class I HDAC Clr3 suppresses the stress sensitivity of Δ*gcn5* cells. Both proteins regulate the same K9/14 residues of H3 (Fig. 16C and 27), and it has been reported before that H3K14 mutant is sensitive to KCl stress (Johnsson *et al*., 2009). The double deleted strain does not restore H3K14/9 acetylation levels in total extracts (data not shown), meaning that the rescuing of Δ*gcn5* is not due to a global effect in the genome. We have confirmed that CESR transcription upon stress is clearly induced if we delete Clr3 in cells lacking Gcn5 (Fig. 29A) and, correlating with this result, H_2O_2 detoxification ability is restored also (Fig. 29B).

Interestingly, Clr3 has been implicated in the regulation of stress-related genes (Wiren *et al*., 2005) and is related with the heterochromatin formation at the *mat* locus, process that also requires Atf1 and Pcr1 transcription factors (Bjerling *et al.,* 2002). Cells lacking Clr3 have been reported to be non sensitive to KCl or 36ºC (Johnsson *et al.,* 2009), resistant to cadmium (Kennedy *et al*., 2008) and we add the oxidative stress resistance to this repertoire of phenotypes upon several stresses (Fig. 26C). Clr3 has not been the only HDAC implicated in the resistance to H_2O_2 imposition; also Δ*hos2* strain showed us better growth fitness in these conditions, an observation that do not fit with the recently published

severe sensitivity of this strain to KCl treatment (Johnsson *et al.,* 2009). Nevertheless, deletion of *hos2* was not sufficient to rescue Gcn5 deletion as Clr3 does (Fig. 28AB).

Preliminary ChIP experiments analyzing the presence of Clr3 in the CESR genes suggest its binding before and after stress exposure, even more, Clr3 is slightly recruited upon H_2O_2 treatment (data not shown). In order to understand the role of this HDAC in the stress response we have to analyze the H3 acetylation levels and total H3 levels in the absence of Clr3 and in cells lacking both, Clr3 and Gcn5 proteins.

12. Sin3, A PUTATIVE ELONGATOR FACTOR, IS NECESSARY FOR THE STRESS RESPONSE

Transcription elongation, as initiation, is a key step in gene expression, but it is less well understood compared to transcription initiation. Sin3 is a putative elongator subunit that possesses histone acetyltransferase activity, suggesting that Elongator may promote efficient transcription elongation by modifying nucleosomes on coding regions. Indeed, we have indirectly confirmed its HAT activity on residues K9 and 14 of histone H3. We have successfully detected Sin3-HA occupancy on transcribed and terminal regions of the stress-dependent genes tested, while no binding has been found in promoters (Fig. 31C). The same genes present an impaired transcription induction upon oxidative stress when Sin3 is deleted (Fig. 31B). However, the role of Sin3 in transcription elongation could be controversial.

In *S. cerevisiae*, ELP3 is predominantly cytoplasmic under several stressful conditions, and few tries to immunoprecipitate it bound to chromatin, even by ChIP on ChIP, have failed (Pokholok et al., 2002). Some authors suggest other functions for this HAT, for instance elongator

may well belong to a class of cytoplasmic B-type histone acetyltransferases that are thought to catalyze acetylation events linked to transport of newly synthesized histones from the cytoplasm to the nucleus (Marmonstein *et al*., 2001; Pokholok *et al*., 2002). Other reports indicate that Sin3 participates in tRNA modification, affecting translation (Esberg *et al*., 2006). We have discarded all this roles as explanations for the Δ*sin3* stress-sensitive phenotype. Even Sin3 has been described as a cytoplasmic protein also by overexpression of GFP-Sin3 (Matsuyama *et al.,* 2006), this localization does not eliminate the possibility that Elongator enters into the nucleus and binds to chromatin, as we have demonstrated by ChIP experiments (Fig. 31CD), whereas in yeast no direct transcriptional targets of the Elongator complex have been identified thus far. Regarding the role as cytoplasmic B-type HAT, we also investigated the localization of histone H3.2-GFP and no differences were detected between wild type and Δ*sin3* backgrounds (data not shown). About translation defects in cells lacking Sin3, we analyzed the protein levels of the main transcription regulators upon activation of the stress pathway (Atf1, Pcr1 and the kinase Sty1), and no difference was detected compared to a wild type (data not shown). Nevertheless, the severe sensitivity of Δ*sin3* to 6-AU on plates strongly suggests its role as part of the Elongator. We have further demonstrated that other mutants lacking putative elongator genes (Δ*iki3*, Δ*elp4*, etc.) are also very sensitive to 6-AU and to oxidative stress (Fig. 32AB). Further experiments to analyze histone H3 acetylation pattern and RNAPII recruitment upon stress should help us to elucidate how sin3 is acting on stress-genes.

CONCLUSIONS

1. **Atf1 and Sty1 are bound to some, but not all, CESR promoter genes, even prior to stress, while the RNAPII is strongly recruited after stress imposition in a Sty1-dependent fashion.**
2. **Nuclear localization of Sty1 is not sufficient to activate transcription.**
3. **The catalytic activity of Sty1 is essential for proper cell cycle, survival in front of stress and transcription activation.**
4. **Atf1 can bind to some promoters in the absence of its partner Pcr1, while no one gene is up regulated upon osmotic stress just by Pcr1.**
5. **Pcr1 bZIP is enough to activate transcription, no other domains of the protein are needed.**
6. **Gcn5 histone acetyl tranferase is necessary for stress-activated transcription.**
7. **Gcn5 probably has a role in promoter clearance, since RNPAII recruitment to CESR promoters is not affected by Gcn5 deletion.**
8. **Deletion of the HDAC Clr3 compensates the transcriptional impairment of Δ*gcn5* strain.**
9. **Sin3 is a new HAT related with stress-transcription and probably involved the Elongator complex.**

MATERIALS AND METHODS

This chapter includes the strains, plasmids and procedures needed for the experimental results presented**.**

STRAINS

Table I. Strains from Results 1.

Name	**Genotype**	**Source**
972	*h-*	Leupold, 1970
PN513	*h- leu1 ura4*	P. Nurse stock
EHH5	*h- leu1 sty1-GFP::kanMX6*	Castillo *et al*., 2003
NT224	*h- leu1 ura4 sty1-1*	Millar *et al*., 1995
AV18	*h- sty1::kanMX6*	Zuin *et al*., 2005
CN011	h^+ *leu1 ura4 rbp1:HÁ:kanMX6*	This work
CN009	h^+ *leu1 ura4 sty1-1 rbp1:HÁ:kanMX6*	This work
MS74	h^+ *leu1 ura4 sty1:GFP: kanMX6*	This work
MS75	h^+ *leu1 ura4 sty1K49R:GFP: kanMX6*	This work

Table II. Strains from Results 2.

Name	**Genotype**	**Source**
972	h^-	Leupold, 1970
MS13	h^- *leu1 atf1::ura4 nmt41x::GFP-HA-atf1::leu1*$^+$	Sansó *et al*., 2008
MS14	h^- *leu1 pcr1::kanMX6 nmt41x::GFP-HA-atf1::leu1*$^+$	Sansó *et al*., 2008
MS15	h^- *leu1 atf1::ura4 nmt41x::GFP-HA-pcr1::leu1*$^+$	Sansó *et al*., 2008
MS16	h^-*leu1 pcr1::kanMX6 nmt41x::GFP-HA-pcr1::leu1*$^+$	Sansó *et al*., 2008
MS7	h^- *leu1 pcr1::kanMX6*	Sansó *et al*., 2008
MS9	h^+ *leu1 atf1::ura4*$^+$ *nmt41x::HA-atf1::leu1*	Sansó *et al*., 2008
EHH5	h^- *leu1 sty1-GFP::kanMX6*	Castillo *et al*., 2003
NT224	h^- *leu1 ura4 sty1-1*	Millar *et al*., 1995
MS48	h^+ *leu1 ura4 pcr1::ura4*$^+$ *atf1::kanMX6*	Sansó *et al*., 2008
JM1066	h^+ *leu1 atf1::ura4*$^+$	Sanchez-Piris *et al*., 2002
JM1821	h^- *leu1 ura4 atf1:HA6His::ura4*$^+$	J. Millar Stock
MS65	h^+ *leu1 ura4 pcr1:ura4 atf1::kanMX6 nmt81::HA-atf1::leu1*$^+$	This work
834	h^- *leu1 ura4 ptc1:: LEU2*	P. Russell Stock
AV15	h^- *atf1::kanMX6*	Zuin *et al*., 2005

AV18	*h⁻ sty1::kanMX6*	Zuin *et al*., 2005
MS5	*h⁻ pcr1::kanMX6*	Zuin *et al.,* 2005
MS38	*h⁻ leu1 ura4 pcr1::kanMX6*	Sansó *et al*., 2008
MS10	*h⁻ leu1 ura4 pcr1::ura4⁺ nmt81x::HA-pcr1::leu1⁺*	Sansó *et al*., 2008
MS36	*atf21::kanMX6 pcr1::ura4⁺*	This work
367	*h⁹⁰ atf21::kanMX6*	P. Russell Stock
MS63	*h⁺ leu1 ura4 pcr1::ura4⁺*	This work
MS72	*h⁺ leu1 ura4 pcr1S122A*	This work
MS73	*h⁺ leu1 ura4 pcr1S122D*	This work
JX26	*h⁹⁰ ade6 leu1 ura4 pcr1::ura4⁺*	Watanabe and Yamamoto, 1996
JA366	*h⁻ leu1*	J. Ayté Stock

Table III. Strains from Results 3.

Name	Genotype	Source
972	*h⁻*	Leupold, 1970
AV18	*h⁻ sty1::kanMX6*	Zuin *et al*., 2005
JM1066	*h⁺ leu1 atf1::ura4⁺*	Sanchez-Piris *et al*., 2002
MS112	*h⁻ gcn5::kanMX6*	This work
MS183	*h⁻ ada2::kanMX6*	This work
MS184	*h⁻ spt8::kanMX6*	This work
HMP47	*h⁻ ade6-M216 leu1 ura4-D18 gcn5:3HA-ura4⁺*	Mitsuzawa *et al.,* 2001
MS176	*gcn5:3HA:ura4 atf1::natMX6*	This work
MS171	*gcn5:: natMX6 atf1:: kanMX6*	This work
CN011	*h⁺ leu1 ura4 rbp1:HÁ:kanMX6*	This work
MS47	*h⁻ gcn5:natMX6 leu1-32 ura4-D18 ade6-M216*	This work
MS185	*h⁻ tfs1::kanMX6*	This work
SPK13	*h⁻ clr3-735*	Hansen *et al*., 2005
SPK19	*h⁻ clr3:: kanMX6*	Hansen *et al*., 2005
SPK28	*h⁻ clr6-1 clr3:: kanMX6*	Hansen *et al*., 2005
SPK29	*h⁻ clr6-1*	Hansen *et al*., 2005
MS186	*h⁻ hos2::kanMX6*	This work
AZ95	*h⁻ sir2::kanMX6*	This work
MS168	*gcn5::natMX6 clr3:: kanMX6*	This work
MS173	*gcn5::natMX6 clr6-1*	This work
MS119	*gcn5::natMX6 hos2::kanMX6*	This work
MS120	*h- sin3:: kanMX6*	This work

MS199	*mts2:: kanMX6*	This work
MS200	*hat1:: kanMX6*	This work
MS59	*h- sin3:3HA:kanMX6*	This work
MS185	*tfs1:: kanMX6*	This work
MS201	*elp2:: kanMX6*	This work
MS202	*elp4:: kanMX6*	This work
MS203	*iki1:: kanMX6*	This work
MS204	*iki3:: kanMX6*	This work
MS205	*SPAC30.02c:: kanMX6*	This work

PLASMIDS

Table IV. Plasmids from Results 1.

Name	Genotype	Source
p178.41x	*pGFP-sty1.41x*	This work
p*sty1*	*psty1-HA-6His.41*	Millar *et al*., 1995
p*sty1·K49R*	*psty1K49R-HA-6His.41*	Zuin *et al*., 2010
pREP.41x	*pnmt41x*	Maundrell, 1993

Table V. Plasmids from Results 2.

Name	Genotype	Source
p179.41x'	*pGFP-HA-atf1.41x'*	Sansó *et al.,* 2008
p171.41x'	*pGFP-HA-pcr1.41x'*	Sansó *et al.,* 2008
p137.41x'	*pHA-atf1.41x'*	Sansó *et al.,* 2008
p138.41x	*pHA-pcr1..41x*	Sansó *et al.,* 2008
p151.41x	*pHA-atf1.41x*	Sansó *et al.,* 2008
p139.81x'	*pHA-pcr1.81x'*	Sansó *et al.,* 2008

Table VI. Plasmids from Results 3.

Name	Genotype	Source
p151.41x	*pHA-atf1.41x*	Sansó *et al.,* 2008

PRIMERS

Table VII. Primers for Northern blot probes

Name	Role	Oligo sequence	Effector
gpx1	glutathione peroxidase	O203: cgggatccatgtctcatttctacgacttg O204: cggaattcttaaaggacactctcgatatcg	Sty1
ctt1	catalase	O188: ccgctcgagatgaactcgaaagactcaa O189: cggcccgggtcagatctaaattgcctg	Sty1/Pap1
zym1	metallothionein	O108:cgggatccatggaacacactacccaatgtaa	Sty1

cta3	calcium ATPase transporter	O109: cggcccgggttacttcgaagcacatttgc' O310: cggggatccatggtaaccattaatatctcg O311: cggcccgggttaggctcttcgagactgc	Sty1
hsp9	heat shock protein 9	O523: cggggatccatgtctgatcccgcaagaaag O524: tcccccgggttacaacttgtcatcaacaaag	Sty1
gpd1	glycerol-3-P-dehydrogenase	O72: cggagatctatgtctggatatggtcaacaagg- O73: cggcccgggttactcggtttcagtaccg	Sty1
srx1	sulfiredoxin	O272: cgggatccatgacttcgattcacactggc- O273: tcccccgggctaatcactatccaaaaatt-	Sty1/Pap1
hsp16	heat shock protein 16	O240: ccctactgtcaacgacttg O241: gcaaggtgacagtcaatagg	Sty1
ntp1	neutral trehalase	O350: ccgctcgagatgccttcgaaattttcc O351: cggcccgggttaatttttatgaatg	Sty1
atf1	cAMP-dependent TF	O192: ccgctcgagatgtccccgtctcccg O193: tcccccgggctagtaccctaaattg	Sty1
ptc1	PP2C	O486: cggagatctatgaagggaagccatcc O487: cggcccgggctaataatagtcattac	Sty1
pcr1	CREB TF	O280:cggggatccatgactgccaaaaaaaaag O281: cggcccgggtcagatgggcccaccaagg	Sty1
trr1	Thioredoxin	O74: cggggatccatgactcacaacaaggttg O75: cgggaattcctaatcggtatcttccaattc	Pap1
pap1	*pombe* AP-1-like	O1: cggggatccatgtccggacaaactgaga O2: cggcccgggtcaacatcgtctttcatcgag	Pap1

Table VIII. Primers for conventional PCR ChIP

Name	Oligo sequence
gpd1	O320: ctatcatccttcgttttcattaccc O321: aagctaagtatttatgtatgagccc
srx1	O322: gaataagctactccaactaccttac O323: cgtttcctgttgtactaatgatctg
hsp9	O482: gctatctcgctttgcaaatc O483: cgtcagttagcattagaaac
cta3	O562: cggcatatgaaaattgcaaaagtatac O563: cagcaaagcttgcctggggaatttaac
cdc18 ORF	JA133: ccgaacttgttaatggataaatc JA134: ggtgacttgagaaaagcattag

Table IX. Primers for Real-Time PCR ChIP

Name	Oligo sequence
gpd1 promoter	O548: ttacgtaatttttcctctttgcttc O549: tgacgtaaaaacactacacttggtc
gpd1 ORF	O862: gagaaactcggtatttactgtggtg O863: aggagggttgaaaccaatagtagtc
gpd1 terminal	O864 : cttgatgcatagatcgaggttgtt O865: gataacaagagactcacaaagcaca
ctt1 promoter	O923: ataatgatgctctttggctcactaa O924: ctggtgtagaattaccaacgtcata

ctt1 ORF	O925: atcgctacaacattttcgatcttac
	O926: aattggtaggattctgattcaaggt
ctt1 terminal	O927: caagtcttctgccgtaaatttctta
	O928: ctcatgaatttgcttttaagctttc
hsp9	O550: tggaaatagcttacgtagctcagaa
	O551: atagctcgctatccaatcagaca
srx1	O552: tactaagaacacaaacccagacctc
	O553: tgtaaaaatttgtcagcatgttagc
control IGR	JA488: tttgtcaaaaagttttcccacatac
	JA489: tttgtcaaaaagttttcccacatac

EXPERIMENTAL PROCEDURES

Solid and liquid sensitivity assay.

For survival on solid plates, *S. pombe* strains were grown in liquid MM or YE5S medium to an optical density at 600 nm (OD_{600}) of 0.5. Cells were then diluted in water, and 10 to 10^5 cells per dot in a final volume of 5 µl (hand made) or 3 µl (metal replica plater) were spotted onto minimal or rich medium plates containing (or not) a stressor. The spots were allowed to dry, and the plates were incubated at 30ºC for 2 to 4 days. To determine survival in liquid cultures, cells were grown in YE5S to an OD_{600} of 0.5. Acute doses of 1 or 2 mM H_2O_2, as indicated, were then added for 60 min. Similarly, other flasks were shifted to 45ºC for 15 or 30 min, as appropriate. Cells were then washed, diluted, and plated on rich medium agar plates to determine survival.

RNA analysis

Total RNA from *S. pombe* cultures was obtained, processed, and transferred to nylon membranes as described previously (Vivancos *et al*., 2004). Membranes were hybridized with the [α-^{32}P] dCTP-labeled *gpx1, ctt1, zym1, cta3, hsp9, gpd1, srx1, hsp16, ntp1,* or *cdc2* probe (see Table VII for specific primers).

Preparation of *S. pombe* trichloroacetic acid (TCA) extracts

S. pombe cultures (5 ml) at an OD600 of 0.5 were pelleted just after the addition of 10% TCA (from a 100% stock) and washed in 20% TCA. The pellets were lysed by vortexing for 5 min, following the addition

of glass beads and 100 µl 12.5% TCA. Cell lysates were pelleted, washed in iced acetone, and dried at 55℃ for 15 min. Pellets were resuspended in 50 µl of a solution containing 1% SDS, 100 mM Tris-HCl (pH 8.0), and 1 mM EDTA. Samples were electrophoretically separated by 8% SDS-PAGE and immunodetected with anti-Atf1. As a loading control, we used monoclonal anti-tubulin (Tub2; Sigma).

Preparation of native extracts

S. pombe cells were grown in MM to OD600 of 0.5 and harvested by centrifugation. For detection of plasmid derived HA-Pcr1, native extracts were obtained. Pelleted cells were washed once with distilled water and resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 120 mM KCl, 5 mM EDTA, 0.1% NP-40, 10% glycerol). Cell suspensions were disrupted by adding glass beads and lysing with three 30-s pulses in a bead beater (Fast Prep; Bio 101) at setting 4.5 and at 4℃. Lysates were then centrifuged to remove cell debris. The protein concentration was determined by using the Bradford protein assay (Bio-Rad).

Preparation of boiled extracts

For detection of endogenous Pcr1, it was necessary to obtain boiled extracts. Pelleted cells were washed once with distilled water and resuspended in HB buffer (25 mM morpholinepropanesulfonic acid [pH 7.2], 60 mM β-glycerolphosphate, 15 mM p-nitrophenyl phosphate, 15 mM $MgCl_2$, 15 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol, and 170 mg/liter phenylmethylsulfonyl fluoride (PMSF)). The cell suspension was boiled for 6 min at 100℃ and then disrupted by glass beads and lysed with two 30-s pulses in a bead beater (Fast Prep; Bio 101) at setting 5.5

and at 4℃. The protein concentration was determined by using the Bradford protein assay (Bio-Rad). One hundred micrograms of protein extracts were loaded onto gels.

Dephosphorylation assay

For dephosphorylation of HA-Pcr1 protein, native extract lysates were incubated with 15 U/μg protein of lambda phosphatase (New England Biolabs) for 60 min at 30℃ before being subjected to SDS-PAGE. When indicated, phosphatase inhibitors were added at the following final concentrations: 1 mM sodium fluoride, 1 mM β-glycerolphosphate, 1 mM sodium pyrophosphate, and 0.2 mM activated sodium orthovanadate.

Protein carbonylation assay

30 ml of *S. pombe* cultured in MM at OD_{600} of 0.5 were centrifuged at 3000 rpm_s for 3 min. Pellet was washed with H2O and resuspended in 250 μl of carbonylation buffer (50 mM Tris-HCl pH 7.5,2 mM EDTA, 0.05% NP-40, 1 mM PMSF, 5 mM benzamidine, 5 mg/ ml aprotinine). Cell suspensions were disrupted by adding glass beads and lysing with three 25-s pulses in a bead beater (Fast Prep; Bio 101) at setting 4.5 and at 4℃. Lysates were then centrifuged for 10 min at 13.200 rpm_s to remove cell debris. The protein concentration was determined by using the Bradford protein assay (Bio-Rad). 10 μg of total extract was boiled for 3 min in 1 vol of SDS 12% to a final vol of 6% SDS. Samples were mixed with 1 volume of 10 mM 2,4-ditrophenylhydrazine (DNPH) in TFA 10% and left them react for 10 min at 25ºC. Reaction was stopped by adding 1 volume of 2 M Tris base, 30% glycerol, 15% β-

mercaptoethanol. Samples suffer a pH change and colour turns from yellow to orange.

Samples were analyzed by Western blot in 10% SDS-PAGE. Carbonylated proteins were visualized by anti-DNPH (Sigma D 9656).

Western blot

Desired quantity of protein extract is mixed with loading buffer and run on a SDS-PAGE gel (sodium dodecyl sulfatepolyacrilamide gel electrophoresis, 30:0.8 acrilamide:bisacrilamide) which percentage depends on the protein to analyze. Gel is transferred to a nitrocellulose membrane 1 hour at 400 mA. The membrane is blocked with 3% BSA (for phospho-p38 antibody) or milk (for the other antibodies) for 1 hour and is incubated over night with the primary antibody. After 3 washes with TBS-T, the membrane is incubated with the secondary antibody, washed 3 times again with TBS-T and developed with ECL reagent kit.

Transformation of *S. pombe*

Cell culture should be grown in YE5S until a concentration of 10^7 cells per ml. Cells should be washed with water and resuspended in lithium acetate (LiAc-TE: 0.1 M litium acetate, 10 mM Tris pH 7.5, 1mM EDTA). $2x10^6$ cells are mixed with 4 µg of carrier DNA (SIGMA Herring Sperm DNA (D-7290)) and 1 µg of the transformant DNA. After incubating the cells for 10 minutes at room temperature, 260 µl of PEG/LiAc-TE (LiAc-TE, 40% PolyEthilenGlycol) are added and incubated for 1 hour at 30ºC. 43 µl dimethyl sulfoxide are added and cells are heat shocked at 42ºC for 5 minutes. After 2 water washes, cells are plated on selective media.

Microarray experiments and data evaluation.

Global expression analysis utilized custom-designed *S. pombe* microarrays. Array construction, sample labelling, and hybridization were carried out as described previously (Sinibaldi et al., 2001). The arrays consisted of 8,785 70-mer oligonucleotides designed to measure gene expression (http://research.stowers-institute.org/microarray/S_pombe/). Briefly, total RNA was prepared from strains 972 (WT), AV15 (Δ*atf1*), and MS5 (Δ*pcr1*), treated (or not) for 20 min with 0.4 M KCl. Polyadenylated RNA was extracted from total RNA by purification with an oligo(dT) cellulose column. RNA quality was assessed on a Bioanalyzer 2100 machine (Agilent). RNA (2 μg per sample) was converted to cDNA by priming with oligo(dT18) and poly(dN9) in the presence of aminoallyl-dUTP (Ambion), followed by conjugation to Cy5 or Cy3 fluorescent dye. Samples were prepared from three biological replicates, and all samples were dye swapped for further technical replication. Labelled samples were mixed for comparative hybridization on poly-L-lysine-coated microarrays printed with 70-mers representing all known *S. pombe* reading frames. The microarrays were scanned with a GenePix 4000B scanner and the images analyzed using the GenePix Pro 6.0 software program (Molecular Devices, Union City, CA). Data analysis was performed with the R programming language. Data were normalized via the print-tip loess method, and differential expression was assessed using the LIMMA software package (Smyth, 2004). Genes exhibiting at least a twofold difference between the wild-type and mutant or treated versus untreated, with unadjusted P values of less than 0.05, were considered differentially expressed.

Microarray data accession number.

Microarray data are available at Array-Express (www.ebi.ac.uk/arrayexpress/) under accession number E-TABM-447.

Chromatin immunoprecipitation

For immunoprecipitation of HA-tagged Atf1 and Pcr1 proteins linked to DNA promoter regions, cells were grown in liquid MM to an OD_{600} of 0.5 and formaldehyde (1.5% vol/vol) was added for 30 min at 25°C. Cross-linking was stopped by adding 187.5 mM glycine. After 5 min, cells were collected by centrifugation and washed twice with cold distilled water. Pellets were resuspended in 250 µl breaking buffer (0.1 M Tris- HCl [pH 8.0], 20% glycerol, and 1 mM PMSF) and lysed with glass beads in a bead beater (Biospect Products). Pellets were collected, washed twice with lysis buffer (50 mM HEPES-NaOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF), and resuspended in 250 µl lysis buffer. Lysates were then sonicated in a Bioruptor (Diagenode) sonicator with eight 30-s high-sonication pulses at 4°C and 1-min pauses between pulses, yielding chromatin fragments with an average size of 500 bp. Lysis buffer was added up to 1 ml, and samples were centrifuged at 16.000 g for 30 min at 4°C. Fifty microliters from the soluble chromatin samples were kept as inputs, and the remaining was immunoprecipitated with monoclonal anti-HA antiserum overnight at 4°C, followed by addition of protein A-Sepharose beads and incubation for 4 h at 4°C. Immunocomplexes were washed once in lysis buffer, twice in lysis buffer containing 0.5 M NaCl, twice in washing buffer (10 mM Tris-HCl [pH 8.0], 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 1 mM PMSF), and

finally once in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Beads were pelleted, and DNA was eluted in 100 µl elution buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA [pH 8.0], 1% SDS) during 20 min at 65℃. Beads were repelleted, the supernatants were transferred to fresh tubes, and any remaining DNA was eluted from the beads by washing it once in 150 µl TE–0.67% SDS. Corresponding elution supernatants were pooled, and formaldehyde cross-linking of both the 50 µl of soluble chromatin and immunoprecipitated chromatin was reversed by overnight incubation at 65℃. DNA was cleaned up by incubation for 2 h at 37℃ with 0.3 mg/ml proteinase K and 0.04 mg/ml glycogen and was then purified by phenol-chloroform extraction and precipitated with ethanol and NaCl. DNA was resuspended in 100 µl TE. Recovered DNA was PCR amplified with specific primers (see Table VIII), and products were resolved on ethidium bromide-containing 2% Tris-borate-EDTA agarose gels. Chromatin immunoprecipitation experiments from Results 3 were carried out as described before, with some modifications. Cells were grown in rich media until OD_{600} 0.5. HA-tagged proteins were cross-linked with 1% of formaldehyde for 20 min at 25ºC. Cross-linking was stopped by adding 125 mM glycine. Recovered DNA was analyzed by Real Time PCR (Light Cycler 480, Roche) with specific primers (see Table IX) and SyberGreen Taq mixture (Roche).

Cell fixation and DAPI staining

Approximately 10^7 cells are harvested from the culture, centrifuged and resuspended in 70% Ethanol (EtOH). Cells can remain fixed for a long time. To stain the cells, they should be previously rehydrated. When the desired amount of cells is put on the slide they are dried and 5mg/ml 4', 6'-diamine-2-phenylindole (DAPI) is applied. Under a

florescence lamp in the microscope and with a UV filter, nuclei can be easily observed.

Ferrothiocyanate assay

In vitro H_2O_2 concentration has been determined by a spectroscopic method, which quantifies a red product (ferrothiocyanate), formed by the oxidation of Fe^{2+} and SCN^- promoted by H_2O_2. Cells grown in YE5S until reach an OD600 of 0.5 were treated or not with 1 mM of H_2O_2 for the desirable times points. A sample of 1 ml was centrifuged 1 min at 13.200 rpm in order to eliminate the cells and obtain free cell medium. Dilution 1/10 in mQ water of each sample was prepared (the lineal range of H_2O_2 concentrations that can be determined with the following method is of 10-150 µM) 100 µl of the diluted medium where mixed with 14.28 µl of TCA 100%, 26.9 µl Fe 10 mM and 13.44µl of SCN 2.5 M. The solution must become reddish once reaction is occurring normally. The measurement of the H_2O_2 concentration was made with spectrophotometer at OD of 480nm. Blank measurement must be made with fresh YE5S medium also dilute 1/10.

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