Regulation of gene expression program by the MAPK Sty1 and the transcription factor Atf1

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A la meva família,

ABSTRACT

In *Schizosaccharomyces pombe*, the MAPK Sty1 is activated upon several stress situations, like osmotic and oxidative stress, UV radiation, heat shock or stationary phase. Since the modulation of gene expression is one of the main outputs of this response, we focused this Thesis work on the characterization of Sty1 pathway as a sensor of oxidative stress and the different molecular events leading to its activation.

A *Schizosaccharomyces pombe*, la MAPK Sty1 s'activa en front diferent tipus d'estrés, així com estrés osmòtic, estrés oxidatiu, radiació ultraviolada, estrés per calor o fase estacionària. Tenint en compte que la modulació de l'expressió génica és un dels principals objectius, em centrat el treball d'aquesta Tesis en la caracterització de la ruta d'Sty1 com a sensor d'estrés oxidatiu i els diferents events moleculars que resulten en la seva activació.

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INTRODUCTION

1 Reactive oxygen species and oxidative stress

All organisms are exposed to reactive oxygen species (ROS) during their metabolic processes as respiration or cause by exposition to ultraviolet (UV) radiation or heavy metals. ROS are defined as a variety of molecules and free radicals derived from oxygen (O_2). The oxygen molecule is itself a stable di-radical but can go through partial reduction to form a number of ROS including the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). (**Fig. 1**)

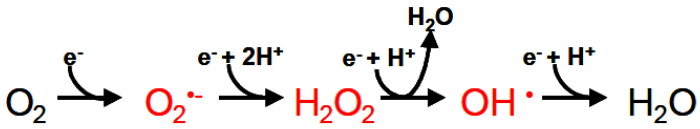

Figure 1: Reactive species oxygen (ROS), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) are based on partial reduction of oxygen molecule (O_2).

Oxidative stress reflects an imbalance between the production of ROS and the biological systems ability to detoxify the reactive intermediates or to repair the resulting damage in DNA, proteins or lipids. Furthermore, some ROS act as cellular messengers in redox signaling.

Superoxide anion, $O_2^{\cdot-}$: one electron reduction state of O_2 formed majority by the electron transport chain. However, due to its instability and to its inability to diffuse in cellular membranes, $O_2^{\cdot-}$ does not react as easily with other cellular components. Nevertheless, $O_2^{\cdot-}$ can release iron from iron-sulfur proteins and ferritin.

Hydrogen peroxide, H_2O_2: two electron reduction state, formed by dismutation of $O_2^{\cdot-}$ or by direct reduction of O_2. The main toxicity is due to its reduction to hydroxyl radical (see below). The stability of its oxygen-oxygen single bond limits its reactivity. However, its reactivity towards

protein thiols can increase depending on the protein environment. Due to its capacity to diffuse through membranes is widely used trigger oxidative stress in research.

Hydroxyl radical, $OH^{\cdot}$: three electron reduction states, formed by the Fenton reaction. $OH^{\cdot}$ is highly reactive and short-lived. It is also produced during UV light dissociation of H_2O_2 and likely in Fenton chemistry, where traces of reduced transition metals catalyze peroxide-mediated oxidations of organic compounds.

In the Fenton reaction, Fe^{2+} is oxidized by H_2O_2 to Fe^{3+} forming and $OH^{\cdot}$ and a hydroxide ion in the process. Fe^{3+} is then reduced back to Fe^{2+} by another molecule of H_2O_2, forming $O_2^{\cdot-}$and a proton.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$$

The Fenton reaction was suggested by Haber and Weissin 1930s and previously a Fenton reagent was developed by Henry John Horstman Fenton as an analytical agent (Fenton H.J.H. et al., 1894).

1.1. Targets and sources of ROS

During aerobic growth, the main source of ROS is the mitochondrial respiration through the oxidative phosphorylation were the electrons of reduced flavins (FADH) and NADH, coming from the Krebs cycle and glycolysis, go over the oxygen through the mitochondrial respiration chain (see **Fig. 2**). Similarly, peroxisomal fatty acid degradation in β-oxidation pathways or the uses of O_2 as a terminal electron acceptor during oxidative protein folding in the ER are also sources of ROS [1].

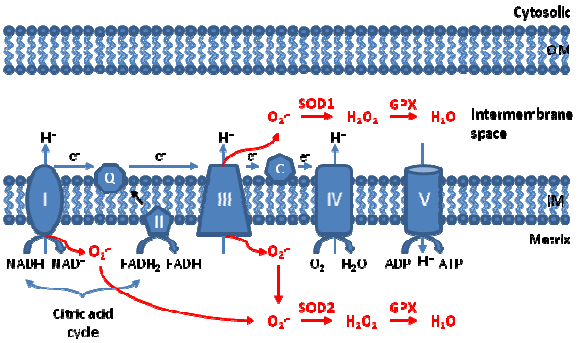

Figure 2: Electrons donated from NADH and FADH2 pass through the electron transport chain and ultimate reduce O_2 to form H_2O at complex IV.

Cells are also under exposure of exogenous ROS. Ionizing radiation, xenobiotics or drugs are a known source of ROS [2].

A variety of exogenous and endogenous factors are responsible for ROS production from the mitochondria and other cell compartments. Cellular levels of ROS are controlled by a complex network of antioxidant systems. ROS act as modulators in several signaling pathways implicated in stress responses, but they can also promote damage to lipids, DNA and proteins.

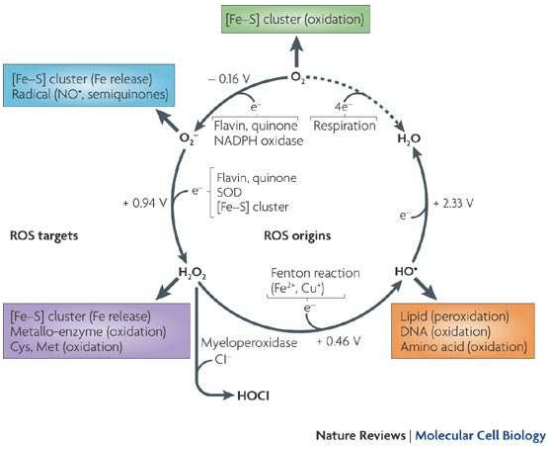

Figure 3: ROS origins and targets. [3]

1.2. ROS damage to biomolecules

Lipid peroxidation

Lipid peroxidation refers to the oxidative degradation of lipids. It comprises a series of chain reactions in which free radicals react with the electrons from the lipids in cell membranes. The lipids most highly susceptible to oxidation by ROS are the polyunsaturated fatty acids present, mostly, in biological membranes.

The reaction is usually iniciated by $O_2^{\cdot-}$, $OH^{\cdot}$ and transition metals. Under aerobic conditions, O_2 will be added to the carbon radical to produce lipid peroxyl radical, $LOO^{\cdot}$. Once synthesized, $LOO^{\cdot}$ can propagate the peroxidation chain reaction by abstracting a hydrogen atom from a new unsaturated fatty acid triggering lipid hydroperoxide.

Inside the larger set of lipids, membrane phospholipids are major targets of ROS, since O_2 and its derivates are more soluble in lipid environment than in solution. Peroxidized membranes are rigid, lose permeability and integrity[4].

Lipid peroxidatic products can also react with amino groups of deoxyguanosine, deoxyadenine, and deoxycytosine to form alkylated products. These DNA damage markers are mutagenic and carcinogenic with effects on signal transduction pathways [5].

DNA damage

ROS can lead to formation of DNA strand breaks, base and nucleotide modifications, particularly in sequences rich in guanosine [6]. Oxidative modifications induces a robust repair response, characterized by excision of modified bases and nucleotides. After DNA damage, cell cycle checkpoints are activated. Checkpoint activation pauses the cell cycle and gives to the cell activation of repair mechanisms. Checkpoint is controlled by two masters kinases: ATM and ATR. ATM kinase responds to double-strand breaks caused by ROS and others insults.

The best known DNA oxidative damage is the formation of 8-hydroxy-2'-deoxyguanosine, an oxidative modification of DNA produce by hydroxylation in the C-8 position of deoxyguanosine residues by $OH^{\cdot}$ [7].

Protein oxidation

ROS oxidation of proteins leads to amino acid residue modifications, cleavage of peptide bonds and formation of covalent protein-protein cross-linked derivatives. Side chain amino acid oxidation may result in reversible or irreversible modifications. Reversible modifications include some types of cysteine (Cys) and methionine (Met) oxidation. Specific enzymatic activities are involved in their repair. However, most of the oxidative modifications results in their definitive damage, protein loss of function and/or protein degradation. It could occur that the accumulation of oxidative damaged proteins overcomes the proteolitic cellullar capabilities, accumulating in the form of toxic aggregates. These aggregates are related with neurodegenerative diseases and aging in mammals. It is still unclear if this aggregation contributes to cell signaling or not, since aggregates do not accomplish the reversibility principle required for cell signaling events.

1.3. Antioxidant defences

Although oxidation reactions are crucial for cell survival, they can also cause damage. Several organisms have evolved complex systems of antioxidant defence mechanisms, which include enzymatic and non-enzymatic activities. Enzymes such as catalase, superoxide dismutase (SODs), glutathione peroxidases (GPxs) and thioredoxin peroxidases or peroxiredoxins (Prxs) interact with ROS leading to their decomposition. Other molecules such as glutathione, vitamine E, vitamine C are non-enzymatic antioxidant defences. Inhibition or insufficient levels of antioxidants cause a disequilibrium in the cell, leading to oxidative stress (**Fig. 4**).

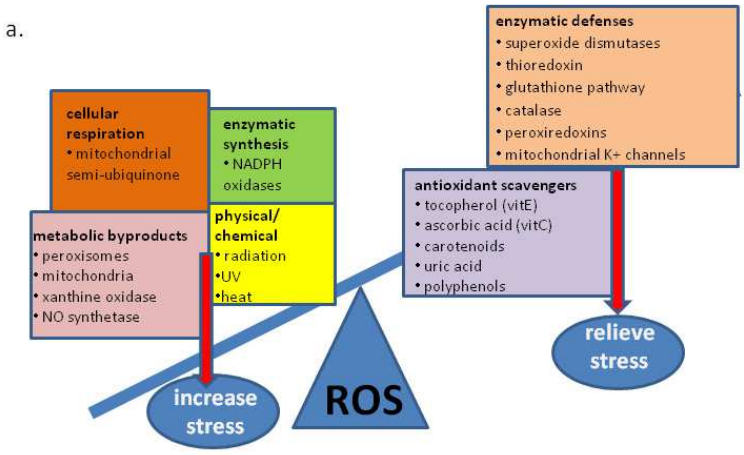

Figure 4: Oxidative stress emerges when the equilibrium between ROS generation and scavenging is disturbed.

1.3.1. Non enzymatic antioxidant activities

Glutathione, ascorbic acid and vitamin E are the non enzymatic antioxidants in the cell. Ascorbic acid and vitamin E are the most abundant hydrosoluble and lyposoluble vitamins respectively, thus there are the only ones believed to have a role in the antioxidant defense.

Glutathione (GSH): The glutathione/glutaredoxin system consists of glutaredoxin (Grx), glutathione (GSH) and NADPH-dependent glutathione disulfide (GSSG)-reductase (GR). γ-glutamyl-cystenyl-glycine (GSH) is the best known example of non-enzymatic defense system. GSH is the most abundant low-molecular-weight compound in most organism. It is an essential metabolite in yeast and was proposed to be required as a reductant during normal growth conditions [8]. GSH acts as a radical scavenger with the redox-active sulfydryl group reacting with oxidants to produce GSSG, its oxidized form.

The synthesis of GSH involves two ATP-dependent enzymatic reactions, catalysed by γ-glutamylcysteine synthetase (Gsh) and glutathione synthetase (Gs). During the first reaction, Gsh1 catalyzes the

formation of dipeptide γ-Glu-Cys form glutamate and Cys. The second step is catalized by Gsh2 and ligates γ-Glu-Cys with glycine to yield GSH [9]. The enzymes for reduction of GSSG to GSH are glutathione reductase (Glr), which are consider essential antioxidant enzymes. Gsh1 enzyme is inhibit by GSH in a typical feedback negative loop and *gsh1* gene expression is regulated by cellular concentrations of GSH, plus sulfur aminoacid biosynthesis [10].

GSH is an important antioxidant molecule in yeast. Cells lacking GSH are very sensitive to peroxides and other compounds such as cadmium and methylglyoxyl [11-13]. Under normal aerobic conditions, the relation between reduced and oxidized form of GSH is 10-15:1 indicating that the reduced form is predominant in yeast and other eukaryotes due to the constitutive activity of Glr1.

It has been recently proposed, however, that the essential role of GSH is due to its participation in the iron-sulfur cluster assembly pathways [14, 15].

Ascorbic acid: Ascorbic acid is a hydrosoluble molecule also known as vitamin C. It acts as a redox couple with GSH in many eukaryotes. In yeast is not clear its function as a antioxidant since it's found in very low concentrations. However, cells lacking an enzyme that catalyze the last step of its synthesis are sensitive to H_2O_2 and hydroxyl radical [16, 17].

Vitamin E: Vitamin E acts as a lipid-based radical chain breaking molecules with scavenging capacity for free radicals including lipid peroxyl, alkoxyl and $OH^{\cdot}$ [18].

1.3.2. Enzymatic ROS detoxification

Catalases

Catalases are ubiquitous heme-containing enzymes that catalyze the dismutation of H_2O_2 into H_2O and O_2.

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Their origin is monophyletic and group in three different families. Two of the protein families are heme enzymes: typical catalases and catalase-peroxidases. Typical catalases comprise the most abundant group in many organisms and they are divided based on having large or small subunits. Catalases-peroxidases are not present in plants or animals and show catalytic and peroxidatic activities. The third group is a minor bacterial protein family with a dismanganese active site. Although all families catalyze the same reaction they differ in the active-site architecture and the mechanism of reaction [19, 20].

Saccharomyces cerevisiae (S. cerevisiae) has two catalases: catalase A encoded by *CTA1*, which is present in peroxisomes, and catalase T encoded by *CTT1* which is cytosolic. CTA1 is involved in the detoxification of H_2O_2 generated by acyl-CoA oxidase during fatty acid β-oxidation at peroxisomes [21]. Contrarily, CTT1 plays a more general role as an antioxidant upon oxidative stress, since its expression is induced by various stress insults including heat, osmotic, nutrient starvation and H_2O_2 stress.

Schizosaccharomyces pombe (S. pombe) has only one catalase called Ctt1, which is cytosolic and its expression under oxidative stress depends on Pap1 and Prr1 [22] and also on Sty1-Atf1 pathways [23-25]. Cells lacking *ctt1* shows an increased sensitivity to H_2O_2 but they do not have any growth defects under basal conditions. This suggests that the level of H_2O_2 generated during aerobic growth is low and does not affect the growth of cells lacking this H_2O_2 detoxification enzyme and that other H_2O_2-detoxification enzymes may be controlling these low levels of peroxides. Ctt1 is not able to detoxify low levels of peroxides due to its low Km; however its catalytic cycle requires the interaction of two

molecules of H_2O_2 inside its active center [26]. In addition, cells over expressing catalase activity show increase resistance to H_2O_2 [27].

Glutathione peroxidases (GPx)

GPx reduces lipid hydroperoxides to their corresponding alcohols and H_2O_2 to water. In this process, two Cys residues of GPxs are oxidized to a disulfide bond; electrons required for the reduction of the disulfide to the thiol form will be provide by the reduced cofactor NADPH, with the intervention of GSH (**Fig. 5**) [28].

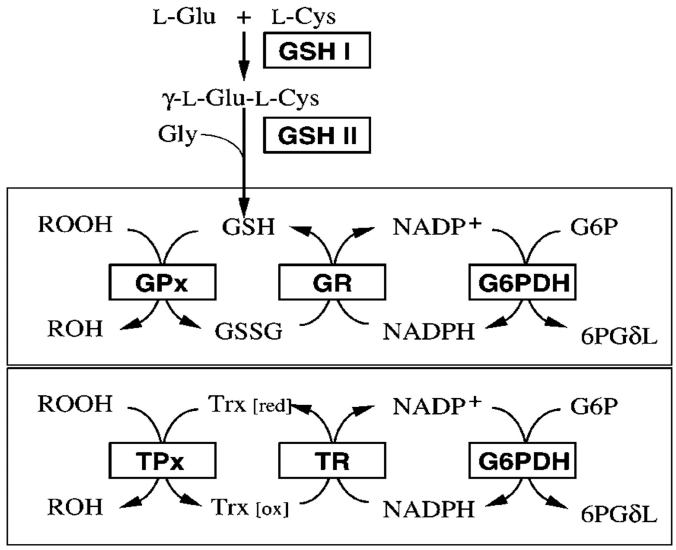

Figure 5: **Catalytic cycles of GPx and TPx reactions.** GSH is synthesized by a sequential reaction of γ-glutamylcysteine synthetase (*GSH I*) and glutathione synthetase (*GSH II*). *GR*, glutathione reductase; *G6PDH*, glucose-6-phosphate dehydrogenase; *TR*, thioredoxin reductase; *GSH*, glutathione (reduced form);*GSSG*, glutathione disulfide; *Trx*, thioredoxin; *ROOH*, peroxides. [29]

Curiously, *S. pombe* and *S. cerevisiae*, do not express any classical GPx. So, GPxs from yeast are classified as atypical 2-Cys peroxiredoxins, they form an intra-molecular disulfide bound in their catalytic cycle, which is then reduced by thioredoxin (Trx) [30, 31].

In *S. pombe* only one GPx (SPBC32F12.03c) has been identified. GPx acts as a Trx-dependent peroxidase in stationary phase, and it degraded H_2O_2 using reduced Trx (ref***).

Peroxiredoxins

Peroxiredoxins (Prxs) are a ubiquitous family of proteins, highly express in cells (up to 1% of total protein), that function as major antioxidant enzymes but also as mediators of H_2O_2 signaling in eukaryotes [32, 33]. These groups of peroxidases catalyze the reduction of H_2O_2, alkyl hydroperoxides and peroxy nitrite into H_2O, with the presence of Trx1, Trr1 and NADPH [34, 35].

Prxs are localized in the cytosol, mitochondria, chloroplasts, peroxisomes, nucleus and membrane. All have in common an universally and conserved sequence motif at their catalytic site, which closely resembles a Trx fold. Some evidences suggest that Prxs have evolved from a Trx-like precursor, so the unique difference between the Trx and the Prx is a Tyr replacing the catalytic Cys. Prxs share the same mechanism to reduce peroxides. It involves a conformational step and three chemical steps: peroxidation, reduction and recycling (see **Fig. 6**).

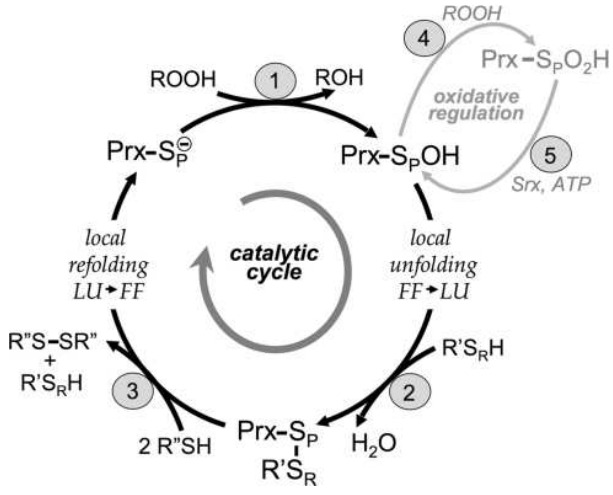

Figure 6: The catalytic cycle of peroxiredoxins (Hall et al., 2011)

So, the first step of the catalytic cycle (step 1 in **Fig. 6**) the peroxide substrate (could be an alkyl hydroperoxide or H_2O_2) enters the fully-folded (FF) conformation of the substrate binding pocket, where it reacts with the peroxidatic Cys (C_p). In consequence, the peroxide becomes reduced to its corresponding alcohol and Cp is oxidized to a sulphenic acid form (S_pOH). In the second step, call the resolution step (step 2 in **Fig. 6**), a free resolving thiol (S_RH) present in the same or in another subunit of the Prx, attacks the S_pOH to form a disulfide bond and release H_2O. To make more accessible the Cp is necessary a local unfolding of the active site. Once the disulfide is formed it keeps in this local unfolded (LU) conformation, finishing the catalytic cycle. Recycling step requires a protein or small molecule thiol, but for most of the Prxs this step involves a Trx-like protein or domain [36].

In addition with the resolution step, there is an over oxidation of the Cp to a sulphenic acid. In presence of more peroxides, this reaction can be followed by a sulphinic (S_pO_2H) or sulphonic (S_pO_2H) acids. This overoxidation (steps 4 and 5 fig.) generates an inactive enzyme, however, S_pO_2H can be recycled by sulfiredoxins to S_pOH in an ATP-dependent manner. It has been proposed that this inactivation could have a role in peroxide signal transduction [36].

In *S. pombe*, the Prx Tpx1 is the major intracellular scavenger of H_2O_2, as is essential for aerobic growth. It is classified as a typical 2-Cys Prx, belonging to the subfamily of PrxI, and it is inactivated upon doses of 1 mM H_2O_2 [37]. In *S. pombe* there are two independent pathways activated depending on the intracellular concentrations of H_2O_2: the Pap1 and the Sty1/Atf1 pathways. I will explain with more details in section 1.1.4.2.

Superoxide dismutases (SODs)

Superoxide dismutases are enzymes that catalyze the dismutation of O_2^- into O_2 and H_2O_2. Thus, they are an important antioxidant defense in cells exposed to oxygen. Next step is to reduced the H_2O_2 generated into H_2O by catalases or peroxidases.

The SOD-catalyzed dismutation of O_2^- is described in the following half-reactions

$$M^{(n+1)} - SOD + O_2^- \rightarrow M^+ - SOD + O_2$$

$$M^+ - SOD + O_2^- + 2H^+ \rightarrow M^{(n+1)} - SOD + H_2O_2.$$

where if M=Cu n=1; and if M=Mn, Fe or Ni n=2

The enzyme activity is dependent on redox cycling of the bound metal cofactor. This means that SOD1 activity needs an intermolecular disulfide bond. The maturation of SOD1 enzyme requires the copper chaperone Ccs1, which participates in copper delivery to the SOD1 apoprotein [38].

S. cerveisiae and *S. pombe* contain two SODs, a cytoplasmatic Cu, Zn-SOD (named SOD1) which is also localized in the mitochondrial inner membrane space. It has a role in the detoxification of O_2^- generated from respiration [39]. The other is a mitochondrial matrix manganese-containing SOD (MnSOD or SOD2) which seems to play different roles during oxidative stress [40].

1.4. Oxidative stress responses: general and specific

Cells use specific sensory and signaling systems to obtain and transmit information from the environment to adjust cellular metabolism, growth and survival. Unicellular organisms have to cope with a wide range of fluctuations, such as variations in nutrient availability, pH, temperature, osmolarity, UV radiation, but also exposition to a range of toxic compounds. They have developed sophisticated systems to sense fluctuations in the intensity, concentrations or presence of such variables to adapt in order to survive and proliferate. Low levels of stress often trigger adaptative responses which result in transient resistance to future higher levels of the same stress, and that can also lead to increase resistance to other types of stresses. This phenomenon is known as cross-protection and suggests that common defense mechanisms are

able to provide a basal level of protection, leading to the concept of a general stress response [41, 42].

Global approaches have been decisive to elucidate the molecular responses of cells to diverse environmental stresses. Whole genome approaches revealed that the transcriptional profiles of the budding and fission yeast show a transcriptional response that is common in the majority of stress conditions. This core response is very conserved between both species. The transcripts which are similarly up regulated involve genes related with carbohydrate metabolism, detoxification of ROS, protein folding and degradation, vacuolar and mitochondrial functions, autophagy, and metabolite transport. Repressed genes are involved in RNA processing, transcription and translation, and ribosomes and nucleotides biosynthesis. This stereotypical response has been termed as “environmental stress response” (ESR) in budding yeast or common environmental stress response (CESR) in fission yeast [41-44].

To regulate their core stress response, cells use different strategies, concerning differential regulation of signal transduction pathways and translating extracellular signals into intracellular ones. In most eukaryotic cell types, the stress-specific Mitogen Activated Protein Kinase (MAPK) pathways are the most important key regulators of these common stress responses. (See section 3)

2. Stress responses in *Schizosaccharomyces pombe*

2.1. Fission yeast as a model system

The fission yeast *S. pombe* is an excellent model to study stress responses and other cellular processes, for example, cell cycle, splicing, etc, at the molecular level. This single cell eukaryote is non-pathogenetic and can be easily grown and manipulated in the lab. Fission yeast has one of the smallest numbers of genes of a known genome sequence for a eukaryote, and has only three chromosomes (**Fig. 7**).

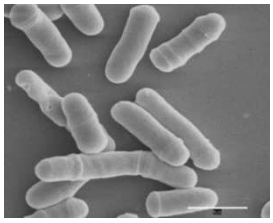

Figure 7: Scanning electron microscope image of S. pombe.

Fission yeast was isolated in 1893 by Paul Lindner from East African beer (the word “pombe” means beer in Swahili language). The sequence of *S. pombe* genome was published in 2002 by a consortium led by the Sanger Institute. This has fully unlocked the power of this organism with many gene homologous to human diseases being identified. Surprisingly, *S. pombe* differs phylogenetically from *S. cerevisae* as much as from humans [45, 46].

Importantly for studying oxidative stress and other cellular processes and pathways, is the capacity of this organism to grow in a haploid state. As a consequence, non-essential genes can be easily deleted by classical techniques of DNA recombination.

S. pombe has many genes which deletion will contribute to enhance or decrease endogenous ROS production. For example, Tpx1 [47] is one enzyme involved in detoxification of endogenous oxidative stress, and also required for aerobic growth. Also mitochondrial deletion mutants were shown to be required for aerobic growth of this organism in aerobic conditions. The transcription factors Pap1, Prr1 and Atf1 are activated upon oxidative stress.

2.2. Responses to oxidative stress: the Sty1 MAPK pathway and the Tpx1-Pap1 pathway

In response to H_2O_2, *S. pombe* activates different signalling pathways depending on the severity of the stress exerted, mediate at least by two transcriptional factors, the AP-1 like Pap1 and the bZIP Atf1 controlled by the mitogen-activated protein kinase (MAPK) Sty1 (**Fig. 8**).

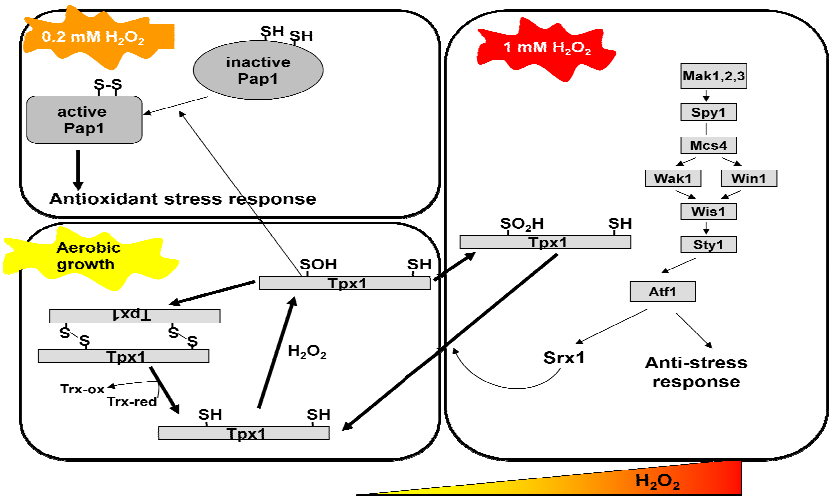

Figure 8: levels of oxidative stress in *S. pombe* [48]

Pap1 pathway: *S pombe* AP-1 transcription factor Pap1 is the homologue of yeast YAP1 and mammalian Jun, and is activated by low levels of ROS. The activation of Pap1 is regulated by its nuclear localization and its oxidation. Pap1 is constantly imported to the nucleus and exported by interaction with the nuclear exportin Crm1 [49]. Upon low levels of H_2O_2, Pap1 suffers a conformational change due to the formation of at least one intramolecular disulfide bond, preventing its interaction with Crm1 [50, 51]. The nuclear export of Pap1 depends on Hba1, a protein which function as a cofactor in the export of Pap1 and Sty1 [52].

Pap1 has two clusters of Cys residues (CRD, Cysteine Rich Domain) important for its activation, one located at the centre of the protein (n-CRD) with four Cys and the second at the carboxy-terminal region (c-CRD) with three Cys and where the NES (Nuclear Export Signal) is located [49, 53]. The treatment with low doses of H_2O_2 induce the formation of an intramolecular disulfide bond between the Cys 278 (n-CRD region) and the Cys 501 or 532 (at c-CRD) [54, 55]. The disulfide bond leads to a conformational change, which impairs the access of Crm1, causing to its dissociation from the Crm1-Hba1 nuclear export machinery and its nuclear accumulation [56]. Reduction-inactivation of Pap1 is probably performed by the thioredoxin system; Trx1 contributes to maintain Pap1 in a reduced state in basal conditions. The deletion of the *trr1* gene, necessary for Pap1 reduction, results in a constitutive oxidation and nuclear localization of Pap1.

Once Pap1 is stress-activated and accumulates at the nucleus, it induces a transcriptional stress program. There are two types of responses triggered by low H_2O_2 doses in a Pap1-dependent manner [54]. The target genes of Pap1 includes genes encoding for detoxifying proteins, like *ctt1* (catalase), *trr1* (thioredoxin reductase), *pgr1* (glutathione reductase) and *tpx1* (thioredoxin peroxidase); and also genes coding for membrane transporters as *hba2* and *pmd1*. All these genes are part of the cellular adaptive response to oxidants. Therefore, the ability of Pap1 to be activated by low levels of oxidants prepares the cell to survive to

higher levels of the oxidative stress [57]. Upon higher levels of stress is the MAPK Sty1 pathway the responsible to allow cells to survive.

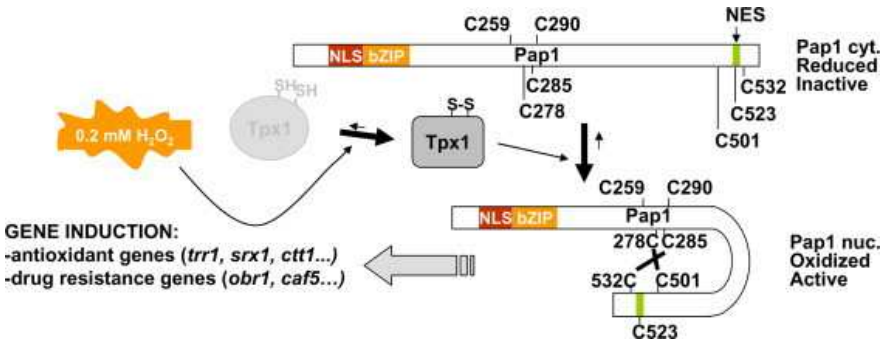

Figure 9: Schematic representation of Pap1-dependent transcription activation by H_2O_2. Upon low levels of H_2O_2 (0.2 mM in the culture media), Tpx1 mediates disulfide bond formation in Pap1, which hinders the nuclear export signal (NES). Nuclear accumulation of oxidized Pap1 triggers transcription of both antioxidant and drug resistance genes. The relative positions of the seven Cys residues in Pap1 are indicated.

Interestingly, the oxidation of Pap1 upon H_2O_2 is mediated by the Prx Tpx1 (see **Fig. 9**). Upon low levels of stress, Tpx1 mediates the formation of at least one disulfide bond in Pap1.

In fission yeast, the Pap1 and Sty1 pathways constitute the key protective responses to oxidative stress. Pap1 is more sensitive to H_2O_2 than the MAPK Sty1, so Pap1 induces adaptation whereas Sty1 promotes survival responses. Sty1 seems to be required for Pap1 activation at high doses of the oxidant due to the role of Prx Tpx1 in the H_2O_2-dependent activation of Pap1 [56].

Tpx1 is a 2-cys Prx that scavenges H_2O_2 in fission yeast, and it is therefore required during aerobic metabolism [47]. Tpx1 is the real sensor and acts upstream of the Pap1 pathway; it transfers the redox signal to Pap1 under low stress. Pap1 can only be oxidized in cells expressing Tpx1. Upon higher levels of stress the formation of a sulphinic acid in Tpx1 inactivates the peroxiredoxin. This means that Tpx1 provides the molecular basis for the temporary shutdown of Pap1 pathway in presence to elevated levels of H_2O_2 (**Fig. 8**). The inactivation of Tpx1 postpones

Pap1 activation until Sty1-dependent transcriptional response is activated. This hyper oxidation of Tpx1 is resistant to reduction by Trx [58], however can be reserved to a thiol group by ATP-dependent sulfiredoxin Srx1. The Sty1 response program induces the *srx1* gene: Srx1 restores the Tpx1-Pap1 redox relay and Pap1 activation by recycling oxidized Tpx1. (***paper review)

Sty1 pathway: The MAP kinase Sty1 pathway becomes activated upon several kinds of stresses: heat shock, stationary phase, osmotic stress and oxidative stress. When this pathway is active, the transcription factor Atf1 is phosphorylated and some some CESR genes are transcribed. Little is known regarding how the distinct inputs enter the pathway, nor which components are required for each specific activation (for more details see section 3).

2.3. Other environmental stress responses in *S. pombe*

2.3.1. Responses to heat stress

The heat stress is defined as the exposition to a temperature different to the temperature for optimal growth of the cell. Heat stress damage causes loss of membrane integrity and protein aggregation. Higher variations of the temperature cause cell death while lower variations induce a response against the heat stress, including the activation of heat shock proteins (HSP), with chaperone activity.

Heat stress activates the Sty1-dependent common stress responses, as stop of cell cycle, changes wall composition or increases the ATPase activity. However, the most important response to heat shock is the activation of the transcription factor Heat Shock Factor (HSF), which increases synthesis of HSPs. This transcription factor binds to Heat

Shock Elements (HSE) in genes necessary for the response in an Atf1-dependent manner [59, 60].

Contrary to the exposition to osmotic and oxidative stress where the activation of Sty1 is relying on wis1, upon heat stress are the tyrosine phosphatases Pyp1 and Pyp2 the main responsible for its activation. These phosphatases inactivated Sty1 by dephosphorylation of Tyr-173, however upon heat stress, Pyp1 and Pyp2 are inhibited. In this condition, the inhibition of phosphatases together with a moderate activity of Wis1 leads to a strong activation of the MAPK Sty1 [61].

2.3.2.Responses to heavy metals

Iron is probably the most important micronutrient used by microorganism. But this metal is a double-edge sword for the cell due to its capacity to trigger Fenton reaction in the presence of H_2O_2 to generate $OH^{\cdot}$. That is why iron import and export is tightly regulated in all organisms.

In *S. pombe* two mechanisms are involved in metal adquisition when iron is scarce: a) iron import by siderophores proteins that allow synthesis of ferrochrome, an organic compound that binds to oxidized iron (Fe^{3+}) and keep it oxidized; b) the other mechanism the iron is reduced by reductase protein Frp1, followed by the import of iron thought proteins Fio1 and Fip1. The inactivation of Fep1 and activation of Php4 repressors mediate the cellular response to iron depleted conditions. When concentrations of iron decreases, Fep1 is released from promoters involved in iron uptake [62], while Php4 accumulates at the nucleus and represses transcription of genes coding for iron consuming or iron storage proteins [63]. Upstream of these transcriptional repressors is the glutaredoxin Grx4, the real sensor of iron deprivation. Grx4 contains an iron-sulfur center which may be involved in this response to iron.

One of the goals of this thesis is to understand whether pathways responding to oxidative stress or iron starvation would regulate each other

to avoid the toxicity of ROS and reduced metals. This section is presented in a manuscript in chapter results (section 3B).

2.3.3.Responses to osmotic stress

Like all eukaryotes organism, osmotic stress in fission yeast is controlled by MAPK pathways.

Under hyper osmotic situation, there is a synthesis of glycerol through the activity of glycerol-3-phoshate dehydrogenase. In *S. pombe* there are two genes that codify for this enzyme: *gpd1* and *gpd2*. The expression of *gpd2* is constituve while the expression of *gpd1* is Sty1-dependent [64, 65]. Cells lacking the MAPK Sty1 are hypersensitive to osmotic stress, confirming the participation of the kinase in the response to this stress situation.

Another gene involved on osmotic stress is *cta3*, which codes for an intracellular cation transporter and its regulation is achieved positively by Sty1 and negatively by the repressors Tup11 and Tup12. Other protein involved in the regulation of *cta3* is Prr1. Prr1 plays a role in oxidative stress and, upon high levels of potassium, is important for the induction of *gpx1* and *ctt1* in a Sty1-independent manner [66].

Finally, other important gene for the transcriptional activation upon osmotic stress is *gcn5,* which is involved in acetylating histones and chromatin remodelers. A subgroup of genes expressed upon osmotic stress is under the regulation of Gcn5, and mostly are CESR genes [67].

2.3.4.Responses to nitrogen deprivation

In normal condition growth, *S. pombe* adopts a mitotic haploid cell cycle, known as vegetative growth. Upon these conditions, fission yeast shows a standard cell cycle: DNA is replicate in S phase and during M phase

chromosomes are segregated and settle into two haploid cells. However, nutrient availability can modify this cycle of proliferation.

During vegetative growth, *ste11* transcripts are kept low and the phosphorylations of Ste11 (Thr-173 and Ser-218) by Pat1 kinase inhibits its nuclear accumulation.

Another pathway involved in signaling nutrient starvation is the Tor1 pathway. The TOR (target of rapamycin) protein is a Ser/Thr kinase, which regulate cell growth in response to environmental changes, such as nitrogen deprivation. Fission yeast has two TOR kinases, Tor1 and Tor2. Two TOR complexes, namely TORC1 and TORC2 have been identified. *S. pombe* TORC1 contains the kinase Tor2 and the TORC2 complex the Tor1. These complexes regulate different aspects of cell growth in response on environmental changes. TORC1 is sensitive to rapamycin, and regulates features involved in cell growth, such as translation, ribosome biogenesis, autophagy, transcription and metabolism. TORC2 is insensitive to rapamycin and have opposite functions regarding the G1 arrest and sexual development.

S. pombe tor1 is not essential for cell growth, however, deletion of *tor1* results in lack of G1 arrest under nutrient starvation and cells are sterile. They show an elongated morphology and are unable to survive to different stresses [68, 69]. Tor1 is also involved in the regulation of leucine uptake [69]. Weisman and colleges showed that growth of auxotrophic cells in leucine display sensitivity to rapamycin. This rapamycin sensitivity was suppressed by replacement of wild-type *tor1* with a putative rapamycin binding defective allele.

Contrary to Tor1, fission yeast Tor2 is essential for vegetative growth. Deletion of *tor2* results in cell death [68, 69]. In addition, it has been shown that *ste11* and *mei2,* critical for sexual development are induced by the loss of Tor2 function [70]. These results suggest that inactivation of Tor2 mimics a situation of nitrogen deprivation.

It has also been described a relation between the Tor1 pathway and Sty1 pathway. Upon nutrient starvation, TOR pathway is inhibited which promotes the activation of Gcn2, a negative translational regulator

of Pyp2. Low expression of Pyp2 allows the activation of Sty1 and, consequently, phosphorylation of Cdc2 which allows the cell to progress into mitosis albeit its small size [71].

3. The Sty1 MAPK cascade in *S. pombe*

3.1. Generalities about stress-dependent MAPK cascades; the HOG1 and p38 cascades

MAPKs constitute a large and highly conserved of proteins family. However, not all members of this family are able to sense stress. In fact, the members whose activity is triggered by stress are also known as Stress-Activated Protein Kinases or SAPKs. I will explain shortly how eukaryotes respond to stress through these classes of kinases, and the specific substrates they regulate.

Saccharomyces cerevisiae

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

SLT2 is a component of the PKC pathway and belongs to the ERK family of protein kinases. It maintains cell wall integrity during heat shock, low salt concentrations, cell wall damage and oxidative stress. The High Osmolarity Glicerol (HOG) pathway is homologue to mammalian p38 MAPK and to the *S. pombe* Sty1. HOG1 is responsible to sense osmotic shocks, and the activation of the pathway leads to induction of osmoadaptative genes responses.

The HOG1 MAPK module comprises three MAPKKK, SSK2, SSK22 (Maeda et al., 1995) and STE11 [72], which activate the MAPKK PBS2. Once is activated, PBS2 phosphorylate HOG1. This pathway is

controlled by two independent mechanisms. One involves a two-component osmosensor composed by the SLN1-YPD1-SSK1 proteins, which activates the MAPKKK SSK2 and SSSK22. Whereas the second mechanism acts through the transmembrane protein SHO1, the MAPKKK STE11, the STE11 binging protein STE50, the STE20 p21-activated kinase (PAK) and the GTPase CDC42 [73-75].

Mammals

In mammals five families of MAPK have been characterized (**Fig. 10**): the Extracellular-Regulated Kinases (ERKs) ERK1 and ERK2, the Jun N-terminal Kinases (JNKs), the p38 MAPKs, ERK3 and ERK4, and ERK5. The best extensively known MAPKs are ERK1/2, the activation motif TEY and which are activated by growth; and the JNKs and p38 kinases, which are able to respond to stress conditions and contain the TPY and TGY motifs respectively (Parson *et al.,* 2001; Roux and Blenis,2004). We will briefly describe these last two.

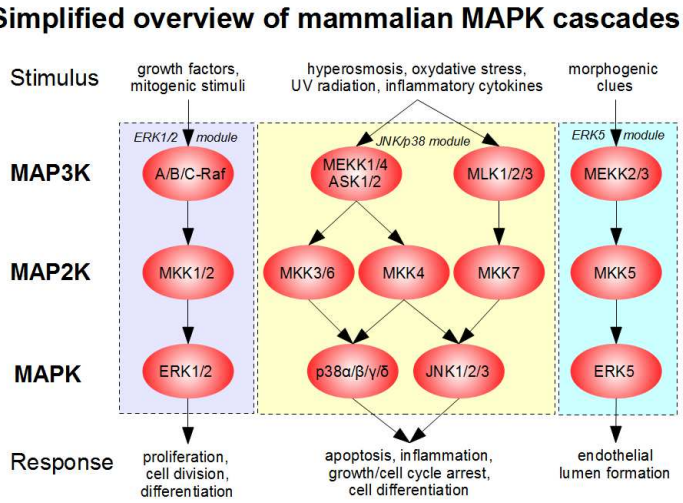

Figure 10: A simplified overview of MAPK pathways in mammals organized into three main signaling modules (ERK1/2, JNK/p38 and ERK5).

The JNK pathway is heavily activated upon ionizing radiation, oxidative stress, heat shock inflammatory cytokines, growth factors and reperfussion injury (Kyriakis and Avruch, 2001). Once the kinase is activated it accumulates into the nucleus. JNK is phosphorylated by two different MAP2K: MKK4 and MKK7, which activation profile do not overlap but cooperate. The collection of Ser/Thr kinases acting as MAPKKKs upstream the JNK kinase shows as the variety of different stimuli that activates that pathway (reviewed by Johnson and Nakamura, 2007).

The p38 isoforms are MAPK that are responsible to respond stress stimuli, such as cytokines, UV radiation, heat shock, and osmotic shock, and are involved in cell differentiation, apoptosis and autophagy (Kyriakis and Avruch, 1996). It responds to the same stimuls than JNK except in ischemia-reperfusion: whereas 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 of which are well studied: p38a, b, g, and d. One of the p38 substrate 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 broken when ATF2 interacts with other proteins or when it is phosphorylated by MAPKs, increasing its transcriptional activity (Li and Green, 1996).

3.2. Components of the Sty1 cascade

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) complete the phosphorelay system. It is still unclear how the sensor histidine kinases recognize the different types of stress. However, it seems that the phosphorelay protein Spy1 is activated by phosphorylation and negatively regulates the response regulator Mcs4 by binding and transferring its phosphate group. At the end, the anchor of the phosphorelay Mcs4 binds to the MAPKKK and initiates the activation of the Sty1 cascade.

It is know that Tdh1 (glyceraldehyde-3-P-dehydrogenase) is necessary for the activation of the pathway upon H_2O_2. Tdh1 interacts physically with Mcs4 and promotes phosphorelay signalling to Mcs4. Mcs4 also plays a role in the osmostress signalling, while Tdh1 dissociates from Mcs4 upon osmostress, while oxidative stress promotes their association [76]. It has been recently published that the Mcs4 response regulator is an integral component of the complex Wis4-Win1 MAPKKK and this complex is disrupted in cells lacking *mcs4*. Both MAPKKK are in a ternary complex with Wis1 keeping the kinase inactive, under oxidative stress, due to phosphorelay cascade, Wis1 is released from the complex and phosphorylates Sty1 (Morigasaki and Shiozaki 2013).

The MAPK module is composed of the MAPK Sty1, which is activated by the MAPKK Wis1 phosphorylating Sty1 at threonine 171 (Thr-171) and at tyrosine 173 (Tyr-173) residues, and two MAPKKK Wak1 (also known as Wis4) and Win1, which are functionally redundant. Their only known substrate is Wis1, which becomes phosphorylated in serine 469 (Ser-469) and Thr-473 (Shiozaki and Russell 1196; Wilkinson et al., 1996; Samejima et al., 1997; Shiozaki et al., 1997).

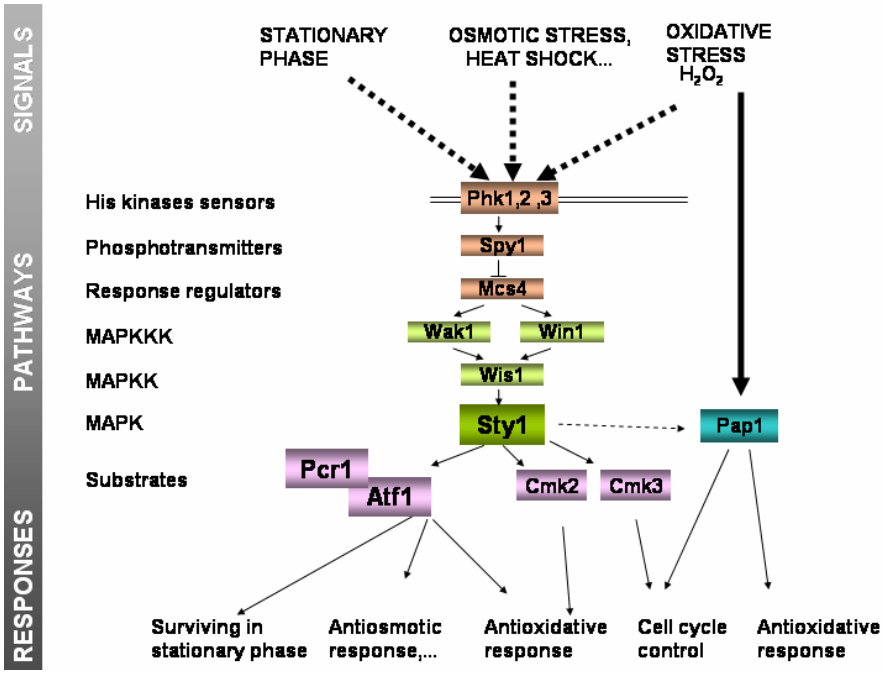

Figure 12: *S. pombe* stress response pathways.

3.3. Regulation of the activity of the Sty1 kinase

The activity of Sty1 kinase has to be precisely regulated. *S. pombe* achieves this by three different means: the kinases that transmit the environmental signals, some changes in sub cellular localization and phosphatases that are responsible to turn off the pathway.

Phosphorylation

The MAPKK Wis1 is physically associated with Sty1 until some stress activates the pathway. When this activation takes place, 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. Cells lacking *wis1* show same phenotype 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 to migrate to the nucleus resulting in stress sensitivity and

elongated morphology of the cell (Gaits et al., 1998). The temporal regulation of the phosphorylation cascade, as well as the spatial distribution of the elements of the pathway, occurs due to the rapid transmission of information from the membrane to the nucleus.

Nuclear migration

Once Sty1 is phosphorylated, it accumulates into the nucleus, where most of its stress role is focused. Actually, in cells lacking *wis1*, Sty1 does not accumulate in the nucleus upon stress [77].

The mechanism by which Sty1 goes to the nucleus is still unknown, so Sty1 does not have a typical nuclear localization signal. It has been proposed that Sty1 nuclear translocation could depend on its dimerization like other mammalian kinases (Khokhalatchev et al., 1998).

Sty1 has one NES between amino acids 122 and 132. The export of Sty1 from nucleus by Crm1, and the cofactor Hba1, has been well characterized [52, 78]. Lack of function of Crm1 or Hba1 results in Sty1 being constitutively nuclear.

Importantly, when Sty1 is artificially forced to be sequestered into the nucleus, and physically separated by the module of kinases, the protein is still accessible to activation by stress due to the ability of Wis1 to travel into the nucleus and phosphorylate its targets [79].

Phosphatases

The dephosphorylation of Thr-171 and Tyr-173 of Sty1 is achieved by different phosphatases. The Tyr-173 is dephosphorylated by two tyrosine-specific phosphatases: Pyp1 and Pyp2 [80]. Thr-171 dephosphorylation is achieved by two specific Ser-Thr phosphatases of the 2C type class (PP2C), Ptc1 and Ptc3 [81]. Since the activation of Sty1 needs 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 consequence of the hyperactivity of Sty1 [81]. Pyp2 expression is enhanced as a part of the CESR response, showing a mechanism of negative feedback regulation

[82, 83]. On the other hand, *pyp1* expression is constitutive and not regulated by stress suggesting a role in the basal dephosphorylation of Sty1 [23].

3.4. Nuclear roles of Sty1-Atf1: regulation of transcription, recombination and heterochromatin assembly

Several substrates of Sty1 have been identified. Srk1, for instance, is a serine/threonine kinase with homology with mammalian calmodulin kinases. Srk1 and it has been localized in a complex with Sty1 (López-Avilés et al., 2008). Upon stress, Srk1 translocates from the cytoplasm to the nucleus in a Sty1 dependent manner. Sty1 has been shown to regulate translation through the modulation of Gcn2 kinase, even thought the specific effectors are still unknown (Dunand-Sauthier et al., 2005). However, most of the effects of Sty1 on chromatin structure and gene expression are accomplish by the bZIP containing protein Atf1.

When the MAPK Sty1 pathway becomes active triggered upon stress by upstream components, leads to its transient accumulation to the nucleus. The main substrate of Sty1 is the bZIP TF Atf1 (see section 4.1), which remains constantly nuclear. Sty1 nuclear localization leads to phosphorylation of Atf1 promoting its stabilization and accumulation [84]. However, cells lacking *sty1* have a decrease level of Atf1 protein, the transcription factor can be expressed from a heterologous promoters, and these cells shows not recovery of transcription activation, suggesting us that Sty1 is required for activation of Atf1 as a TF [85].

Atf1 regulates the transcription of genes forming a heterodimer with another small bZIP TF, Pcr1, which can also affect stability of Atf1 [84, 86, 87].

Several lines of evidence suggest a potential role of Atf1 in chromatin remodeling. When *S. pombe* cells enter meiosis homologue chromosomes recombine at higher frequency than during mitosis. These

recombination events occur localized in the recombinant hotspots. About 50% of the natural hotspots are characterized by the presence of DNA sequences that are recognized by TF [88].

One of the best known recombination hotspot is *ade6-M26*, constituted by a point mutation in the *ade6* gene. This mutation creates a nonsense codon and a cAMP-responsive-element (CRE) like sequence. This sequence acts as a binding site for Atf1-Pcr1 heterodimer [87, 89]. In response to an osmotic stress signal, but no other stressors, the binding of Atf1-Pcr1 activates the transcription of an *ade6* minigene [90]. In addition, components of the MAPK pathway are also needed, as Wis1 and Sty1 activated kinases [91].

Full induction of all these chromatins modifications is facilitated by a histone acetyltransferase (HAT) Gcn5, and a Swi2/Snf2-like ATP-dependent chromatin remodeling factor (ADCR), Snf22 [92]. More ADCRs involved in this role are Hrp1 and Hrp3 [93], where Hrp3 is required for full activation of meiotic chromatin alteration at *ade6-M26*, whereas Hrp1 is required for the suppression of chromatin changes before the onset of meiosis.

In *S. pombe*, heterochromatin encloses three important regions: centromeres, telomeres and a 20-kb-long mating-type region called *mat2/3* locus. This heterochromatin prevents abnormal chromosome segregation, anomalous recombination in repetitive sequences in telomeres and gene expression in the mating locus during mitosis. These DNA regions contain deacetylated histone H3, what allows H3 methylation at the same residue by the methylase Clr4, and this modification gives the binding site for Swi6; both Clr4 and Swi6 participate in spreading heterochromatin to neighbouring regions. It has shown that the RNAi pathway is also responsible to initiate heterochromatin at these loci.

Furthermore, the TFs that are substrates of Sty1 are deeply implicated in this genome wide regualation. Atf1 and Pcr1 heterodimer also binds to two CRE sites located near the *mat2/3* locus, and initiate heterochromatin assembly via a parallel pathway [94, 95]. Cells lacking *atf1* or *pcr1* are defective for initiation of heterochromatin formation,

epigenetic inheritance and silencing of artificially inserted marker genes, such as *ade6*. Two histones deacetylases (HDAC) have been proposed as essential to mediated heterochromatin formation by Atf1-Pcr1: Clr6 [95] and Clr3 [96].

Deletion of *wis1* or *sty1* has the opposite effect to mating locus architecture: they show wild-type levels of silencing of heterochromatin and expression of an inserted *ade6* marker gene was lower than wild-type cells. This suggests that inactive/unphosphorylated Atf1-Pcr1 have the ability to recruit repressive chromatin activities to the mating locus, therefore Atf1-Pcr1 could also have a role in repression transcription. This has been postulated for the catalase coding gene *ctt1.* Only Δ*sty1* cells, but not Δ*atf1* or Δ*atf1* Δ*sty1* display extremely low levels of *ctt1* expression; both proteins are required for stress-dependent induction of the gene [97]. The euchromatin gene silencing under basal conditions of *ctt1* depend on Atf1, Pcr1 and on the HDAC Clr6 [95].

4. Regulation of the stress transcriptional program by Sty1-Atf1

In *S. pombe*, genome wide transcription studies revealed that there is a large number of genes, about 4970, whose expression levels changed two fold or higher after one stressor out of the five that had been analyzed: H_2O_2, sorbitol, cadmium, heat shock and MMS [43, 44]. The authors of this study found that in four out of five of those stresses, 140 genes were commonly induced and 100 repressed.

This outcome was called the Common Environmental Stress Response (CESR). CESR up regulated genes are implicated in carbohydrate metabolism (*tps1, ntp1, fbp1,* etc.), signaling and transcriptional regulation (*pyp2, pcr1, atf1*, etc.), lipid or fatty acid metabolism, antioxidants (*ctt1, gpx1, srx1*, etc.), DNA repair, protein degradation and folding and others. More than 70% of CESR genes are Sty1-dependent and the majority of these are Atf1-dependent as well.

The MAPK Sty1 controls more genes than Atf1, suggesting that Sty1 regulates additional TFs.

4.1. The heterodimer Atf1-Pcr1

Atf1 belongs to the ATF/CREB family. This family of proteins binds to the same DNA sequence, the cAMP response element (CRE) (consensus sequence: TKACGTCA). To function as TFs these proteins have to dimerize.

In mammalian cells, the formation of bZIP TFs heterodimer has been proposed to be the basis of the majority of transcriptional regulatory interactors, increasing in that way the diversity of gene responses [98].

In vitro studies and co-immunoprecipitation experiments in *S. pombe* reported that Atf1 dimerize with Pcr1, another small bZIP TF (Whals and Smith, 1994; Kanch et al., 1996). Cells lacking *atf1* show similar phenotype than cells with mutations in *sty1* which includes sensitivity to osmotic, oxidative, heat shock and heavy metals stress and their viability is reduced upon stationary phase (Nguyen et al., 2000; Quinn et al., 2002; Zuin et al., 2010). Atf1 has nuclear localization even prior to stress [77].

Cells lacking Pcr1 share many stress related phenotypes with Sty1 and Atf1 deletions. Both Δ*pcr1* and Δ*atf1* cells are cold sensitive, inefficient in mating and spore formation upon nitrogen starvation, and show no transcriptional activation of some nutrient depletion induced genes, such as *ste11.* The heterodimer binds to hotspots more efficiently than single proteins Atf1 or Pcr1 [87, 89].

Pcr1 role in stress response is still unclear. Some authors suggested that Atf1 but not Pcr1 was required for resistance to extracellular osmotic stress mediated by NaCl. However, Pcr1 is able to bind to stress promoters.

The Atf1-Pcr1 heterodimer is also involved in the heterochromatization of the *mat* locus. *atf1* deletion or mutations in the

ATF/CREB-binding site of the *mat3-M* locus have nearly undetectable effects on silencing heterochromatin. Moreover, the heterodimer is required for histone deacetylation and H3 Lys-9 together with histone deacetylases (HDAC) Clr6. Surprisingly, a similar activity has been reported on *ctt1* stress promoter gene [95].

Atf1 contains 11 potential MAPK sites (S/TP, a serine or threonine residue followed 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.

The group of Nic Jones reported that the Atf1-11M phospho-mutant protein, which lacks intact MAPK sites, displays decreased stability compared to its wild type counterpart and accumulates to a lesser extent upon stress. Nevertheless, there is still robust activation of Atf1-dependent gene expression. Therefore, and according to this report, Atf1 phosphorylation was not required for stress-induced activation of Atf1-target genes but rather served to positively regulate the stability of the Atf1 protein. Consistent with this, *atf1-11M* does not share the stress sensitivities displayed by an *atf1* deletion mutant (Lawrence et al., 2007). These results posed a dilemma: Atf1 is phosphorylated by Sty1; its role in stress-induced activation of gene expression is Sty1-dependent, yet phosphorylation of Atf1 by Sty1 does not appear to be critical for transcriptional activation *per se*.

One of the purposes of this thesis work is to try to decipher the role of Sty1 and Atf1 in the transcriptional response (see section results 2).

4.2. Role of the SAGA complex in the regulation of the Sty1-, Atf1-dependent gene expression program

Transcription, recombination, repair and replication are, at the end, regulated by chromatin structure. There are three mechanisms to modify chromatin and make it accessible for these events. The first involves the incorporation of histone variants which sequences differ from the canonical histones. The second mechanisms use ATP to remodeler the nucleosome pattern along the chromosome. Lastly, chromatin-dependent factors can bind covalently to histones and modified them. A great number of histone tail modifications have been identified such as acetylation, methylation, phosphorylation and ubiquitination. These histone marks involve regulation of DNA-to-nucleosome association or are used as hallmarks for TF or other chromatin remodelers.

An extensively studied, a post-translational modification of chromatin is the reversible lysine acetylation of histones H3 and H4, which is related to transcription activation. Histone acetylation is carried out by histone acetyltransferases (HATs), which catalyze the transfer on an acetyl group to lysine residues at the amino-terminal tails of histones. On the other hand, deacetylation of the same residues is an active process catalyzed by histone deacetylases (HDACs). This dynamic balance not only regulates transcription, but also affects centromeric cohesion, proper chromosome segregation during cell division, DNA replication and repair of double strand-breaks [99-101].

HAT enzymes are divided into five families, but *S. pombe* only has homologues to members of three of these families: the *S. pombe* HATs Gcn5, Sin3/Elp3 and Hat1 belong to the family GNAT (Gcn5-related N-acetyltransferases); Mst1 and Mst2 are essential genes belonging to the MYST family; and Taf111 is homologous to members of TAF family (TATA binding protein-associated factors).

The principal stress-related HAT is Gcn5, a GNAT super family protein and a component of the SAGA co activator complex. SAGA was first discovered in *S. cerevisiae* [8]. The first association between Atf1-Pcr1 and Gcn5 was its role in the *ade6-M26* meiotic hotspot. Cells lacking *gcn5* shows a decrease in H3 acetylation around M26 and

chromatin remodeling is slowed down. Moreover, H3 and H4 are hyper acetylated in an Atf1 and Pcr1-dependent manner in early meiosis [100].

Gcn5, was also shown to regulate the expression of a subset of osmotic stress genes, which promoters where enriched by ATF/CREB sites and half of them belong to CESR genes [67].

Finally, Gcn5 was directly related with the stress response, where its recruitment to CESR genes upon oxidative stress is Atf1 and Sty1-dependent, promoting H3K9 and K14 acetylation and nucleosome eviction. Deletion of the Gcn5-coding gene, but also of more components of SAGA complex, like Ada2, Hfi1 or Spt8 render cells sensitive to oxidative stress due to a impaired transcription activation of Atf1-dependent genes [102].

4.3. Role of elongator in the translation of stress mRNAs

The elongator complex, including the histone acetyl transferase Sin3/Elp3, was isolated as an RNA polymerase II-interacting complex [103]. This protein harbours motif found in the GNAT family of HATs [104]. Together, this data formed the basis for the idea that Elongator assists the RNA polymerase II (Pol II) transcriptional machinery during transcript elongation, acetylating nucleosomes in the path of elongating polymerase. However, after the initial discovery, Elongator and its cellular function have generated some controversy. First, it has been found that most Elongator is actually found in the cytoplasm [105]. Second, attempts to detect Elongator at active genes in yeast by chromatin immunoprecipitation failed [105]. Third, it was reported that yeast Elongator is involved in exocytosis and, finally, Elongator have a role in tRNA modification [106].

This new role in tRNA modification arises from *S. pombe* antisupressor screening, in which *sin3-193* mutant showed reduced levels of modified nucleoside mcm^5s^2U from a subset of tRNAs [107].

Importantly, most defects initially associated with a role of Elongator in transcription and exocytosis was bypassed by elevated levels of tRNAs, those modified by this complex.

The genetic code is degenerated, so most amino acids are encoded by more than one triplet, some of which are more common than others regarding the codon usage of each organism. Up to 75-100 different post-transcriptional nucleoside modifications have been shown in eukaryotic tRNAs [108]. Most modifications occur at the anticodon loop. One example is dual modifications of a U (U34) at the 5' wobble position of the anticodon coding for glutamine, lysine and glutamic acid. This modification, mcm^5s^2U, has been suggested to play a role in either translation efficiency or fidelity, and to be required for viability in yeast.

One of the singularities of the redundancy of the genetic code is that allows organism to choose between alternative codons for the same amino acid, and that may exert some consequences on the efficiency of translation. The amount of tRNA in each species is considered to be proportional's to the copy number of the tRNA-coding genes in the genome.

We have reported that the lack of functional Elongator complex results in stress phenotypes due to its contribution to tRNA modification and subsequent translation inefficiency of certain highly expressed mRNAs required to counteract stress. Many genes highly expressed upon H_2O_2 stress in a Sty1-Atf1-dependent manner contain a biased number of lysine codon, which require one of the tRNAs modified by Elongator. The transcriptional defects of lacking Elongator may be an indirect consequence of deficient translation of a TF, Atf1-Pcr1, and other components of the stress responses. We present the results in this thesis work in a manuscript on Appendix 6.2.

4.4. Nucleosomal architecture of stress promoters by Sty1-Atf1

In *S. pombe*, a genome-wide positioning study indicated that Gcn5 is localized in regions coding for highly express genes, at least under basal conditions, suggesting some role in elongation. Moreover, CESR genes recruit Gcn5 through Atf1 and Pol II into promoters, however the recruitment of Pol II is not abolished in cells lacking *gcn5,* even though transcription is severely diminished [102].

In fact, *S. pombe* stress genes do not require chromatin remodellating at promoters to initiate transcription, because the majority of highly induced genes upon oxidative stress display an unexpectedly large nucleosome depletion region (NDR) at their promoters [102].

In fission yeast stress genes, the pre-initiation complex is assembled on promoters in a Gcn5-independent manner. Upon this situation, the +1 nucleosome acts as a physical barrier for Pol II progression through the gene. Pol II is phosphorylated at Ser5 residues during initiation state, and mainly phosphorylated at Ser2 during elongation [109]. In fact, the phosphorylation status of Pol II carboxy-terminal domain (CTD) will give us some indication of the position of the Pol II. Accordingly, cells lacking Gcn5 have only defects in phosphorylation of Ser2, probably due to the presence of nucleosomes at ORFs and to a defect in elongation [102]. It is important to mention that pre initiation complex formation requires the presence of phosphorylated/activated Atf1.

When nucleosome maps are analyzed at genome-wide level, stress promoters display a common nucleosome organization, with a region relatively free of nucleosomes upstream of the transcription start site bordered by two arrays of well-positioned nucleosomes. According to the literature, NDRs can originate either directly from DNA composition, or from trans factors. Thus, DNA sequences may contribute indirectly to generate such NDR, for example *S. cerevisiae* NDRs are enriched in poly (dA-dT) tracts. Some trans factors may also be involved in NDR

formation: there are some evidences that point out that TF may compete with histones at promoters to generate NDRs.

40 out of 50 most induced oxidative stress genes in fission yeast present an NDR with an average size of 400 bp, thereby there is an open chromatin conformation under basal conditions. Fission yeast promoters do not seem to be enriched in poly(dA-dT) tracts. How are then, the NDRs established? It has been reported that the TF Atf1 and Pcr1 are constitutively bound to CRE-containing stress promoters [85, 110] and remodels chromatin structure [111]. Moreover, it has been reported some inducible NDRs in nitrogen-dependent genes in an Atf1-independent manner [112]. Our lab was recently published evidences demonstrating that Atf1 forces the appearance of NDRs at stress promoters. The loss of Atf1 at promoters of stress genes causes an unlocked +1 nucleosome, an irregular nucleosome array in coding regions and an increase in cryptic transcription events. The TF Atf1 could also be responsible to compete with histones directly or to recruit chromatin regulators to generate these NDRs. We propose that Atf1 competes with histones to bind DNA, and function as a barrier that blocks the +1 nucleosome positioning and in turn allows the proper organization of downstream nucleosome profile [113]. .

OBJECTIVES

OBJECTIVES

Our first goal was to decipher the role of the MAPK Sty1 pathway in the response of fission yeast to oxidative stress, by studying first the participation of different components of the cascade and, secondly, by analyzing the regulation of the cellular transcriptional response by the MAPK Sty1 and the TF Atf1 upon stress imposition in the fission yeast *S. pombe*.

The specific objectives of this PhD project are:

1. Involvement of the different components of the Sty1 pathway in sensing and transmission of the H_2O_2 signal

2. Identification of new substrates or interactors of the MAPK Sty1

3. Regulation of the gene expression program by the MAPK Sty1 and the TF Atf1. Elucidating how Sty1 and Atf1 are activating the transcriptional response.

4. Exploring the role of all the fission yeast H_2O_2 scavengers in the antioxidant stress response, both under basal and stressed conditions.

RESULTS

The results will be divided in three sections:

In the first section we will study the role of different components of the Sty1 cascade in the response to oxidative stress. Thus, we will analyze specifically the role of the MAPKKKs Win1 and Wis4, and of the MAPK phosphatase Pyp1, in the activation of Sty1 upon H_2O_2 stress. We will also describe a proteomic strategy to isolate new regulators of the pathway.

The second section focuses on the regulation of the gene expression program driven by Sty1 and the TF Atf1. We have analyzed the role of Atf1 in transcription regulation, trying to understand which putative MAPK phosphorylation sites and protein domains are important to its function. Moreover, we have designed an heterologous system by replacing the CRE site of *gpd1* and *ctt1* promoters by a GAL4-binding-site (G4BS) and we use the DNA binding domain of yeast GAL4 protein coupled to Atf1 or Sty1 proteins in order to tether both proteins to the promoters, and determine their effect on transcription activation.

Lastly, the third section aims to study some aspects of the antioxidant cell responses in *S. pombe*. Half of this part is presented as a published manuscript.

1 Role of Sty1 pathway components in the cellular response to H_2O_2

Many signaling kinases modulate gene expression through regulatory phosphorylations of their target substrates as TF or other DNA binding proteins. In *S. pombe*, the MAPK Sty1, through the TF Atf1, is required for many of the stress-induced transcriptional changes that occur upon stress. Meanwhile, several items remained unclear.

In this chapter we study the role of some of the components of the Sty1 pathway in stress defence and new features of its role in transcriptional regulation. Moreover, we have used a proteomic approach to find new substrates of Sty1.

1.1. Characterization of components of the MAPK Sty1 cascade; MAPKKK and tyrosine-phosphatases.

Several reports indicate that: (i) H_2O_2 needs the presence of the upstream components Mak2 and Mak3 [114]; (ii) some Cys residues in Sty1 are important for H_2O_2 stress [115, 116]; and (iii) the Mcs4-interacting protein Tdh1 (glyceraldehyde-3P-dehydrogenase) receives the H_2O_2 input and transmits it to the pathway [76]. However, several aspects of each one of these proposals are, at minimum, controversial and mutually exclusive. Moreover, it had not been determined if the kinase activity of the protein was essential for a proper stress response or whether nuclear localization of Sty1 was the only necessary aspect to activate transcription.

Some time ago, our laboratory received an *S. pombe* collection of viable open reading frame deletion mutants, where we obtained cells deleted in almost all genes belonging to the components of the MAPK cascade.

1.1.1. The Wis4 and Win1 MAPKKK are essential for Sty1 activation upon osmotic and oxidative stress

In an attemp to evaluate the role of Wis4 and Win1 in the response to various forms of stress, activation of Sty1 was monitored under osmostress or oxidative stress. We were able to follow these events using Western Blot and a commercial anti-p38-P antibody or a homemade anti-Atf1 antibody. Liquid cultures of wild-type, Δ*wis4*, Δ*win1* and double deleted Δ*wis4* Δ*win1* were exposed to 0.6 M KCl or 1 mM of H_2O_2 during 5 min. In wild-type cells strong activation of Sty1 was observed upon both treatments, but only a small increase was detected in single mutants Δ*wis4* or Δ*win1.* The Sty1 activation was totally abolished when deleting both MAPKKK, Δ*wis4* Δ*win1* (**Fig. 1A**).

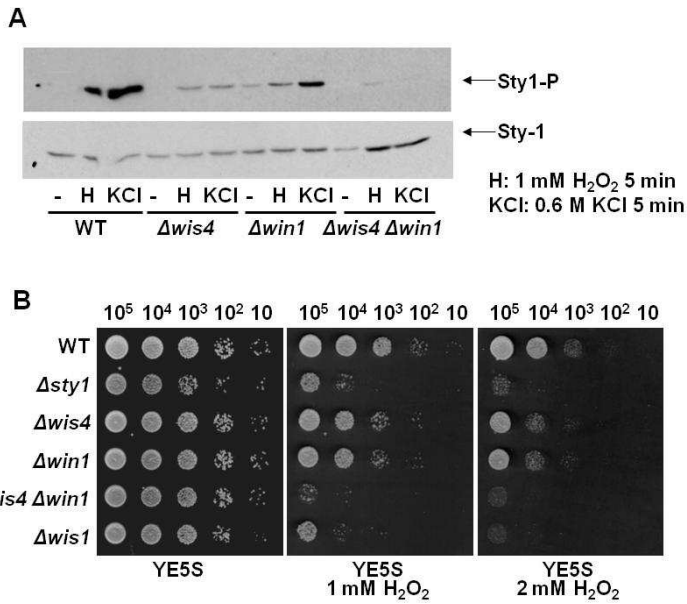

Figure 1: **Wis4 and Win1 MAPKKK are responsible for proper activation of Sty1 pathway upon oxidative and osmotic stress**. **(A)** TCAs extracts from WT (972), Δ*wis4* (EP37), Δ*win1* (EP39) and Δ*wis4* Δ*win1* (EP43) upon 1 mM of H_2O_2 or 0.6 M of KCl **(B)** Survival to grow on H_2O_2 plates of same strains as above and Δ*sty1* (AV18) and Δ*wis1* (EP44).

Accordingly, cells lacking one of the MAPKKK are only slightly sensitive to grow on plates with H_2O_2, but the double mutant is as sensititive to H_2O_2 stress as Δ*sty1* or Δ*wis1* strains. (**Fig. 1B**).

Therefore, both Wis4 and Win1 MAPKKKs are required for robust response to osmotic and oxidative stress. These MAPKKKs to be redundant regarding activation of the cascade by H_2O_2, as previously shown [117].

1.1.2. Both MAPKKK are required for transcriptional induction of CESR genes.

Wis1 phosphorylates Sty1 in two residues: Thr-171 and Tyr-173. These phosphorylations activate Sty1 in front of a stress situation, which results in Sty1 translocation to the nucleus. Once in the nucleus, Sty1 phosphorylates Atf1 and induces the oxidative transcriptional stress response. As shown before, upon oxidative stress both MAPKKKs, Wis4 and Win1, are imperative for Sty1 activation and as a consequence for Atf1 phosphorylation **(Fig. 2A)**. Moreover, Sty1 is not phosphorylated in cells lacking *wis1* upon H_2O_2, which confirms that Wis1 is the only MAP kinase for Sty1.

Next we analyzed the transcriptional activation of some CESR genes by NB in the same strains as 2A. As a reflection of this defective Sty1 pathway activation, these cells failed to response to stress **(Fig. 2B)**. Therefore, *S. pombe* needs both MAPKKK Wis4 and Win1 MAPKKK to trigger the antioxidant response upon peroxides.

It was surprising for us to confirm that the single mutants Δ*win1* or Δ*wis4* display a strong defect in Sty1 and Atf1 phosphorylation, as well as in activation of CESR genes, but they behave as wild-types on survival plates. We speculate that the basal levels of *ctt1* may be different in these strains compared to Δ*wis1* cells (see Discussion for details).

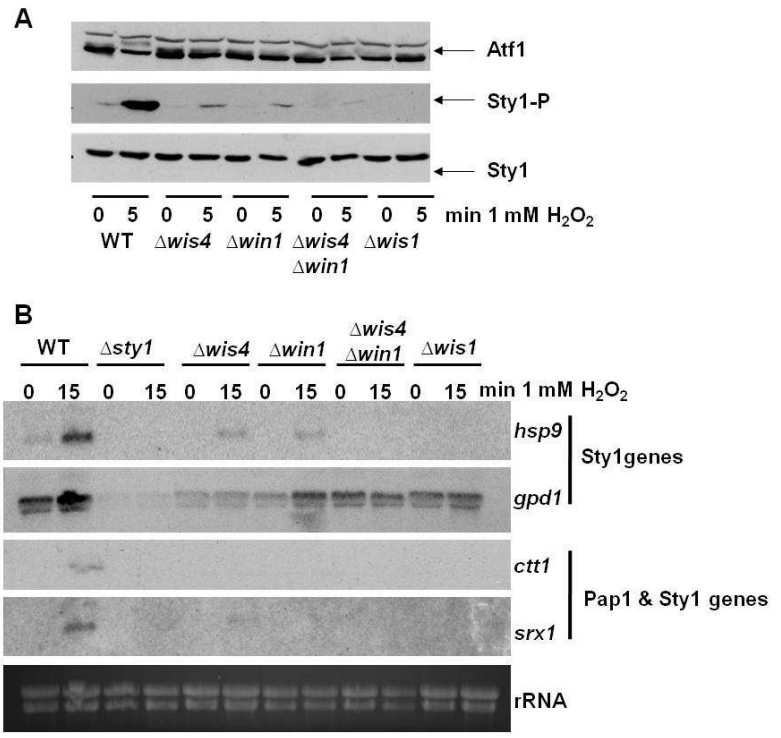

Figure 2: **Strains deleted in Wis4 and Win1 have no Atf1 activation and, therefore no stress genes transcription**. **(A)** TCAs extracts from WT (972), Δ*wis4* (EP37), Δ*win1* (EP39), Δ*wis4* Δ*win1* (EP43) and Δ*wis1* (EP44) upon 1 mM of H_2O_2. **(B)** Cultures of strains as in A were treated or not with 1 mM of H_2O_2 during 5 min. Total RNA was obtained and analyzed by Northern blot with probes for *hsp9, gpd1, ctt1* and *srx1*. Ribosomal RNA (rRNA) are shown as loading controls.

1.1.3. Regulation of Sty1 by tyrosine and serine/threonine specific phosphatases.

Like all MAPK pathways, Sty1 signalling is highly regulated to control the magnitude and duration of signalling. This is achieved through the coordination of positive and negative signals. Positive signals include stress conditions that results in Sty1 phosphorylation and negative signals involve phosphatases that inactivate Sty1 through dephosphorylation. Therefore, Sty1 activity is attenuated by either serine/threonine PP2C

phosphatases (Ptc1, Ptc2, Ptc3 and Ptc4) or tyrosine phosphatases (Pyp1 and Pyp2).

No obvious growth defects result from individual disruptions of *ptc2* and *ptc3* genes, but a Δ*ptc1* Δ*ptc3* double mutant displays aberrant cell morphology that is further accentuated in a Δ*ptc1* Δ*ptc2* Δ*ptc3* triple mutant [118]. Moreover, it was recently published a role of Ptc4 in the mitochondria upon oxidative stress. They suggest that a fraction of Sty1 also localizes to the mitochondria upon oxidative stress, and, thus, Ptc4 is the phosphatase responsible for dephosphorylation of Sty1 [119].

Here we analyzed growth defects from individual deletions of *pyp1, pyp2, ptc2, ptc3* and *ptc4*, on plates containing H_2O_2. Cells lacking *pyp1* display resistance when they were grown into H_2O_2 plates. Deletions of *ptc2* and *ptc3* have no phenotypic effect. However, deletion in *ptc4*, a mitochondrial Ser-Thr phosphatase, is sensitive to H_2O_2, as previously report [119](**Fig. 3A**).

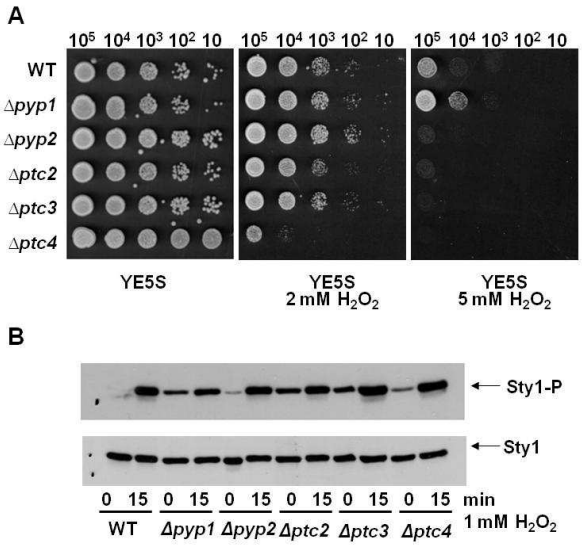

Figure 3: **Deletion of *pyp1* enhances resistance to H_2O_2.** **(A)** Strains WT (972), Δ*pyp1* (EP48), Δ*pyp2* (EP103), Δ*ptc2* (EP98), Δ*ptc3* (EP99) and Δ*ptc4* (EP127) were grown in liquid YE media, and the indicated number of cells were spotted onto plates with H_2O_2 at the indicate concentrations. **(B)** Cultures of strains as in A were treated or not with 1 mM of H_2O_2 for 15 min. TCA extracts were analyzed by SDS-PAGE gel followed by Western blot analysis using polyclonal anti-p38-P and anti-Sty1 antibodies.

Some of these phosphatase-encoding genes, such as *pyp1* or *ptc2*, are constitutively expressed, whereas others, such as *pyp2* or *ptc1* are stress induced in a Sty1-dependent manner. Basal activity of Sty1 is kept low through the action of Pyp1 whereas Pyp2 is proposed to attenuate Sty1 activity upon osmostress [80, 83]. We check by Western blot the phosphorylation of Sty1 in these single deletion mutants of phosphatases. Cells defective in *pyp1*, *ptc2* or *ptc3* display basal activation of Sty1 that its increased upon treatment with H_2O_2, indicating that these phosphatases are the main negative regulators of the kinase (**Fig. 3B**).

1.1.4. Pyp1 is the main phosphatase of Sty1

Sty1 is dephosphorylated by two-tyrosine phosphatases, Pyp1 and Pyp2, with Pyp1 having the major activity [23, 80, 118]. Therefore, we explore in more detail the function of Pyp1. As mentioned before, Δ*pyp1* cells display constitutive activation of the MAPK Sty1 pathway. We assayed Sty1 and Atf1 phosphorylation by H_2O_2 by Western blot. Wild-type and Δ*pyp2* cells display Sty1 phosphorylation only after H_2O_2. However, Δ*pyp1* cells display basal activation of Sty1 and Atf1 prior to stress although this activation is increase upon H_2O_2 (**Fig. 4A**). Moreover, Northern blot analysis and survival experiments revealed that Δ*pyp1* cells, but not Δ*pyp2*, display constitutive activation of the antioxidant response (**Fig. 4B-C**).

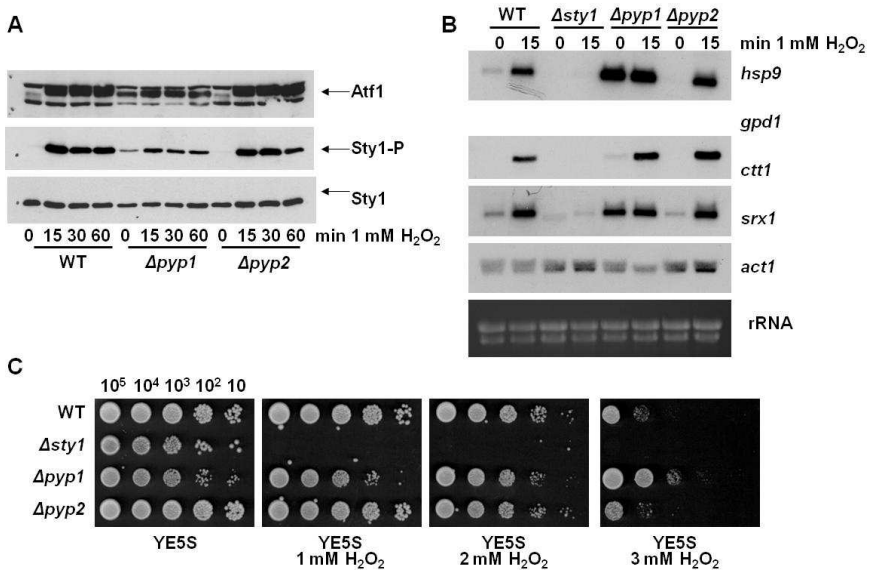

Figure 4: Pyp1 is the major negative regulator of Sty1. **(A)** Western blot analysis of Sty1 and Atf1 phosphorylation upon oxidative stress. Strains WT (972), Δ*pyp1* (EP48) and Δ*pyp2* (EP103) were grown in YE media and were collected before and after treatment of 1 mM of H_2O_2 at the times indicated. TCA extracts were prepared and protein was analysed by Western blot using anti-p38-P (Sty1-P), anti-Atf1 (Atf1) and anti-Sty1 (Sty1). **(B)** Δ*pyp1* strain display constitutive induction of the gene stress program. Strains WT (972), Δ*sty1* (AV18), Δ*pyp1* (EP48) and Δ*pyp2* (EP103) were treated or not with 1 mM of H_2O_2 during 15 min. Total RNA was extracted and analyzed by Northern blotting, with probes for *hsp9, gpd1, ctt1* and *srx1*. Ribosomal RNA (rRNA) and actin (*act1*) are shown as loading controls. (C) Δ*pyp1* cells are resistant to peroxides. Same cell cultures as in B were spotted into YE plates containing or not 1 or 2 mM of H_2O_2.

1.1.5. Constitutive Sty1 activation in Δ*pyp1* cells is Wis1-and-Sty1 dependent.

To determine whether the activity of Sty1 is regulated in cells lacking *pyp1*, we constructed double deletion strains, Δ*pyp1* Δ*wis1* and Δ*pyp1* Δ*sty1*. As expected, the tolerance growth on H_2O_2 plates of Δ*pyp1* is abolished in cells lacking the MAPKK Wis1 or MAPK Sty1, indicating that this resistance is Wis1- and Sty1-dependent **(Fig. 5A-B)**.

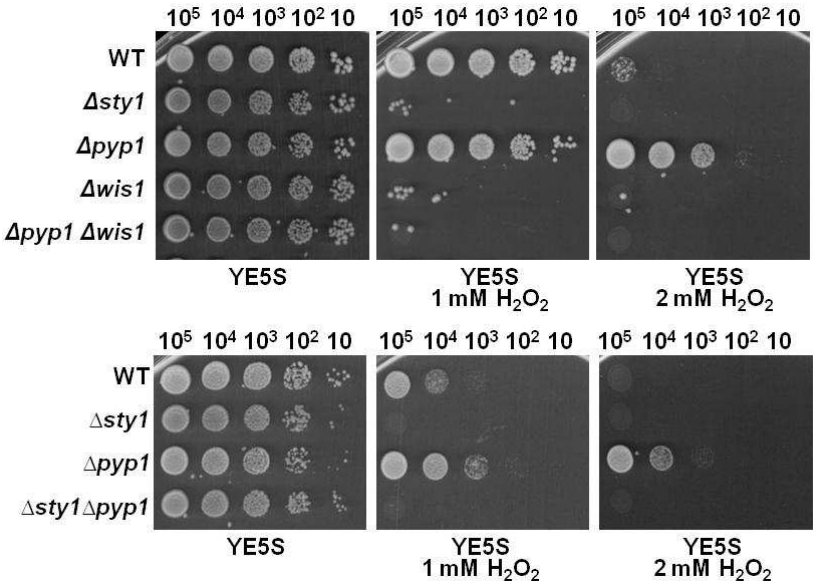

Figure 5: Wis1 and Sty1 are essential for resistance to peroxides. Analysis of the stress resistance of strains WT (972), Δ*sty1* (AV18), Δ*pyp1* (EP48), Δ*wis1* (EP44), Δ*pyp1* Δ*wis1* (EP67) and Δ*pyp1* Δ*sty1* (EP45) were spotted onto plates containing or not 1 or 2 mM of H_2O_2.

1.1.6. A strain deleted in *wis4, win1* and *pyp1* display an hyperactivation of Sty1 under basal conditions

According to the experiment describe above, Wis1 is the only kinase of Sty1: deletion of *pyp1* in cells lacking Wis1 is not sufficient to trigger resistance to peroxides. We then decided to test wether the same applies to the triple MAPKs: do Δ*pyp1* Δ*win1* Δ*wis4* cells display constitutive activation of Sty1? Surprisingly, the answer is yes: the triple knockout strain has basal Sty1 and Atf1 phosphorylation as shown by Western blot (**Fig. 6A**), and constitutive activation of the Sty1-dependent gene expression program (**Fig. 6B**).

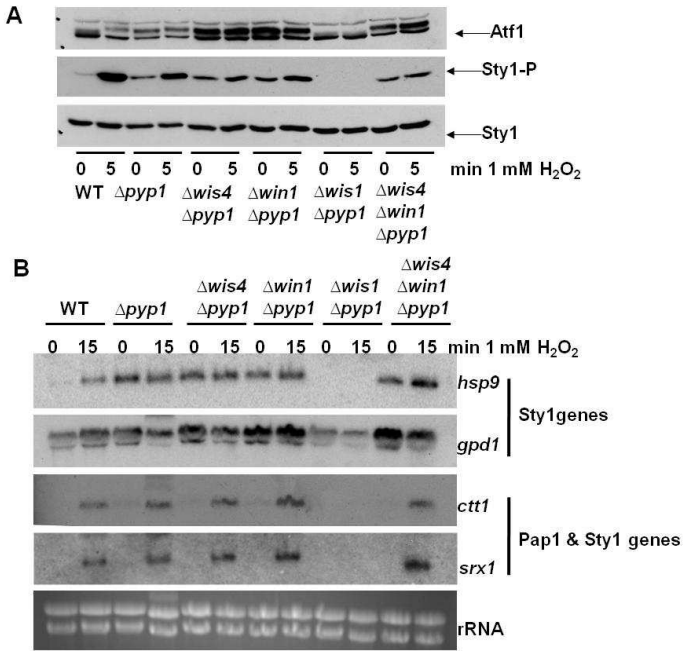

Figure 6: **Hyperactivation of Sty1 is independent of MAPKKK if deleting *pyp1*. (A)** Western blot analysis of Sty1 and Atf1 phosphorylation upon oxidative stress. Strains WT (972), Δ*pyp1* (EP48), Δ*wis4* Δ*pyp1* (EP62), Δ*win1* Δ*pyp1* (EP63) Δ*wis1* Δ*pyp1* (EP67), Δ*wis4* Δ*win1* Δ*pyp1* (EP70), were grown in YE media and were collected before and after treatment of 1 mM of H_2O_2 at the times indicated. TCA extracts were prepared and protein was analysed by Western blot using anti-p38-P (Sty1-P), anti-Atf1 (Atf1) and anti-Sty1 (Sty1). **(B)** Same cells as A were treated or not with 1 mM of H_2O_2 during 15 minuts. Total RNA was extracted and analyzed by Northern blotting, with probes for *hsp9, gpd1, ctt1* and *srx1*. Ribosomal RNA (rRNA) and actine (*act1*) are shown as loading controls.

1.1.7. The MAPKKK Mkh1 cannot phosphorylate Wis1 in the absence of Win1 and Wis4.

The basic architecture of each functional MAPK cascade is composed of three sequentially acting protein kinases. Thus, activation by specific stimuli of MAPK signal transduction pathways is followed by changes in gene expression that play a crucial adaptative role in the adaptation of cells to environmental conditions. In contrast to the six or more MAPK

cascades presents in budding yeast, three distinct MAPK signalling cascades have been identified in *S. pombe*. These include the mating pheromone-responsive MAPK pathway, the stress-activated protein kinase and the cell integrity pathway [120, 121].

The cell integrity pathway consists of a MAPK cascade composed by the MAPKKK Mkh1, MAPKK Pek1/Shk1 and the MAPK Pmk1. Deletion of each kinase causes hypersensitivity and growth arrest in response to hyperosmotic stress or to high temperatures, and morphological defects [122].

To check which kinase is responsible for the induction of Sty1 in the triple mutant Δ*pyp1* Δ*win1* Δ*wis4,* we further deleted *mkh1* in this background. We tested by survival experiments if cells lacking *mkh1* suppresses the H_2O_2 tolerance of our triple Δ*wis4* Δ*win1* Δ*pyp1* mutant, but the fourth deleted mutant displays the same resistant phenotype as the triple mutant (**Fig. 7**).

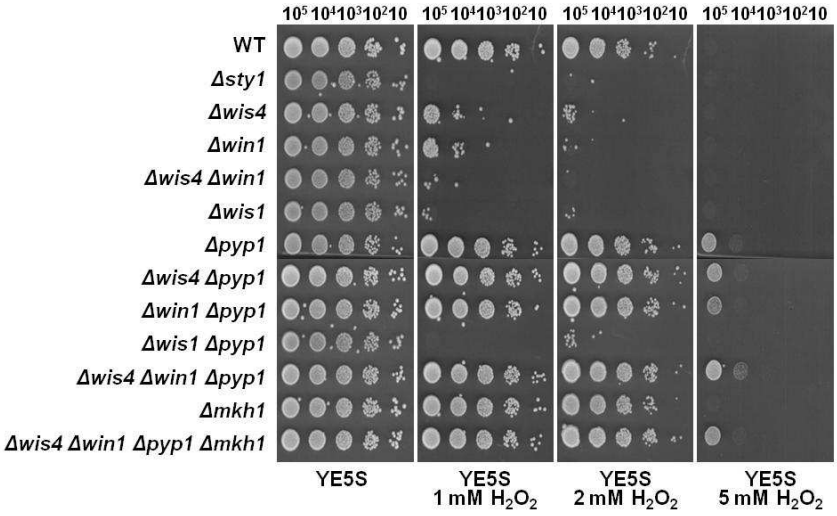

Figure 7: Mkh1, the MAPKKK of the cell integrity pathway is not the alternative Wis1 kinase. Liquid cell cultures of strains WT (972), Δ*sty1* (AV18), Δ*wis4* (EP37), Δ*win1* (EP39), Δ*wis4* Δ*win1* (EP43) and Δ*wis1* (EP44), Δ*pyp1* (EP48), Δ*wis4* Δ*pyp1* (EP62), Δ*win1* Δ*pyp1* (EP63) Δ*wis1* Δ*pyp1* (EP67), Δ*wis4* Δ*win1* Δ*pyp1* (EP70), Δ*mkh1* (EP72) and Δ*wis4* Δ*win1* Δ*pyp1* Δ*mkh1* (EP74), were grown in rich media (YE5S) and the indicated number of cells were spotted into YE5S with the indicated concentrations of H_2O_2.

Similarly, we determined that the MAPK of the pheromone pathway, Pek1, was not able to phosphorylate Wis1, since the quadrupled mutant still displayed resisitance to peroxides (data not shown). Further work will be required to identify the kinase responsible of this phosphorylation.

1.2. Identification of Sty1 interactors

To isolate possible interactors of Sty1, we used a proteomic approach that combines affinity purification with an antibody, followed by mass spectrometry. We tagged Sty1 with an HA epitop at its genomic locus, at the carboxy-terminus, in wild-type and in cells lacking the main phosphatase, Pyp1. The main goal of using these two strains is to find cytoplasmatic and nuclear interactors. As shown in Fig. 9, cells lacking *pyp1* display constitutively active/phosphorylated Sty1 in both untagged and in a *sty1-HA* tagged strain compare Δ*pyp1* and Δ*pyp1 sty1-HA* in **Fig. 9A**. As expected both strains are resistant to peroxide on solid plates (**Fig. 9B**) and Sty1 remains nuclear before and after stress, when tagged at its CTD with GFP (**Fig. 9C**). We used this strain background, *sty1-HA* Δ*pyp1*, to isolate Sty1 nuclear interactors. On the other hand, strain *sty1-HA* is not active/phosphorylated in basal conditions, grows as a wild-type in H_2O_2 plates, and is cytoplasmatic under basal conditions regarding cell localization by GFP microscopy; therefore, we will use this strain background to pull down Sty1 and to find cytoplasmatic interactors.

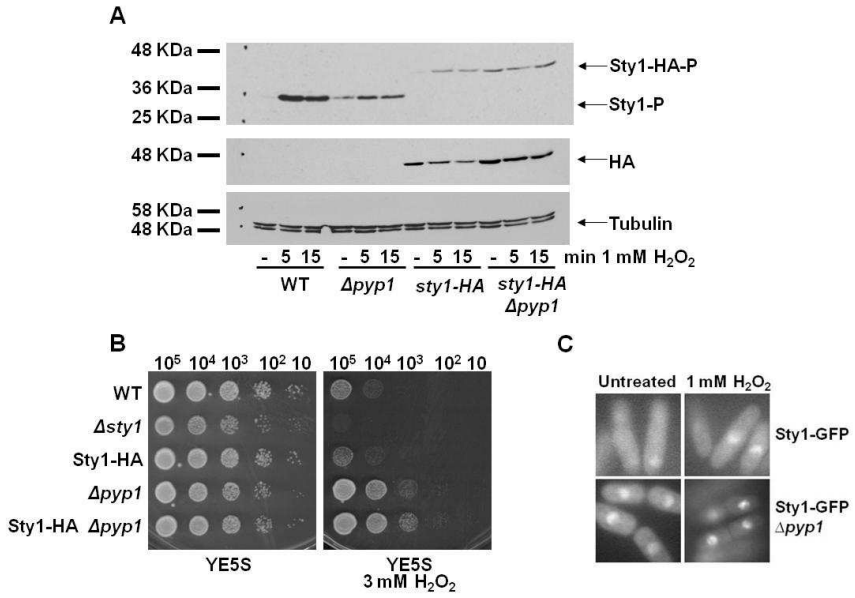

Figure 9: Characterization of strains used in the pull –down experiments. **(A)** TCA extracts from WT (972), Δ*pyp1* (EP48), *sty1-HA* (EP42) and *sty1-HA* Δ*pyp1* (EP49). **(B)** Survival of different strains as in A in response to H_2O_2 exposure. **(C)** Localization of Sty1 in wild-type and Δ*pyp1*. The cellular distribution of Sty1-GFP was determined by fluorescence microscopy.

Briefly, we immunoprecipitated Sty1-HA from cell extracts, and analyzed the purified protein by mass spectrometry using dimethyl labeling quantification (DM).

The purification in a single step should be an efficient protocol, more than tandem affinity purifications and it would increase the number of possible targets. However, we would have an excess of unspecifically purified proteins. To avoid this inconvenience we purified a non-tagged strain and compared the peptide list with our two samples: *sty1-HA* versus *sty1-HA* Δ*pyp1.*

The schematic description of our pull-down experiment is represented in Fig. 10. The first part of the protocol was to obtain native extracts of each of the three cultures under low temperatures not to disturb interactions (see Materials and Methods for more details). We took a sample of native extracts before immune-precipitation to quantify the total amount of protein in each sample, and also saved a small

fraction to quantify by mass spectrometry the relative levels of proteins the three cell extracts. We quantified by Bradford and equal amounts of whole cell protein (50 mg) were immuno-precipitated. There was a previous step of pre-clearing, in which extracts were incubated with a non specific resin (protein G sepharose). The purpose of this extra step of purification was to get rid of proteins that could bind non-specifically to the anti-HA-beads. The pre-cleared extracts were then incubated with a protein G sepharose cross linked to an anti-HA antibody.

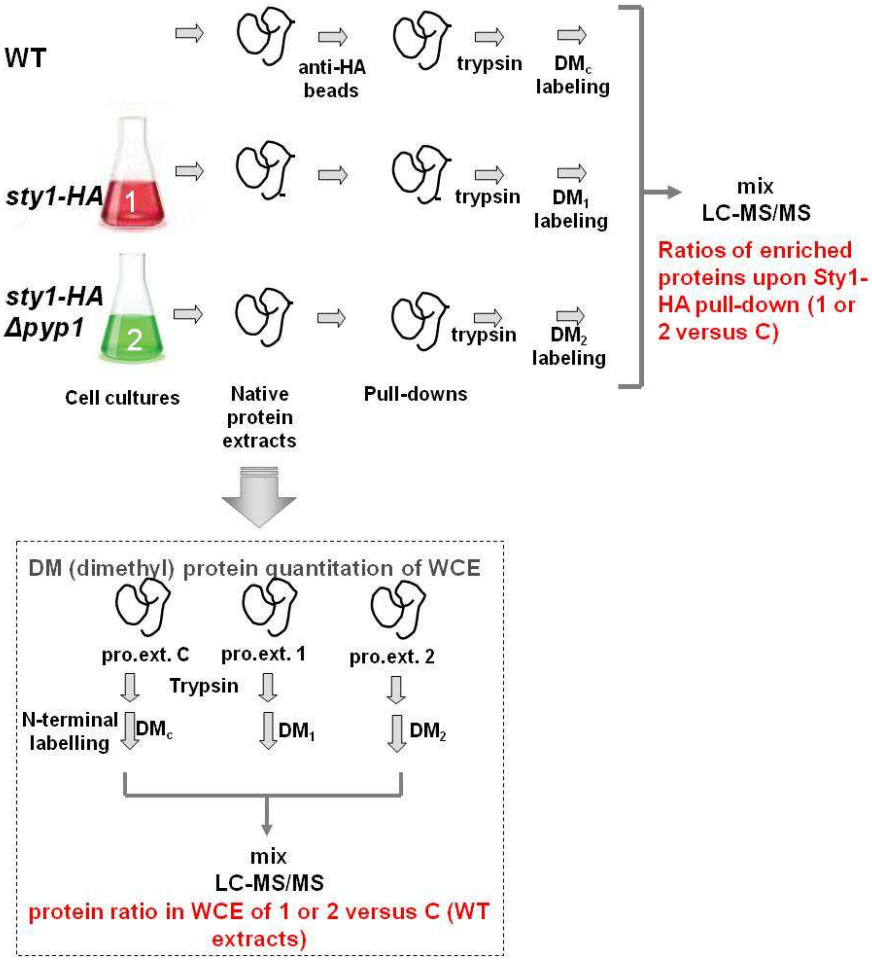

Figure 10: Pull-down strategy (see text for details).

The purification of Sty1-HA was confirmed by Western Blot (**Fig. 11A**) and by silver staining. In the silver gel we loaded 1/10 of the purified fraction (**Fig. 11B**). We couldn't distinguish any differential band among different immune-precipitates, what indicates the presence of many non-specific proteins during our enrichment procedure. The rest of purified fractions (1/9) plus a small fraction of native extracts prior to enrichment were labeled with DM for quantification. To do so we trypsinized the samples and labelled peptides at their NTDs with normal, medium or heavy formaldehyde (see Materials and Methods). Peptides from three pull-downs (untagged, *sty1-HA* and *sty1-HA* Δ*pyp1*) were then mixed and analyzed by LC-MS/MS at the proteomic facility at the UPF/CRG in Barcelona. We take into account only proteins with a ratio DM Sty-HA/DM non-tag or Sty1-HA Δ*pyp1*/DM non-tag >1.

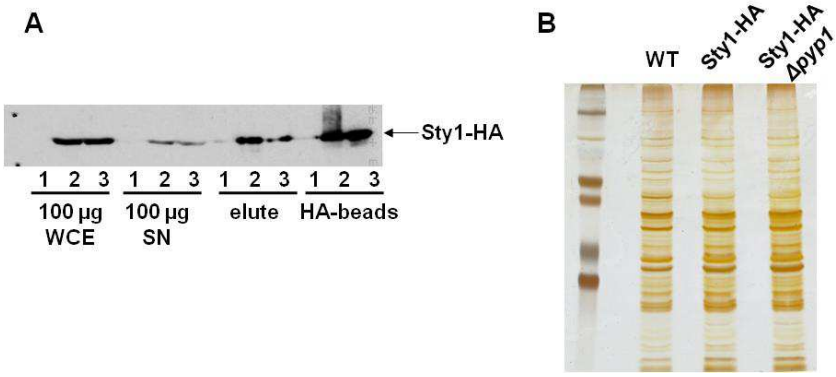

Figure 11: **Purification of Sty1-HA**. **(A)** 50 mg of native extracts were obtained from *sty1-HA* (2), *sty1-HA* Δ*pyp1* (3) and WT (1) (972, non-tagged) and were purified through anti-HA column. In a SDS-PAGE were loaded 100 µg of total extracts, 100 µg of not bound to HA column, elution fraction (1/50 of total elute fraction) and protein not eluted from HA-beads. Protein was analyzed using Western blot with anti-HA. **(B)** 1/10 of the purified fraction was loaded in a SDS-PAGE and silver stained. Equal amount of extracts from the three strains was monitored.

First we analyzed the individual relative protein concentrations in both samples before immunoprecipitation. As shown in Fig. 12, the relative amount of protein in strain *sty1-HA* Δ*pyp1* was significantly

diminished, although it represents a normal distribution. We use this data to normalize the results for the immunoprecipitation.

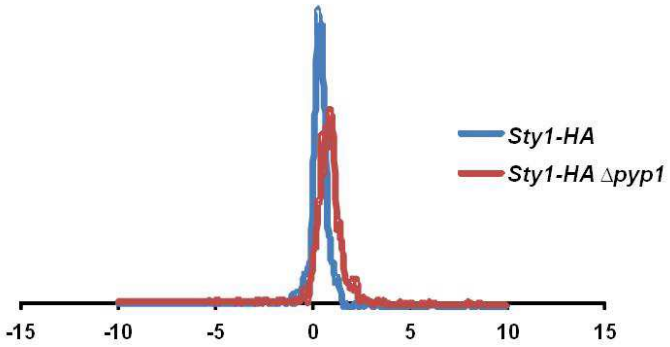

Figure 12: **Metric plot representing the distribution of the DM ratio of total proteome for each sample.**

A total of 500 proteins in the pull-down from *sty1-HA* and 342 proteins from *sty1-HA* Δ*pyp1,* which showed a ratio higher than 1 fold relative to the pull-down from the control strain, were detected by MS. It is not possible to establish the threshold to consider any given peptide as clearly overrepresented in one sample, but higher the ratio is, higher the specificity of the purification (**Fig. 12**). Moreover, some of the peptides belong to the CESR genes, and, as said before, the expression levels are increased in Δ*pyp1* cells.

From proteomic results we got two enrichment data: one belonging from protein quantification of total extract before purification (DM extract) and another from protein quantification after the immunoprecipitation (DM IP). As expected, Sty1-HA, the bait protein of our approach, was clearly overrepresented with a DM ratio of 6 or 10 in each sample. Also other known Sty1 substrates were isolated in the experiment, as Cdc37 or Srk1, with higher ratio, although they were already enrich in the total extracts. This would suggest that the method is not enough good to find substrates. Moreover, almost all proteins enriched in extracts belong to the CESR genes, so, it is difficult to discard these proteins as real substrates o just CESR proteins. In addition, there were some proteins more enriched than Sty1 in the purification: Rds1,

Hrp3, Mrt4, etc. There are several explanations for the fact that these peptides were more abundant than the ones corresponding to Sty1, but the most plausible explanation is that these peptides were more efficiently labeled with the DM reagents.

We have analyzed some of the deletion of these putative Sty1 substrates by analyzing their sensitivity or resistant growth on H_2O_2 plates, and also by checking the upon H_2O_2 stress by Northern Blot

The gene *rds1* (SPAC343.12) codes for a protein of 40 kDa, and it has been described as an adenine repressible gene regulated by glucose, ammonium, phosphate and temperature and that, it has a function in stress-related responses of the cell [123]. Rds1 was also characterized as a Fep1-dependent gene, and its transcription is up-regulated in response to copper deficiency [124]. As shown in **Fig. 13**, cells deleted in *rds1* do not display any phenotypical defect, regarding growth on H_2O_2 plates or any stress or iron-dependent transcriptional defect.

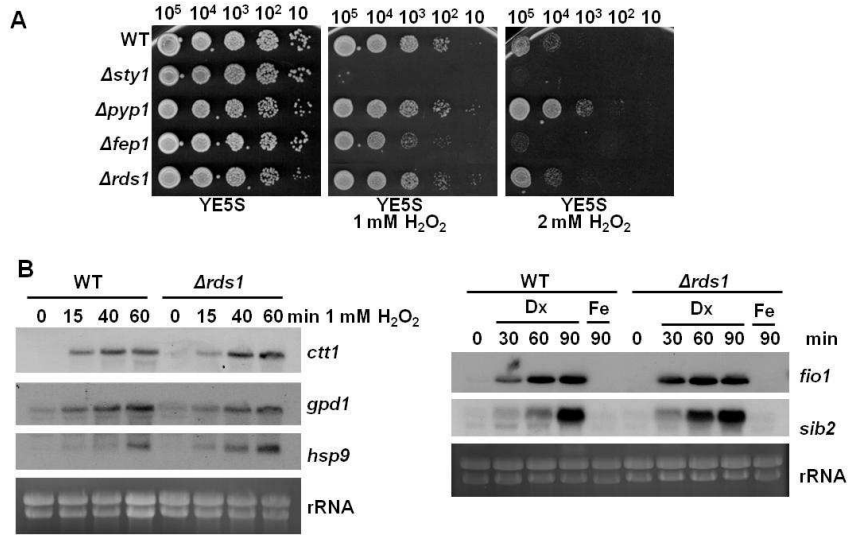

Fig. 13: Δ***rds1* phenotype upon different stresses**. **(A)** Liquid cell cultures from strains WT (972), Δ*sty1* (AV18), Δ*pyp1* (EP48), Δ*fep1* (NG1) and Δ*rds1* (EP140), were grown in YE5S and were spotted in plates containing or not 1 or 2 mM of H_2O_2. **(B)** The same strains as in A were exposed to 1 mM of H_2O_2 or to deferroxamin (Dx) or iron (Fe) at indicated times. Total RNA was extracted and by analyzed by Northern blot with *probes ctt1, gpd1, hsp9, fio1, pcl1* and *sib2*. Ribosomal RNA (rRNA) was used as a loading control.

2. Dissection of Sty1-Atf1 transcriptional activation program

The MAPK Sty1 regulates the transcriptional responses promoting cell survival triggered by different environmental stress in *S. pombe*. Upon stress activation, Sty1 migrates in the nucleus, where it stimulates a gene expression program through the TF Atf1. Cells deleted in *atf1* are sensitive to oxidative stress, osmotic stress and heat shock [85].

Nuclear Sty1 phosphorylates Atf1 at the nucleus. What is the effect of these multiple phosphorylations? Is Atf1 gaining affinity for its CRE sites at promoters? Are the phosphorylations stabilizing the TF, as proposed by the group of Nic Jones [84]? Is the phosphorylated TF recruiting some chromatin modifiers or even Pol II? The fact that the *atf1* gene is also activated by Atf1 in a stress-dependent manner further complicates responding to these questions.

This section, aimed at investigating the exact participation of phosphorylated Sty1 and phosphorylated Atf1 in transcription activation, is divided in two blocks. Block 2.1 presents the results from constitutively expressing HA-Atf1 and different mutant versions in a Δ*atf1* background. We have also expressed only the bZIP domain of Atf1.

In block 2.2, we try to dissect the role of Sty1 and Atf1 on transcription initiation by fusing the GAL4 DNA binding site and analyzing their effect on mutated CESR promoters, in which GAL4 DNA sites have been inserted.

2.1. Expression of HA-tagged Atf1 and mutant versions from the constitutive *sty1* promoter

Within this chapter, we pretend to investigate: (i) the role of the DNA binding domain of Atf1 on transcription initiation, and (ii) the role of Atf1

phosphorylations by Sty1 on the activation of the TF. To do so, we expressed HA-tagged wild-type, truncated forms and phosphorylation mutants in Δ*atf1* cells. All the chimeric genes were integrated at the *leu1* locus of *S. pombe*, and were transcribed from the constitutive *sty1'* promoter. As will be shown later, the levels of HA-Atf1 and mutant derivatives were not significant higher than those of wild-type cells (only 2-5 times more protein in our chimeras).

Atf1 bZIP DNA-domain is located at C-terminus, and it has a homology with members of the ATF/CRE family of mammalian TF and binds specifically to ATF/CRE recognition sites [125]. In order to know the functionality of the bZIP, we constitutively expressed in Δ*atf1* cells either full length, a truncated Atf1 without the bZIP domain or a truncated Atf1 expressing only the bZIP. As we will be shown below, the bZIP domain alone is sufficient for wild-type tolerance to peroxides.

Phosphorylation of Atf1 by Sty1 has been demonstrated *in vitro* and *in vivo*; however the sites of phosphorylation are unknown. It has been long believed that phosphorylation of Atf1 by Sty1 is essential for its activation. However, the group of Nic Jones reported that an Atf1 form lacking all 11 MAPK phosphorylation sites has a similar activity than wild-type Atf1. They also show that the nonphosphorylatable mutant is very unstable, and they suggested that the main role of Sty1-dependent phosphorylation is to stabilize rather than to activate the TF [84]. In this thesis work, we have generated different phosphorylation mutants of Atf1, mimicking the phosphorylatable and the nonphosphorylatable version, expressed them form plasmids under constitutive promoters, and we have seen that these Atf1 mutants were stable and functional.

2.1.1. Cells lacking Atf1, Sty1 or both display different stress-related phenotypes

To test whether Atf1 and Sty1 are both equally required for stress survival, we compared cells lacking or not each protein under a variety of adverse

stress conditions. Sty1 and Atf1 were required for full survival upon oxidative stress, osmotic stress or heat stress. In contrast, double mutant Δ*atf1* Δ*sty1* were able to survival upon heat stress, even though cells lacking *atf1* or sty1 showed severely impaired survival (**Fig. 14A**). Since Sty1 were required for survival on osmotic, oxidative stress or heat stress, we tested whether induction of some Sty1 and Pap1-dependent CESR genes would be impaired in Δ*atf1* Δ*sty1* but not in single mutants. In wild-type cells, the induction of most stress-inducible genes was dependent on Sty1 and Atf1. However, some target genes that are not induced in a Δ*sty1,* such as *ctt1* (encoding for catalase) or *srx1* (encoding the peroxiredoxin reductase Srx1), were induced in a Δ*atf1* Δ*sty1* at same levels as a Δ*atf1* cells (**Fig. 14B**). These results suggest that whereas both Atf1/Sty1 seems to be responsible for general transcriptional activation, Atf1 needs to be phosphorylated by Sty1 to allow the tiny induction of some CESR genes. Most CESR genes, such as *gpd1, hsp9, ctt1* and srx1, require ATf1 and Pcr1 for their stress-dependent induction [85]. We used chromatin immunoprecipitation to analyze the binding of Atf1 to these stress promoters in vivo. Using strains carrying a plasmid with HA-tagged of Atf1 expressed under constitutive promoter, we observed that Atf1 is bound to promoters before stress imposition, but it further accumulated upon H_2O_2 in a Sty1-dependent manner (**Fig. 15C**).

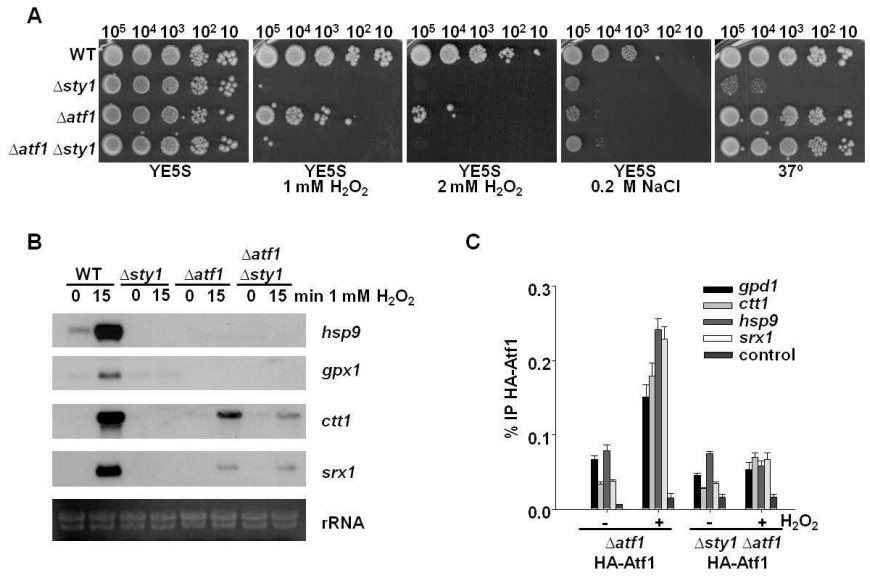

Figure 14: **Sty1 and Atf1 are required for response upon peroxides**. **(A)** Liquid cultures from strains WT (972), Δ*sty1* (AV18), Δ*atf1* (MS98) and Δ*atf1* Δ*sty1* (IV59) were grown on YE media and spotted on plates containing 1 or 2 mM H_2O_2, 0.2 M NaCl or upon heat stress (37ºC). **(B)** Cultures of strains as in B were treated with 1 mM of H_2O_2 during 15 min. Total RNA were extracted and analyzed by Northern blot using probes for *hsp9, gpd1, ctt1*, and *srx1*. *act1* was used as a loading control. **(C)** Cultures of strains Δ*atf1* HA-Atf1 (EP203) and Δ*sty1* Δ*atf1* HA-Atf1 (EP303) were used to perform ChIP experiments using primers at *ctt1, gpd1, hsp9* and *srx1* promoters. An intergenic region was used as a negative control (control). Error bars (SEM) for all ChIP experiments were calculated from biological triplicates.

2.1.2. The DNA-binding domain of the Atf1 TF is sufficient to induce some stress genes

To analyze in more detail the function of the bZIP DNA-domain of Atf1, we expressed in a Δ*atf1* cells either full length, a truncated Atf1 lacking the bZIP domain or a truncated Atf1 expressing only the bZIP (**Fig. 15A**). Interestingly, full length Atf1 and the bZIP domain alone were completely functional and restored the wild-type tolerance to H_2O_2, whereas cells expressing HA-Atf1ΔbZIP domain, are as sensitive as Δ*atf1* cells (**Fig. 15B**). Both proteins Atf1 full length and bZIP were expressed to levels similar to those of wild-type cells (**Fig. 15C**). Regarding transcription

activation of CESR genes, only cells expressing full length HA-Atf1 displayed full induction of the whole gene expression program, as shown by NB (**Fig. 16D**). However, not only HA-Atf1 but also HA-Atf1.bZIP were able to trigger the activation of *ctt1* and *srx1* genes, which are under the control not only Atf1 but also Pap1. An important conclusion of these experiments is that wild-type expression of only the *ctt1* and *srx1* genes seems to be sufficient to restore wild-type tolerance to peroxides, but not to other types of stresses such as NaCl (see **Fig. 17A**).

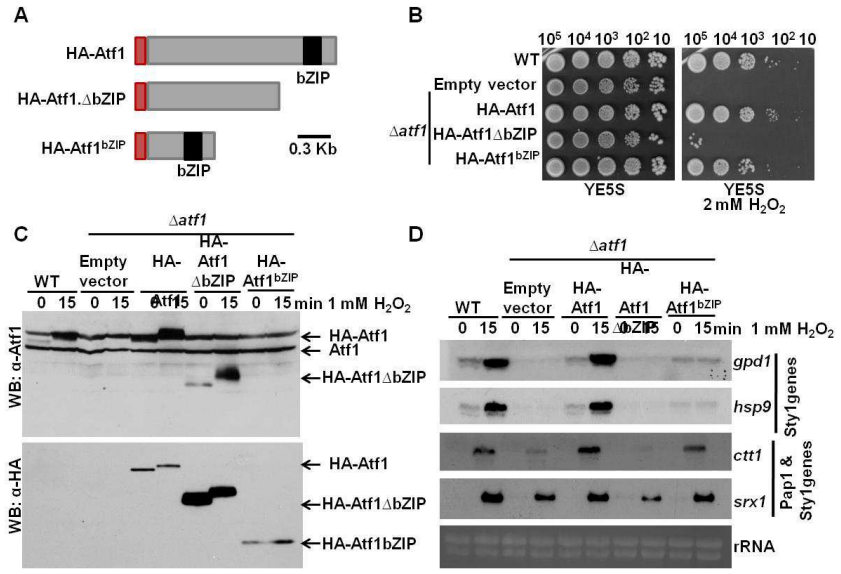

Figure 15: **DNA-binding domain of Atf1 is sufficient to restore the sensitivity to H_2O_2. (A)** Schematic representation of HA-Atf1, HA-Atf1.ΔbZIP and HA-Atf1^{bZIP} proteins. **(B)** Vectors carrying a constitutive expressed wild-type HA-Atf1 or truncated versions HA-Atf1.ΔbZIP and HA-Atf1^{bZIP} and an empty plasmid were integrated in the chromosomes of Δ*atf1* strains. Rich media cultures of strains WT (972), Δ*atf1* + empty vector (EP201), Δ*atf1* + HA-Atf1 (EP203), Δ*atf1* + HA-Atf1.ΔbZIP (EP203.ΔbZIP) and Δ*atf1* + HA-Atf1^{bZIP} (EP203.bZIP) were spotted on plates containing 2 mM H_2O_2 and 0.2 M NaCl***. **(C)** Same cultures as in B, either untreated (0) or treated with 1 mM H_2O_2 during 15 min (15), were analyzed to determine protein levels by Western blot using monoclonal antibody against HA or polyclonal antibody against Atf1. **(D)** Stress dependent transcriptional analysis of strains as in B, were treated or not with 1 mM H_2O_2 for 15 min. Total RNA was analyzed by Northern blot with probes for *hsp9, gpd1, ctt1* and *srx1*. *rRNA* are shown as a loading control.

2.1.3. Atf1^{bZIP} seems to promote proper Pap1 function at *srx1* and *ctt1* promoters

The induction of the stress-inducible genes *ctt1* and *srx1* is Sty1- and Pap1-dependent. To further investigate the role of the bZIP domain of Atf1, we expressed in Δ*atf1* Δ*pap1* cells full length Atf1 or both truncated versions lacking the bZIP or expressing only the bZIP. As expected, these cells were not able to survive on H_2O_2 plates (**Fig. 16A**), although three proteins were expressed to similar levels to Atf1 in wild-type (**Fig. 16B**). Moreover, the transcription of stress genes was abolished in cells lacking both *pap1* and *atf1*, even if they expressed the full length Atf1 or bZIP domain of Atf1 (**Fig. 16C**). These results suggest that we both need the TF Pap1 and Atf1 to induce some CESR genes. Indded, the bZIP domain of Atf1 is sufficient to facilitate Pap1 binding to these two promoters (Patricia Garcia and Elena Hidalgo, unpublished data).

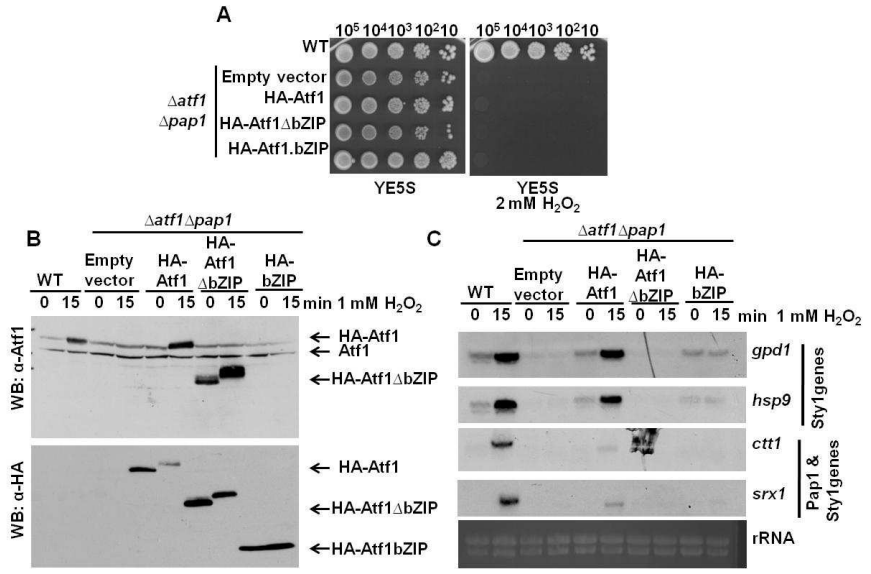

Figure 16: **DNA-binding domain of Atf1 seems to promote proper Pap1 induction on *ctt1* and *srx1* genes**. **(A)** Vectors carrying a constitutive expressed wild-type HA-Atf1 or truncated versions HA-Atf1.ΔbZIP and HA-Atf1^{bZIP} and an

empty plasmid were integrated in the chromosomes of Δ*atf1* Δ*pap1* strains. Rich media cultures of strains WT (972), Δ*atf1* Δ*pap1* + empty vector (EP260), Δ*atf1* Δ*pap1* + HA-Atf1 (EP261), Δ*atf1* Δ*pap1* + HA-Atf1.ΔbZIP (EP261.ΔbZIP) and Δ*atf1* Δ*pap1* + HA-Atf1^{bZIP} (EP261.bZIP) were spotted on plates containing 2 mM H_2O_2. **(B)** Same cultures as in B, either untreated (0) or treated with 1 mM H_2O_2 during 15 min (15), were analyzed to determine protein levels by Western blot using monoclonal antibody against HA or polyclonal antibody against Atf1. **(C)** Stress dependent transcriptional analysis of strains as in B, were treated or not with 1 mM H_2O_2 for 15 min. Total RNA was analyzed by Northern blot with probes for *hsp9, gpd1, ctt1* and *srx1*. *rRNA* are shown as a loading control.

2.1.4. The MAPK phosphorylation site at the Atf1^{bZIP} domain is not required for Atf1 function

The bZIP domain of Atf1 seems to be sufficient to promote wild-type tolerance upon oxidative stress. Atf1 contains eleven phosphorylation sites for the MAPK Sty1, namely a serine or threonine residue immediately followed by a proline, one of these sites is inside to the bZIP domain (**Fig. 17A**). To further investigate the role of the bZIP domain, we mutated this MAPK putative phosphosite to alanine (S438A). Δ*atf1* cells expressing the bZIP domain of Atf1 display the same phenotype as the Ser-to-Ala mutated version of the bZIP. These cells were able to grow upon oxidative but not osmotic stress (**Fig. 17B**). Both proteins were expressed at similar levels as those Atf1 cells in wild-type cells (**Fig. 17C**) and the transcription profile showed induction of *ctt1* and *srx1* independently of the mutation (**Fig.17 D**). These results suggest that the phosphorylation of Ser438 is not important to survive upon peroxides. Again, we noticed that neither the bZIP domain nor the S438A mutant version rescue the stress sensitivity to NaCl, suggesting that wild-type expression of *ctt1* and/or *srx1* is sufficient to promote survival to oxidative stress but not to other stresses.

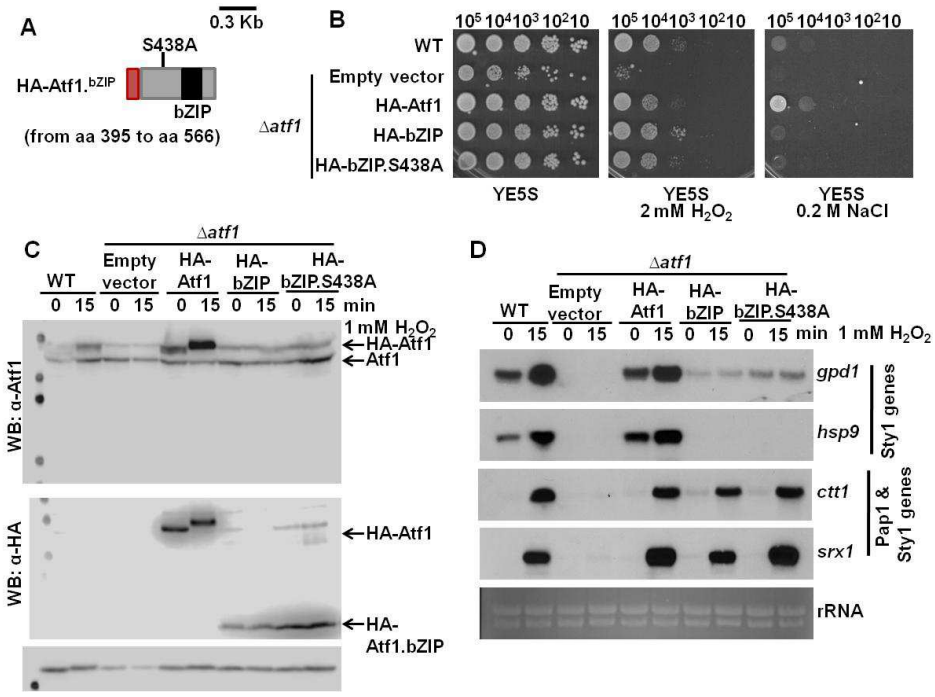

Figure 17: **(A)** Schematic representation of HA-Atf1$^{bZIP.S438A}$ protein. **(B)** Vectors carrying a constitutive expressed wild-type HA-Atf1 or HA-Atf1^{bZIP} and HA-Atf1$^{bZIP.S438A}$ and an empty plasmid were integrated in the chromosomes of Δ*atf1* strains. Rich media cultures of strains WT (972), Δ*atf1* + empty vector (EP201), Δ*atf1* + HA-Atf1 (EP203), Δ*atf1* + HA-Atf1^{bZIP} (EP203.bZIP) and Δ*atf1* + HA-Atf1$^{bZIP.S438A}$ (EP203.bZIP.S438A) were spotted on plates containing 2 mM H_2O_2 and 0.2 M NaCl. **(C)** Same cultures as in B, either untreated (0) or treated with 1 mM H_2O_2 during 15 min (15), were analyzed to determine protein levels by Western blot using monoclonal antibody against HA or polyclonal antibody against Atf1. **(D)** Stress dependent transcriptional analysis of strains as in B, were treated or not with 1 mM H_2O_2 for 15 min. Total RNA was analyzed by Northern blot with probes for *hsp9, gpd1, ctt1* and *srx1*. *rRNA* are shown as a loading control.

Phosphorylation of Atf1 by Sty1 has been well demonstrated; however the phosphorylation sites of Atf1 crucial for its function are still unknown. We decided to test whether the S438A mutation disrupted Atf1 function in a full length context. We expressed a HA-Atf1.S438A in Δ*atf1* cells. We first compared the growth of cells containing the full length of HA-Atf1 and the mutated full length HA-Atf1.S438A on plates containing

H_2O_2 or NaCl, and both grew as a wild-type strain (**Fig.18A**). We next checked the levels of Atf1 protein before and after H_2O_2 to determine whether phosphorylation modulated Atf1 stability; however no changes in phosphorylation of the HA-Atf1.S438A version were observed (**Fig. 18B**). Concomitantly, the HA-Atf1.S438A was able to induce gene activation after stress imposition (**Fig. 18C**). All these results together indicate that phosphorylation Ser438 is not required for the function of Atf1 as a TF.

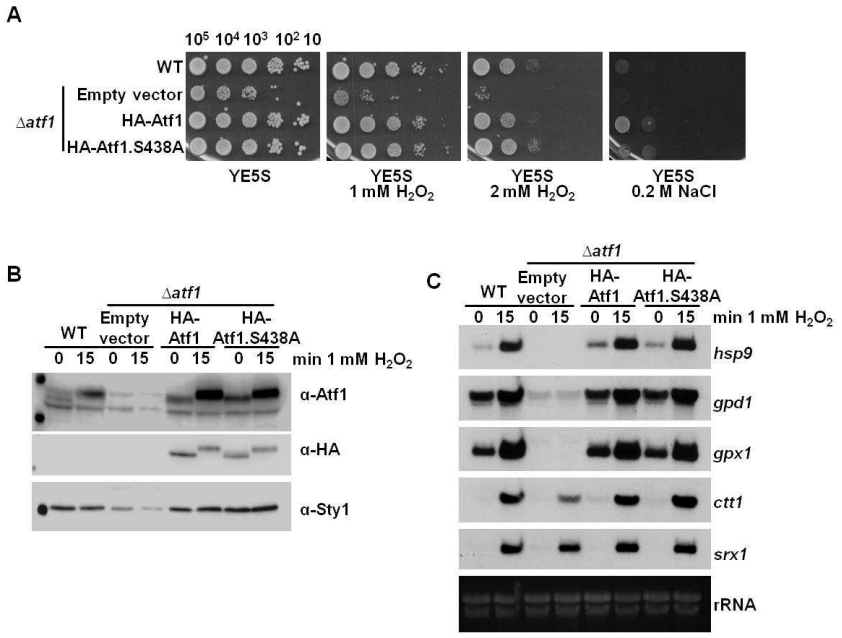

Figure 18: Phosphorylation of Ser438 is not required for the functionality of Atf1. (A) Rich media cultures of strains WT (972), Δ*atf1* + empty vector (EP201), Δ*atf1* + HA-Atf1 (EP203) and Δ*atf1* + HA-Atf1.S438A (EP203.S438A) were spotted on plates containing 1 or 2 mM H_2O_2 and 0.2 M NaCl. **(B)** Same cultures as in A, either untreated (0) or treated with 1 mM H_2O_2 during 15 min (15), were analyzed to determine protein levels by Western blot using monoclonal antibody against HA or polyclonal antibody against Atf1. Sty1 antibody was shown as a loading control **(C)** RNA from same cultures as in A were treated or not with 1 mM H_2O_2 for 15 min. Total RNA was analyzed by Northern blot as described above.

2.1.5. Characterization of the MAPK putative sites of the Atf1 TF

The Atf1 protein possesses eleven potential MAPK sites: S2, S4, T77, S140, S152, S172, T204, T216, S226, T249 and S438. As previously reported, the levels of Atf1 protein in wild-type cells are significantly increased and the mobility of the protein in SDS-PAGE was retarded following stress imposition (**Fig. 20B**). In 2007, a mutant *atf1* gene containing single point mutations of the eleven potential MAPK phosphosites, the *atf1.11M* allele, was integrated into the *atf1* locus by the group of Jones. These multiple mutations had a dramatic effect in the stability of the protein, however major loss of function was not observed. In contrast to cells lacking *atf1,* this *atf1.11M* strain had no sensitivity to H_2O_2 and could still induce some Atf1-dependent genes. In this study, the authors concluded that Atf1 phosphorylation by Sty1 is not required for activation of Atf1 *per se* but rather for increasing its stability [84].

atf1 is also a CESR gene, and therefore the reduced protein levels of the Atf1.11M mutant could be a consequence of a decrease in the expression of the *atf1* mRNA. We were not confident to believe that the main role of Sty1 upon Atf1 was to stabilize the protein. To further investigate the role of the different MAPK putative phosphorylation sites of Atf1, we have generated two mutated versions of *atf1* were all phosphosites, except the one near to the bZIP domain, were mutated to alanine or to isoleucine mimicking unphosphorylatable version (Atf1-10M) and, to glutamic or aspartic mimicking the phosphorylatable version (Atf1-10D) (**Fig. 19A**). The full length synthesis was performed by GeneScript. Again, the HA-Atf1-10M and HA-Atf1-10D mutants were expressed from an integrative plasmid under the control of the constitutive *sty1'* promoter in Δ*atf1* cells. We first analyzed the stability of the protein by Western Blot, and, as we show in **Fig. 19B**, the stability was not affected by the mutations: the levels of Atf1 proteins of the Atf1.10M and Atf1.10D were similar to those of wild-type HA-Atf1. We next compared the phenotypes of cells

expressing These results suggest that Atf1 phosphorylation is not required for Atf1 stability, because Atf1.10M mutant displays same levels of protein as HA-Atf1, once it is expressed from a constitutive promoter. Whether these mutations eliminate the activity of the TF on CESR genes, and participate in wild-type tolerance to environmental stresses, will shortly be determined.

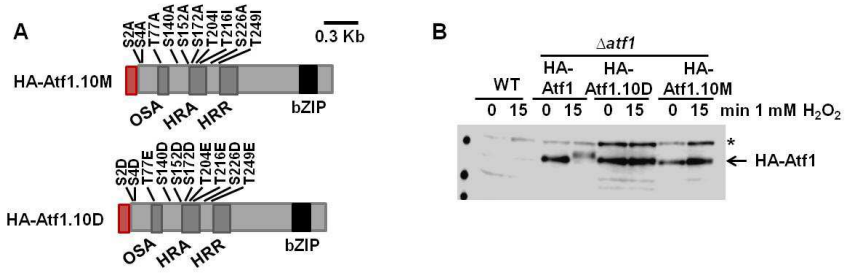

Figure 19: Atf1 phosphomutants under oxidative stress (A) Schematic representation of ten phosphosites residues of Atf1 mutated to mimic the phosphorylatable (HA-Atf1.10D) and non-phosphorylatable (HA-ATf1.10M) proteins. **(B)** Vectors carrying a constitutive expressed wild-type HA-Atf1 or mutants HA-Atf1.10D and HA-Atf1.10M were integrated in the chromosomes of Δ*atf1* strains. Rich media cultures of strains WT (972), Δ*atf1* + empty vector (EP201), Δ*atf1* + HA-Atf1 (EP203), Δ*atf1* + HA-Atf1.10D (EP203.10D) and Δ*atf1* + HA-Atf1.10M (EP203.10M) were spotted on plates containing 2 mM H_2O_2 and 0.2 M NaCl or heat stress (37º).

2.2. Effect of Gal4-Atf1 fusion proteins on transcription of *CRE-to-Gal4BS* mutant promoters

As shown in the above block, the role of Atf1 phosphorylation by Sty1 is, at most, not essential: the *atf1.10M* strain displays wild-type induction of classical stress genes, and it only shows impaired activation of *ctt1* and *srx1*. We decided to design a different heterologous system to dissect the role of Atf1 and Sty1 on transcription activation.

Some time ago, the group of Wayne P. Whals used mutagenesis of DNA elements and chimeric proteins to define new features of Atf1 and Pcr1 on homologous recombination. They focused on two different aspects, one was meiotic recombination hotspot of the *ade6-M26*, which requires both Atf1 and Pcr1 (see introduction), and the other was upon osmotic stress response which is Pcr1 independent. They demonstrated that the DNA budding yeast Gal4 coupled to Atf1 was able to tether Atf1 TF to an engineered *ade6* gene containing a Gal4 site (G4BS) instead the CRE site [126].

We generated different chimeric gene expressing the GAL4 DNA binding domain (G4) fused to different mutants of Atf1 and mutated the CRE site of two stress genes to dissect the Atf1 activity in stress transcriptional responses. We have used this methodology to analyze the role of Atf1 and Sty1 in the induction of transcription of two stress-dependent promoters: *ctt1* and *gpd1*. With this system we could dismiss the effect of Atf1-to-DNA binding in transcription activation, since the chimeras will be constitutively bound to the mutated promoters.

In addition it is still unknown the role of Sty1 in transcriptional regulation. One possibility is that Atf1 is present in unstressed cells as a complex with other factors repressing Atf1 activity. Phosphorylation of these factors or of Atf1 by Sty1 may be enough to disrupt the complex and permit the TF activity. It has been postulated, also, that Sty1 is recruited to the promoters and possesses a transcriptional activation domain. To analyze these possibilities in more detail we have generated G4 chimeric proteins fused to Sty1 to tether artificially the MAPK to promoters and analyzed the possible role of Sty1 as a “transcription factor”.

2.2.1. Expression of G4-to-Atf1 chimeric proteins

We have constructed several integrative plasmids allowing the expression of G4 DNA-binding domain (G4), some of them fused to FLAG and HA tagged, coupled with different mutant versions of Atf1 Again, the

chimeric genes were expressed under the control of a constitutive promoter, the *sty1'* promoter. In particular, we constructed plasmids allowing the expression of G4 DNA binding domain fused to FLAG and HA (G4-HA), fused or not to full length Atf1 or a truncated form lacking the bZIP (G4-HA-Atf1.ΔbZIP) (**Fig. 20A**). We expressed these proteins in a Δ*atf1* strain. Only cells expressing the full length version of Atf1 were able to grow upon H_2O_2, as expected (**Fig. 20B**).

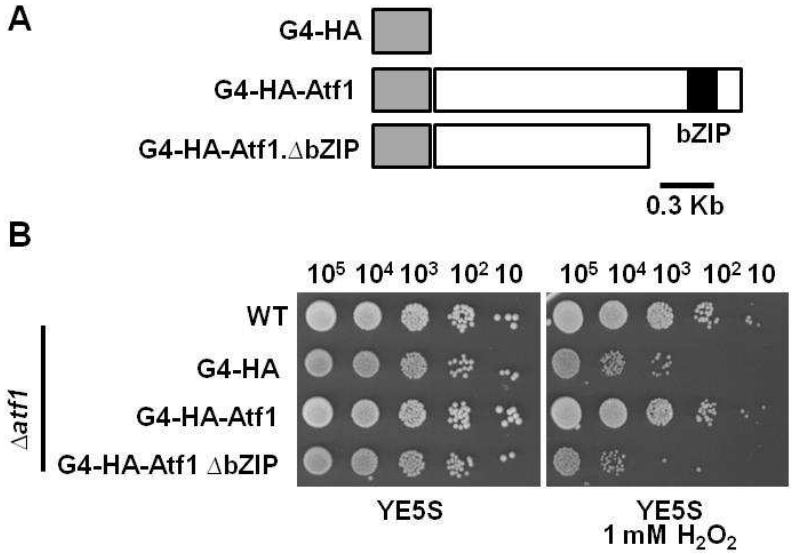

Figure 20: **Schematic diagram of constructs we used in this study**. (**A**) The *sty1'* promoter were used to drive constitutive expression of the indicated chimeric proteins, which contain the G4 and HA tagged, were fused or not to Atf1 full length (G4-HA-Atf1) or to the amino-terminal 395 amino acids of Atf1 (G4-HA-Atf1.ΔbZIP). (**B**) Same plasmids that in A were transformed in Δ*atf1* strain (EP193). Strains Δ*atf1* + pG4-HA (EP207), Δ*atf1* + pG4-HA-Atf1 (EP206) and Δ*atf1* + pG4-HA-Atf1.ΔbZIP (EP206.ΔbZIP) were spotted in plates with 1 mM of H_2O_2

2.2.2. Construction of cells carrying *CRE-to-G4BS* substitutions at the endogenous *ctt1* and *gpd1* promoters

To construct strains carrying substitutions of the CRE sites to G4 binding sites at the endogenous promoters, we followed a protocol recently proposed by Charlie Hoffman to reduce the number of false positives in the negative 5-fluoroorotic acid (5-FOA) (see Materials and Methods). Briefly, we worked with a wild strain auxotrophic for uracil, leucine and lysine (CHP1364 *h*$^-$ *ura5-294 lys7-2 leu1-32*) and we deleted 160 bp of the promoters around the CRE site with a cassette containing both the *ura5*$^+$ *lys7*$^+$ genes as recently described by the group of Hoffman [127]. We replaced such genes by recombination with a linear fragment of 500 bp of the *gpd1* or *ctt1* promoters with the CRE site changed by a G4 binding site (CRE-to-G4BS), and selected uracil auxotrophic clones by resistance to 5-fluoroorotic acid (5-FOA); we double checked lysine auxotrophy in the selected clones, which decreases the number of false positives in the screen. The resulting strains were crossed out to wild-type strains to eliminate auxotrophies.

The CRE site of *ctt1* gene is located at -360 bp from transcription start site (TSS) (**Fig. 21A**). As reported before, cells lacking *ctt1* are extremely sensitive to H_2O_2 [27, 128]. Cells deleted in the promoter of *ctt1* (*ctt1'.ΔCRE*) or cells expressing the CRE-to-G4BS version of the *ctt1* promoter (*ctt1'.G4BS*) are sensitivity to H_2O_2 (**Fig. 21B**). Next, we analyzed the transcription profile of these strains and, as expected; the induction of *ctt1* gene is severely impaired in the ctt1'G4BS strain but not other CESR genes. As expected there is not any gene induction when we deleted *sty1* or *atf1* (**Fig. 22C**).

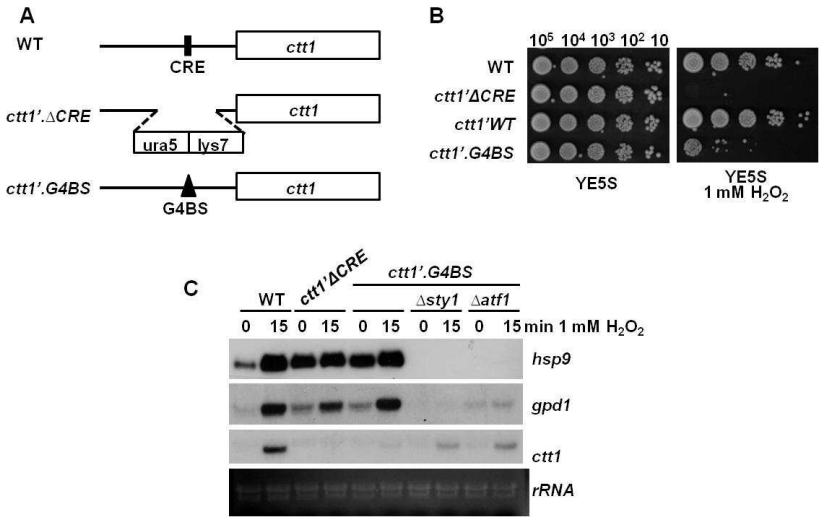

Figure 21: **CRE site substitution by G4-binding site impaired the expression of *ctt1* gene**. (**A**) Schematic representation of CRE site substitution by a G4BS. (**B**) Strains WT (972), *ctt1'ΔCRE* (EP142), *ctt1'WT* (EP144) and *ctt1'.G4BS* (EP184) were grown in liquid rich media (YE5S), and the indicated number of cells were spotted onto plates with or without 1 mM of H_2O_2. (**C**) Same strains as B and strains *ctt1'.G4BS Δsty1* (EP186) and *ctt1'.G4BS Δatf1* (EP187) were treated with 1 mM of H_2O_2 at indicated times. Total RNA were extracted and analyzed by Northern blot using probes for *hsp9, gpd1, ctt1*, and *srx1*. Ribosomal RNA (*rRNA*) was used as a loading control.

Regarding the *gpd1* gene, it contains two CRE putatice sites for Atf1 binding, one located at -320 bp and the other at -367 bp from TSS (**Fig. 22A**). Deletion in the *gpd1* promoter has abolished the induction of *gpd1* gene. However, we need to mutate both CRE sites to a G4BS to inactivate gene expression of *gpd1*. As shown in **Fig. 22B-C**, change of one or other CRE site to G4BS is not enough to impaired transcription. However, strain with both CRE sites substituted by two G4BS recover basal levels of *gpd1* induction but it not display any *gpd1* expression upon H_2O_2. As expected, no induction was observed when deleting *sty1* or *atf1* (**Fig. 22D**).

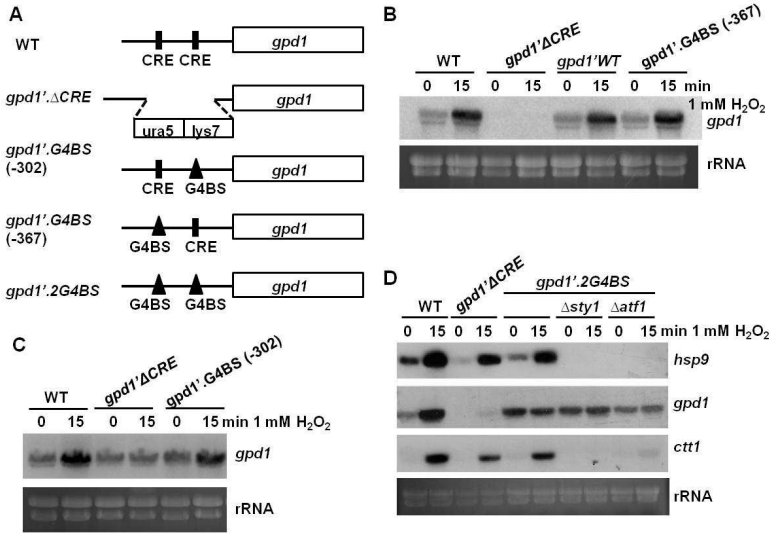

Figure 22: **Two CRE sites substitution by G4BS impaired the expression of *gpd1* gene**. **(A)** Schematic representation of different CRE sites substitution by G4BS. **(B)** Strains WT (972), *gpd1'ΔCRE* (EP143), *gpd1'WT* (EP145) and *gpd1'.G4BS* with second CRE site mutated (EP185) were treated with 1 mM of H_2O_2 at indicated times. Total RNA were extracted and analyzed by Northern blot using probes for *gpd1.* Ribosomal RNA (rRNA) was used as a loading control. **(C)** Strains WT (972), *gpd1'ΔCRE* (EP143), *gpd1'.G4BS* with first CRE site mutated to G4BS (EP255) were treated with 1 mM of H_2O_2 at indicated times. Total RNA were extracted and analyzed by Northern blot using probes for *gpd1.* Ribosomal RNA (rRNA) was used as a loading control. **(D)** Strains WT (972), *gpd1'ΔCRE* (EP143), gpd1'G4BS with both CRE site mutated to G4BS (EP252), gpd1'G4BS with both CRE site mutated to G4BS in Δ*sty1* (EP258) or Δ*atf1* background (EP259) were treated with 1 mM of H_2O_2 at indicated times. Total RNA were extracted and analyzed by Northern blot using probes for *hsp9, gpd1, ctt1 and srx1.* Ribosomal RNA (rRNA) was used as a loading control.

Once we found that changing both CRE sites at the *gpd1* promoter is enough to abolish its stress-dependent transcription, we checked if cells carrying this mutated promoter display any sensitivity to oxidative or osmotic stress. While deletion of the *gpd1* renders cells sensitive to osmotic stress (*gpd1'.ΔCRE*), strain *gpd1'.2G4BS* does not display any relevant phenotype (**Fig. 23**).

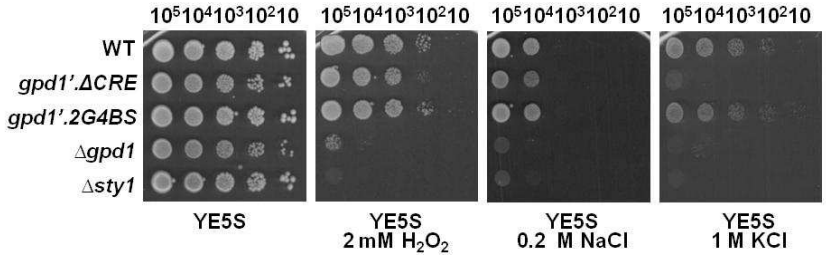

Figure 23: **Sensitivity assay of different *gpd1* mutants**. Strains WT (972), *gpd1'ΔCRE* (EP143), *gpd1'.G4BS* (EP252), Δ*gpd1* (EP199) and Δ*sty1* (AV18) were grown in liquid rich media (YE5S), and the indicated number of cells were spotted onto plates with or without 2 mM of H_2O_2, 0.2 M NaCl or 1 M KCl.

2.2.3. G4-HA-Atf1 and G4-HA-Atf1.ΔbZIP can promote transcription from the *ctt1'.G4BS* promoter in a stress-dependent manner

We express the G4 chimeras in *ctt1'.G4BS* cells (**Fig. 24A-B**). Elimination of the bZIP domain prevented G4-Atf1 to bind to other stress promoters or to other CRE sites.

As expected, both G4-Atf1 and G4-Atf1.ΔbZIP restored the ability of *ctt1'.G4BS* cells to grow on H_2O_2 plates, meaning that induction of *ctt1* was restored, and as previously reported, induction of *ctt1* is enough to sustain growth on H_2O_2 plates [128] (**Fig. 24C**). We then analyzed the induction of *ctt1* gene, and both G4-Atf1 and G4-Atf1.ΔbZIP promote transcription (**Fig. 24D**). These results suggest that tethering G4-Atf1 or G4-Atf1.ΔbZIP to *ctt1'G4BS* promoter is sufficient to recover stress-dependent transcription.

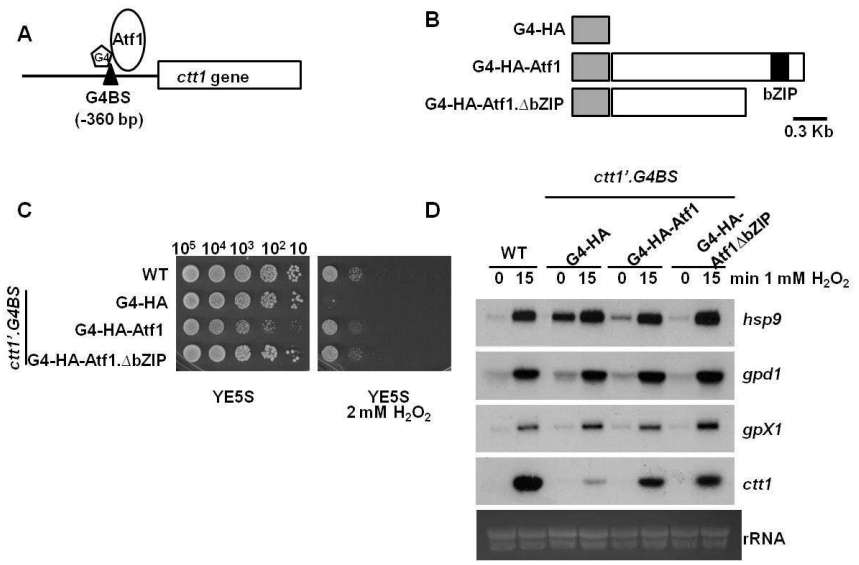

Figure 24: **Induction of *ctt1* is restored when tether artificially G4-Atf1 to *ctt1'.G4BS* promoter in wild-type cells. (A)** Schematic representation of *ctt1'.G4BS* promoter. **(B)** Diagram of vectors carrying the expression of different chimeric proteins **(C)** Cultures of strains WT (972), *ctt1'G4BS pG4-HA* (EP213), *ctt1'G4BS pG4-HA-Atf1* (EP212) and *ctt1'G4BS pG4-HA-Atf1ΔbZIP* (EP212.ΔbZIP) were grown in rich media and spotted in plates containing 2 mM of H_2O_2. **(D)** Same strains as in C were treated with 1 mM of H_2O_2 at indicated times. Total RNA were extracted and analyzed by Northern blot using probes for *hsp9, gpd1, ctt1 and gpx1.* Ribosomal RNA (rRNA) was used as a loading control.

2.2.4. Tethering G4-Atf1 to *gpd1'.2G4BS* promoters allows the induction of the *gpd1* gene in a stress-dependent manner

Once we have checked that *ctt1'.G4BS* promoter is induced when we tether G4-Atf1, we want to analyzed if the same situation occurs in the *gpd1'.2G4BS* promoter, which have both CRE sites substituted by G4BS (**Fig. 25A**). To do that, we transformed *gpd1'.2G4BS* cells with a plasmid expressing G4 or G4-Atf1 proteins (**Fig. 25B**). The transcription of *gpd1* is recovered when we tethered artificially G4-Atf1 to the *gpd1* promoter, and again this transcription was stress-dependent (**Fig. 25C**).

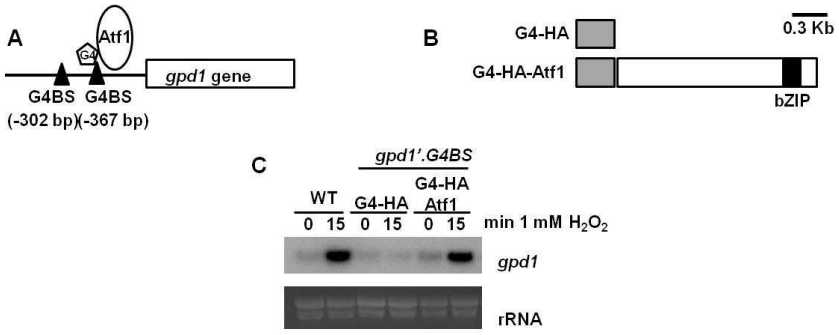

Figure 25: **Induction of *gpd1* is restored when tethering G4-Atf1 to *gpd1'.2G4BS* promoter**. (**A**) Schematic representation of CRE site substitution by a G4BS. (**B**) Representation of both chimeric proteins, which contain the G4 and HA tagged, were fused or not to Atf1 full length (G4-HA-Atf1). (**C**) Cultures of strains WT (972), *gpd1'.2G4BS pG4-HA* (EP262), *gpd1'.2G4BS pG4-HA-Atf1* (EP307) were treated with 1 mM of H_2O_2 at indicated times. Total RNA were extracted and analyzed by Northern blot using probe for *gpd1*. Ribosomal RNA (rRNA) was used as a loading control.

As a conclusion, the induction of *ctt1* and *gpd1* mutant promoters by the G4-Atf1 chimeras is stress-dependent, that is, we need imposition of stress to induce transcription. We next checked if both, G4-HA and G4-HA-Atf1 are bound to promoters prior to stress and if their recruitment is H_2O_2-dependent. As shown in **Fig. 26,** chromatin immunoprecipitation (ChIP) assay showed specific binding of the three G4 chimeras proteins (G4-HA, G4-HA-Atf1 and G4-HA-Atf1ΔbZIP) to *ctt1'.G4BS* or *gpd1'.2G4BS* promoters, and the binding is not increase upon treatment with H_2O_2, suggesting that an event other than recruitment to DNA is occurring in these artificial systems.

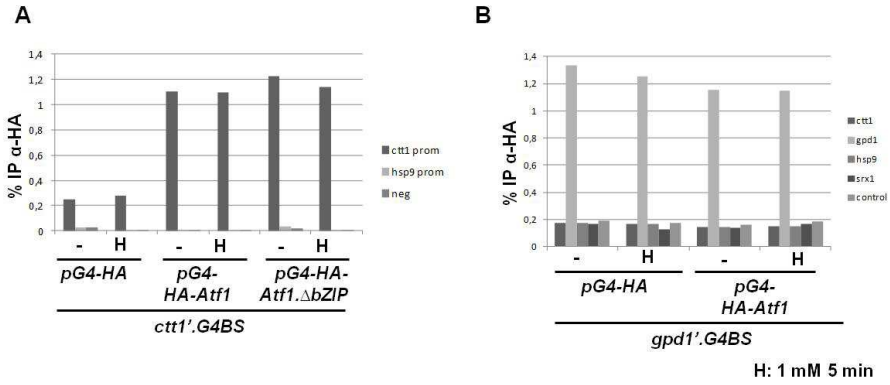

Figure 26: **G4 bound physically to *ctt1'.G4BS* or *gpd1'.2G4BS*.** (**A**) Cultures of strains *ctt1'G4BS pG4-HA* (EP213), *ctt1'G4BS pG4-HA-Atf1* (EP212) and *ctt1'G4BS pG4-HA-Atf1ΔbZIP* (EP212.ΔbZIP) were used to perform ChIP experiments using primers at *ctt1* and *hsp9* promoters. An intergenic region was used as a negative control. (**B**) Cultures of strains *pG4-HA* (EP262), *gpd1'.2G4BS pG4-HA-Atf1* (EP307) were used to perform ChIP experiments as in A, using primers at *ctt1, gpd1, hsp9* and *srx1* promoters. An intergenic region was used as a negative control.

2.2.5. Design and expression of G4-Sty1 chimeras

Regulation of gene expression by MAPK is essential for proper adaptation to extracellular insults. Exposure of fission yeast to various stressors results in rapid activation of Sty1, which coordinates the transcriptional program required for cell survival. The mechanisms by which Sty1 regulates gene expression are not completely understood. HOG1, the homologue of Sty1 in budding yeast, has been found to be associated with some osmostress promoters. HOG1 seems to be recruited to promoters by specific TFs. Once there, HOG1 can recruit more components of the transcriptional machinery, such as Pol II or some histone deacetylases [129-131].

In *S. pombe*, Sty1 has been found recruited to promoters of stress-induced genes where the kinase activity of Sty1 seems to be required for their recruitment [132].

To dissect the role of Sty1 in transcription activation we have several plasmids allowing the expression of G4-FLAG-HA coupled to different mutant versions of Sty1 (**Fig. 27A**), with the aim of tethering artificially the MAPK Sty1 to *ctt1'.G4BS* and *gpd1'.2G4BS* promoters.

First, we have checked the functionality of these chimerics Sty1 proteins transforming a strain lacking *sty1*. All the Sty1 chimeric proteins (G4-Sty1, G4-HA-Sty1 and HA-G4-Sty1) expressing Sty1 were able to rescue sensitivity to peroxides Δ*sty1 cells*. In contrast, the catalytically inactive chimeric protein G4-HA-Sty1.K49R, which harbours a K-to-R mutation in the predicted ATP binding site (Lys-49-Arg replacement) was not able to complement the oxidative stress defects of Δ*sty1* cells (**Fig. 27B**).

We have also generated a chimeric Sty1 harboring an analogue sensitive mutation: the Sty1.T97A [133], fused to the G4-HA tag (G4-HA-Sty1.T97A). The threonine-to-alanine modification in the ATP-binding pocket of the kinase renders a protein with wild-type properties, until the ATP analogue 3MB-PP1 is added to the growth media. This inhibitory analogue can only bind to this mutant kinase, allowing specific inactivation of the modified kinase *in vivo*. As shown in Fig. 28C, addition of 3MB-PP1, specifically blocked the catalytic activity of the mutant MAP kinase Sty1.T97A, and inhibited the phosphorylation and accumulation of Atf1 [134]. All Sty1 chimeric proteins were expressed at same levels, and they were totally functional as determined by monitoring Sty1 and Atf1 phosphorylation by Western Blot (**Fig. 27 C-D**).

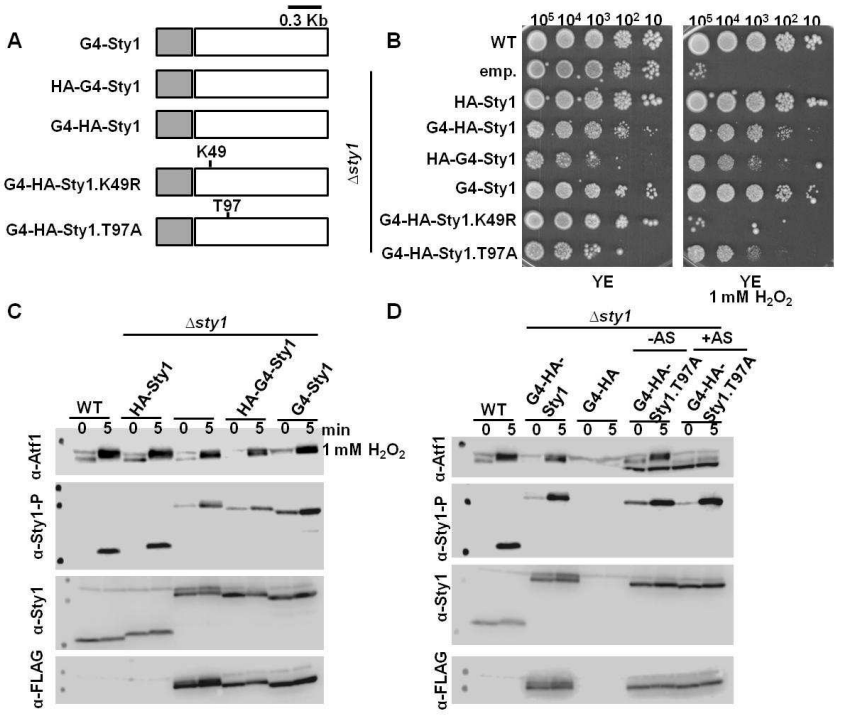

Figure 27: Expression of Sty1 chimeric proteins in Δ*sty1* cells. (A) Schematic representation of G4-Sty1 proteins. **(B)** Liquid cultures of strains WT (972), Δ*sty1* + emp. (EP312), Δ*sty1* + HA-Sty1 (EP313), Δ*sty1* + G4-HA-Sty1 (EP314), Δ*sty1* + HA-G4-Sty1 (EP315), Δ*sty1* + G4-Sty1 (EP316), Δ*sty1* + G4-HA-Sty1.K49R (EP314.K49R) and Δ*sty1* + G4-HA-Sty1.T97A (EP314.T97A), were grown in rich media and spotted in plates containing 1 mM H_2O_2. **(C-D)** TCA extract of same strain as in A were left untreated or treated with 1 mM H_2O_2 of during 5 min, and were analyzed by Western blot with antibodies against Atf1, Sty1-P, Sty1 and FLAG.

2.2.6. Binding of G4-Sty1 to *G4BS*-containing *ctt1* and *gpd1* promoters

Once we have analyzed that tethering G4-Atf1 to *ctt1* and *gpd1* promoters was enough to trigger transcription and allow survival, we wanted to test if Sty1 was capable too. To do that we have transformed strains carrying both mutated promoters, *ctt1'.G4BS and gpd1'.2G4BS*, with the chimeric plasmids allowing the expression of the G4-Sty1 chimeras.

We have shown before that G4-Atf1 chimeric protein rescues the phenotype of strain *ctt1'.G4BS* and recovers the induction of the *ctt1* gene. The G4-Sty1 chimeric protein recovered the ability to growth upon H_2O_2 plates (**Fig. 28A**). Protein expression of both chimeras was slightly different (**Fig. 28B**) but the induction of *ctt1* was restored by both chimeras to the same extend (**Fig. 28C**). These results suggest that tethering G4-Sty1 to *ctt1'.G4BS* promoter is sufficient to promote the *ctt1* transcription.

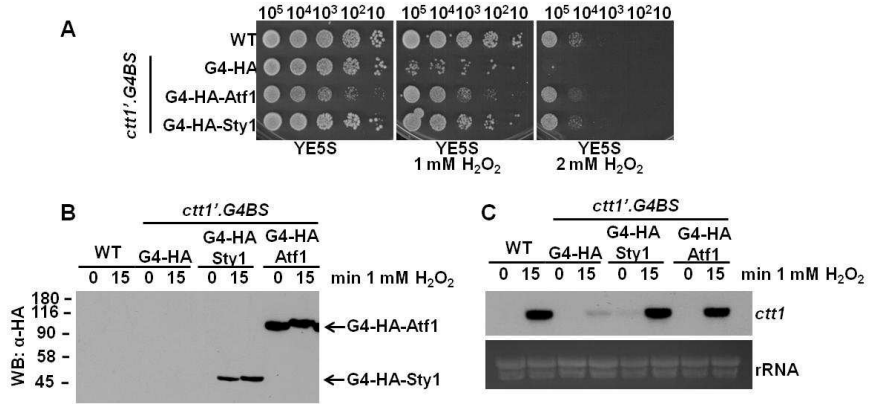

Figure 28: G4-Sty1 chimera is able to restore *ctt1* induction of *ctt1'.G4BS* promoter as the G4-Atf1 does. (A) Cultures of strains WT (972), *ctt1'G4BS pG4-HA* (EP213), *ctt1'G4BS pG4-HA-Atf1* (EP212) and *ctt1'G4BS pG4-HA-Sty1* (EP215) were grown in rich media and spotted in plates containing 1 or 2 mM H_2O_2. **(B)** Cultures of strains as in A were treated 1 mM H_2O_2 during 15 minutes. TCA extracts were loaded in a gel and were analyzed by Western blot with antibodies against HA. **(C)** Same strains as in A were treated with 1 mM of H_2O_2 at indicated times. Total RNA were extracted and analyzed by Northern blot using probes for *ctt1.* Ribosomal RNA (rRNA) was used as a loading control.

Regarding the *gpd1'.2G4BS* promoter we have transformed the strain *gpd1'.2G4BS* with a plasmid expressing G4-HA, G4-Sty1, G4-HA-Sty1 or HA-G4-Sty1 proteins. The transcription of *gpd1* is recovered when we tethered artificially any of the chimeric Sty1 proteins to the *gpd1* promoter (**Fig. 29**).

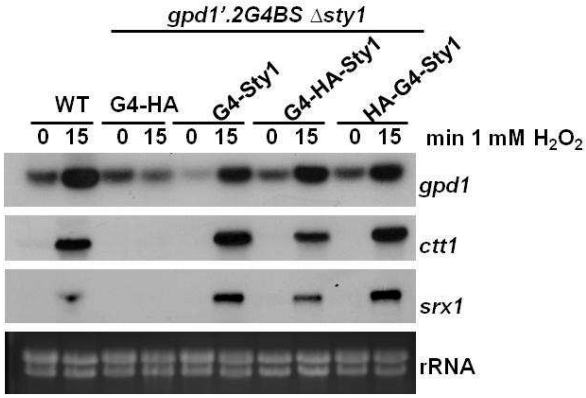

Figure 29: Tethering of different G4-Sty1 chimeras to the *gpd1'.2G4BS* promoter. Stress transcriptional analysis of strains WT (972), *gpd1'.2G4BS* + G4-HA (EP262), *gpd1'.2G4BS* + G4-Sty1 (EP264), *gpd1'.2G4BS* + G4-HA-Sty1 (EP272) and *gpd1'.2G4BS* + HA-G4-Sty1 (EP271), treated or not with 1 mM H_2O_2 for 15 min. Total RNA was analyzed by Northern blot with probes for *gpd1, ctt1* and *srx1*. rRNA is shown as loading control.

3. Antioxidant cell responses

Up to 500 genes are up-regulated in a Sty1- and Atf1-dependent manner upon H_2O_2 stress, and the specific role of their gene products in the antioxidant cellular defense is unknown. During the course of this thesis project, we have studied two aspects related to the antioxidant cell response. First, we have demonstrated the essential role of catalase over-expression in the development of tolerance to extracellular peroxides (see section 3.1). Second, we have investigated whether H_2O_2 stress participate in iron homeostasis (see section 3.2).

3.1. Characterization of *S. pombe* H_2O_2 scavengers

Previously in the laboratory, the Prx Tpx1 was characterized as the main H_2O_2 scavenger in aerobic conditions, because cells lacking Tpx1 are not

able to grow on plates under aerobic conditions. We found that the deletion of the gene coding for thioredoxin reductase, *trr1*, is a suppressor of the sensitivity to aerobic growth of Δ*tpx1*. We show in this results section, presented as a published manuscript, that deletion of *trr1* suppresses the anaerobic phenotype of Δ*tpx1* cells, due to the constitutive activation of Pap1, which triggers the expression of up to 80 genes, including catalase (17-fold increase). We demonstrate that overexpression of Ctt1 is sufficient to allow the growth of Δ*tpx1* cells under an aerobic atmosphere.

Moreover, we also show that overexpression of catalase is sufficient to rescue, totally o partially, respectively, the sensitivity to peroxides of Δ*atf1* or Δ*pap1* cells, even though these two TFs are able to trigger many different genes in response to peroxides.

Paulo E, García-Santamarina S, Calvo IA, Carmona M, Boronat S, Domènech A, Ayté J, Hidalgo E. A genetic approach to study H2O2 scavenging in fission yeast--distinct roles of peroxiredoxin and catalase. Mol Microbiol. 2014 Apr;92(2):246-57. doi: 10.1111/mmi.12548.

3.2. Cross-induction of the iron depletion regulon by oxidative stress

Iron represents a paradox: on one hand it is a vital micronutrient for all living organisms and on the other hand an excess of iron may be toxic due to its ability to mediate Fenton reactions in combination with H_2O_2. As shown before in other cell types refs***, we hypothesized that there should exist a regulated cross-talk between the oxidative stress pathway and the iron regulon in *S. pombe* to impede the synergistic toxicity between ROS and iron, as exists for others microorganisms.

In *S. pombe,* the iron starvation response has been characterized by the group of Simon Labbe [63]. Briefly, the inactivation of Fep1 and activation of Php4 transcriptional repressors mediate the cellular response to iron deficiency [135]. Thus, when iron is limiting Fep1 is released from promoters of genes involved in iron uptake [62], while Php4 accumulates at the nucleus and represses transcription of genes coding for iron-consuming or iron-storage proteins [62]. Upstream of these transcriptional repressors is the glutaredoxin Grx4, which seems to be the real sensor of iron deprivation, probably through an iron-sulfur center [63, 136] (**Fig. 30**).

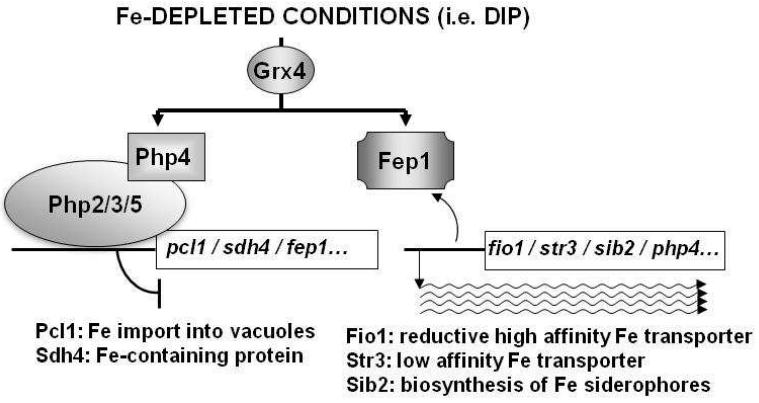

Figure 30: Scheme depicting the iron starvation regulon in *S. pombe.* Iron starvation triggers down-regulation of genes coding for iron storage and iron-containing proteins (in a Php4 repressor-dependent manner), and up-regulation of genes coding for iron uptake (in a Fep1 repressor-dependent manner).

Previous work in the lab demonstrated that, unexpectedly, H_2O_2 triggers the transcriptional iron-starvation response, promoting iron import and decreasing iron usage (**Fig. 31**). Sty1 and Atf1 are required for this activation.

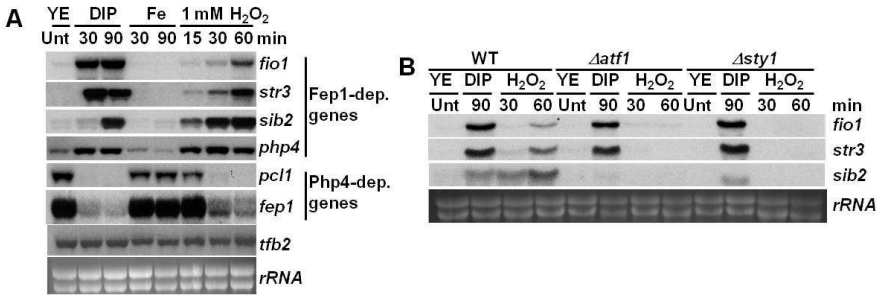

Figure 31: The iron-starvation response is activated by H_2O_2 in a Sty1-Atf1 dependent manner. (A) Total RNA from strain 972 (WT) was obtained from cultures growing in YE treated or not with 250 µM DIP, 100 µM of $FeCl_3.6H_2O$ (Fe) or 1 mM H_2O_2 at indicated times. Northern blot membranes were hybridized with the [α-^{32}P] dCTP-labelled *fio1, str3, sib2, php4, pcl1, fep1*probes containing the complete ORF. tubulin gene (*tfb2*) and ribosomal RNA were analyzed as loading controls. **(B)** The H_2O_2–driven activation of the iron starvation gene response relies on Sty1 and Atf1. Same as in A, but using RNA from strains 972 (WT), AV15 (Δ*atf1*) and AV18 (Δ*sty1*).

As will be shown here, we suspect that upon oxidative stress conditions, the over-expression of the heme-containing catalase in combination with the toxic effect of H_2O_2 in iron-containing proteins deprives all the intracellular available iron and this triggers the induction of the whole iron-starvation pathway. The late activation of iron import upon H_2O_2 stress contributes to growth resumption.

3.2.1 Over-expression of the iron-containing catalase as part of the H_2O_2-dependent stress response may contribute to iron depletion

We speculated that one or several of the stress gene products would contribute to trigger an iron deprivation situation by sequestering most of the intracellular available iron. One of the genes most highly expressed by H_2O_2, *ctt1*, encodes the heme-containing catalase (**Fig. 32**). Indeed, the gene is up-regulated in a Sty1- and Atf1- dependent manner by any environmental situation tested, but reaches its maximum fold-induction, 35-fold, only upon high doses of peroxides [43]. We tested our hypothesis by analyzing whether cells lacking ctt1 would induce the iron starvation response after H_2O_2 exposure. Induction of genes such as *fio1* or *sib2*, or down-regulation of *pcl1* by H_2O_2 was abolished in Δ*ctt1* cells (**Fig. 32**).

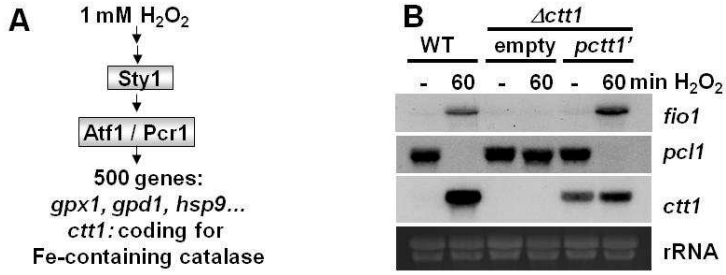

Figure 32: Over-expression of catalase after H_2O_2 stress causes activation of the iron regulon. (**A**) Scheme of MAPK Sty1 pathway showing that more than 500 genes are induced upon 1 mM of H_2O_2. (**B**) H_2O_2 does not trigger the iron starvation response in cells lacking *ctt1*. Northern blot analysis as in Figure A, but with RNA from strains 972 (WT), EP197 (Δ*ctt1* empty) and EP231 (EP198 carrying the integrative plasmid p422'; Δ*ctt1 pctt1'*).

Catalase is highly over-expressed upon treatment of cells with extracellular H_2O_2, and our finding suggests that at least upon peroxide exposure the protein sequesters most of the available iron. This may jeopardize cell recovery, since many proteins may have lost their iron as a

result of H_2O_2 reactivity, which in combination with depletion of available iron by catalase may invoke the iron deficiency response. We rationalized that even in the absence of H_2O_2 stress, expression of the catalase gene could be a target of iron starvation- and Php4-dependent down-regulation, as previously reported for other metallo-proteins such as aconitase, succinate dehydrogenase or sulfite reductase. A previous report of the transcriptome of cells subjected to the iron chelator DIP [137] demonstrated than *ctt1* is among the genes down-regulated by this stress in a Php4-dependent manner. We also show here by Northern blot that the basal levels of *ctt1* are repressed by DIP (**Fig. 33A**). Furthermore, our chromatin immuno-precipitation analysis demonstrates that the Php4 repressor is recruited to the *ctt1* promoter upon DIP treatment as it is to the well characterized Php4 target *pcl1* promoter (**Fig. 33B**), decreasing the presence of the large subunit of RNA polymerase II, Rpb1, at both promoters (**Fig. 34C**). As expected, Php4 recruitment and Rpb1 release are both dependent on the presence of the iron deprivation sensor Grx4 (**Fig. 34B, C**).

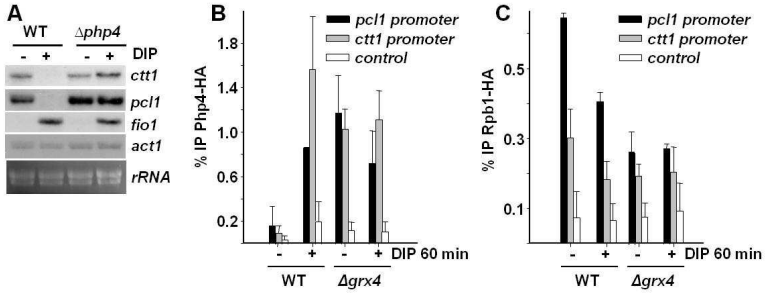

Figure 33: Expression of *ctt1* is down-regulated upon iron starvation in a Php4-dependent manner. (A) The mRNA of *ctt1* is down-regulated by Php4 after the addition of DIP. Total RNA from 972 (WT) and NG40 (Δ*php4*) strains was obtained from cultures growing in YE treated or not with 250 µM DIP during 90 min, and analyzed as described in Figure 1B. **(B)** Php4 is recruited to *ctt1* and *pcl1* promoters upon iron starvation. Strains NG123 (Php4-HA WT) NG131 (Php4-HA Δ*grx4*), expressing Php4-HA, were cultured and treated (+) or not (−) with 250 µM DIP during 60 min. ChIP experiments using anti-HA antibodies, coupled to quantification by real-time PCR, were performed using primers covering only promoter regions (*ctt1* and *pcl1*). Primers of an intergenic region were used as a negative control (control). Error bars (SEM) were calculated from biological duplicates. **(C)** Same as in B, but using strains NG129 (Rbp1-HA WT)

and NG135 (Rbp1-HA Δ*grx4*). ChIP experiments were done by Javier Encinar del Dedo.

Our experiments suggest that oxidative stress triggers a situation of iron deprivation, probably as a consequence of both toxic and signaling events: oxidation of free and protein-bound iron, and over-expression of the iron-consuming catalase. Toxic doses of extracellular peroxides, such as 1 or 2 mM, are able to transiently halt the growth of *S. pombe* rich media cultures and delay growth resumption by 3-6 hours, respectively. This toxicity exerted by H_2O_2 is partly due to the inactivation of metabolically essential iron-sulfur enzymes, as described in *E. coli* [138]; these clusters will have to be repaired to accomplish cell resumption. We speculated that the synthesis of proteins such as the heme-containing catalase may partially deplete the pool of labile available iron, so that an extra burst of iron import would be required to repair the pool of damaged iron-containing proteins. To test this hypothesis, we decided to limit iron import after a burst of oxidative stress, with the expectation that it may exacerbate the time lag for growth resumption after peroxide exposure. Since there are multiple alternative pathways to promote iron entry (see Introduction), the use of mutants lacking one specific importer would not avoid iron entry, and mutant carrying multiple deletions in alternative iron import genes have not been described. We therefore largely limited the pool of extracellular iron by using different concentrations of the iron chelator DIP, which did not significantly affect the growth of *S. pombe* cultures. However, these concentrations of DIP impaired cell recovery after H_2O_2 stress in a concentration-dependent manner (data not shown). This result indicates that part of the toxicity associated to peroxide stress causes a transient iron deprivation, and that activation of iron import is required to facilitate growth resumption.

DISCUSSION

In our laboratory we work trying to elucidate signaling processes mediated by ROS. Thus, when we add H_2O_2 to the cells, two signal transduction pathways are induced in response to moderate doses (Pap1 pathway) or high or toxic doses (Sty1 pathways) of peroxides.

Both MAPKKK, Wis4 and Win1, are essential for CESR gene induction but not for tolerance growth to peroxides

The MAPK Sty1 is largely known to be essential in several cellular programs such as environmental stress defense, mating, stationary phase survival, meiotic recombination and normal cell cycle progression. Indeed, cells lacking Sty1 are sensitive to a wide range of stresses: oxidative and osmotic stress, heat shock, stationary phase, etc. How are many different stress stimuli transmitting to a single MAPK? Two possible explanations may be considered: i) multiple receptors with different stress specificity converge into the same MAPK; ii) all stress conditions are sensed by the same receptor which is in connection with the MAPK.

The Sty1 pathway is composed by a His-to-Asp phosphorelay system which acts upstream, and by a MAPK module composed by the MAPKKKs Wis4 and Win1, the MAPKK Wis1 and the MAPK Sty1. Upon a stress situation, Sty1 is phosphorylated by Wis1, and migrates to the nucleus, where it regulates the initiation of alternative gene expression programs to cope with these new conditions. Some reports have described a different role of each MAPKKK depending on the stress suffered. Indeed, the MAPKKK Wis4 has been involved in osmotic-stress signal, thus cells lacking *wis4* are highly defective in osmotic-stress induced by Sty1 [139]. Moreover, it has been reported that Mcs4 acts as a response regulator protein transmitting the oxidative stress signals to Sty1 pathway promoting their association with both MAPKKKs [57 {Morigasaki, #87, 114, 140]}. We have demonstrated that both Wis4 and Win1 MAPKKK are required for robust response to oxidative and osmotic stress, thus deletion of both proteins are defective on Sty1 activation upon

both stresses. However, both MAPKKK are essential for proper transcriptional induction of CESR genes but not for normal tolerance to peroxides on plates. We speculate that the basal levels of *ctt1* could be higher in cells deleted in *wis4* or *win1* compared to cells deleted in *sty1* or *wis1* and, this basal expression of Ctt1 may be enough to restore grow under peroxides. As will be fully described below, expression of Ctt1 is the most important factor governing tolerance to peroxides.

Characterization of *S. pombe* phosphatases

While 5-6 MAPK pathways have been described in *S. cerevisiae*, three distinct MAPK pathways have been identified in *S. pombe*: the cell integrity (Pmk1) [141], the pheromone signaling (Spk1) [121] and the SAPK (Sty1) pathways [80, 118]. The fission yeast MAPK pathways are tightly connected with each other. For instance, the Pmk1 and the Sty1 pathways share the negative regulators Pyp1, Pyp2 and Ptc1, and they also target the same TF Atf1 to regulate gene expression. Although the stress signal is most often related to the Sty1 activation through MAPKKKs, there are some evidences that postulate Sty1 activation independent of the MAPK module. It has been shown that upon cadmium or low glucose treatments, Sty1 is activated bypassing the MAPKKKs through an oxidation-dependent inhibition of the Pyp1 phosphatase, which dephosphorylates Sty1 under normal growth condition [142]. Furthermore, upon heat stress Sty1 is activated due to the inhibition of Pyp1 and presumably Pyp2 [81]. On the other hand, osmotic and oxidative stress signals from the phosphorelay system are related to downstream proteins of the MAPK pathway in a MAPKKK-dependent manner [114, 143].

In this thesis work we have confirmed that Pyp1 is the main negative regulator of Sty1 under basal conditions, since deletion of this phosphatase leads to constitutive activation of Sty1, regarding basal Sty1 phosphorylation, resistance to H_2O_2 on plates and basal induction of CESR genes prior to stress. As expected, this constitutive activation of the pathway is Wis1-and Sty1-dependent, indicating that both MAPKK

and MAPK are required for normal stress induction. According to the literature and to our data, Wis1 is the only kinase of Sty1, since deletion of *pyp1* in cells lacking Wis1 does not trigger the antioxidant response. We aimed to test if deletions of the triple MAPKKK in combination with deletion of *pyp1* display the same phenotype. Surprisingly, triple knockout strain, Δ*wis4* Δ*win1* Δ*pyp1*, show basal activation of the Sty1 pathway as the same extends as Δ*pyp1* cells does. The fact that Sty1 could be activated in cells lacking both MAPKKKs confirms that Sty1 pathway activation may be also due to the inactivation of their main negative regulator Pyp1. Which may be the kinase phosphorylating Wis1 and therefore Sty1 in this triple Δ*wis4* Δ*win1* Δ*pyp1* mutant? We first speculated that other MAPKKKs (i.e. Mkh1 or Pek1) could be able to phosphorylate Wis1 in the absence of its natural kinases. However, that was not the case. Further work will be required to identify which kinase can exert this role.

Identification of Sty1 interactors

At the beginning of this thesis work, our main objective was to understand the sensing and signaling events that may occur in the Sty1 pathway upon oxidative stress. We used a proteomic approach that combines affinity purification with anti-HA antibody followed by analyzing the purified protein by mass spectrometry using dimethyl labeling quantification. We were able to purify Sty1 in a wild-type and in a Δ*pyp1* strains, and we indentified many peptides belonging to the CESR genes. Among the putative interactors identified, we isolated some known Sty1 substrates, such as Cdc37 [144] or Srk1 [145]. We analyzed the enrichment of peptides in total extracts and in immunoprecipitation, to distinguish the genes up-regulated due to the strains compared to the real substrates. However, almost all peptides found were enriched in total extracts and in the immunoprecipitation, suggesting that this method is not good enough to identify substrates.

Atf1 and Sty1

The MAPK Sty1 regulates the transcriptional responses promoting cell survival triggered by different environmental stresses in *S. pombe*. Upon stress activation, Sty1 migrates to the nucleus, where it stimulates a gene expression program through the Atf1 TF. It remains still unclear the role of Sty1 and Atf1 in transcription regulation. Moreover, the phosphorylation sites of Atf1 crucial for its function are still unknown. In this Thesis we provide new evidences of the characterization of Sty1 and Atf1 upon environmental stress in the regulation of the expression of CESR genes.

First of all, cells expressing full length or the bZIP of Atf1, a truncated form of the TF lacking two-thirds of the protein and unable to trigger transcription of *gpd1* or *hsp9*, were able to trigger the activation of *ctt1* and *srx1*, which were under the control of Atf1 and Pap1. An important conclusion of these experiments is that wild-type expression of *ctt1* and *srx1* genes is sufficient to restore tolerance to oxidative stress, but not to osmotic stress. The bZIP truncated form of Atf1 has a putative phosphosite for Sty1, Ser438. According to our results, phosphorylation of Ser438 is not required for the function of the TF Atf1. Moreover, the bZIP domain of Atf1 alone is able to displace histones at promoters and to maintain an opened chromatin structure. We speculate that binding of the bZIP to promoters which open the chromatin structure, may facilitate Pap1 binding to *ctt1* and *srx1* promoters (Patricia Garcia and Elena Hidalgo, unpublished data).

Atf1 is a TF with eleven Sty1 phosphorylation sites. It has been reported by the group of Nic Jones that an Atf1 mutant lacking all 11 MAPK phosphorylation sites, called Atf1-11M, has a similar activity than a wild-type protein. They also show that the non-phosphorylatable mutant is very unstable, and they point out that the main role of Sty1-dependent phosphorylation is to stabilize Atf1 rather than to activate it [84]. In an attempt to analyze the role of this different MAPK phosphosites, we have generated different plasmids expressing different phosphorylation mutants of Atf1 mimicking the hyper-phosphorylated or hypo-phosphorylated form.

Importantly, these mutant forms are all expressed from a constitutive promoter and are HA-tagged. Surprisingly, we have found that cells expressing an Atf1.10M mutant, with 10 SP-TP mutated to AP or IP, respectively, or cells expressing an Atf1.10D mutant, with 10SP-TP mutated to DP or EP, respectively, have same protein levels as wild-type Atf1, suggesting that protein levels are not related with phosphorylation, but rather to mRNA expression (the endogenous *atf1* gene is also regulated upon stress in a Sty1- and Atf1-dependent manner Obviously, more experiments need to be done to clarify role of Atf1 phosphorylation by Sty1 on the activity of the transcription factor.

Artificially tethering of G4-Atf1 to G4BS-containing stress promoters restores gene induction

To further investigate the role of Atf1 and Sty1 in transcriptional activation, we decided to design a heterologous system based on replacing the CRE site of *gpd1* and *ctt1* promoters by a GAL4 (G4) binding site (G4BS). We checked first that cells expressing the mutated versions of these two promoters do not display any induction of the corresponding gene. We choose those two promoters because it seemed obvious than *ctt1* and *gpd1* represented the two types of Atf1-dependent genes: (i) those dependent on both Atf1 and Pap1 (i.e. *ctt1*); and (ii) those dependent only on Atf1 (i.e. *gpd1*).

We then constructed two plasmids allowing the expression of the HA-tagged G4 alone or G4 fused to Atf1. We then expressed both proteins in cells with the CRE site located at *gpd1* and *ctt1* promoters replaced by the G4BS. Using this heterologous system we have been able to discard the effect of Atf1-to-DNA binding in transcription activation, thus the chimeras are constitutively bound to the mutated promoters.

Cells deleted in the *ctt1* promoter or expressing the mutated version CRE-to-G4BS of the *ctt1* promoter are extremely sensitivity to H_2O_2. These cells have only affected the induction of *ctt1*, but not other CESR genes. We can conclude that catalase plays a critical role in

scavenging high doses of H_2O_2. The induction of the *ctt1* gene was restored when we artificially tethered the TF G4-Atf1 or G4-Atf1.ΔbZIP, lacking the bZIP domain, and this induction is stress-dependent. These results demonstrated that recruitment of the Atf1 TF to the *ctt1* promoter is not important for its H_2O_2-dependent activation.

We have also generated a *gpd1* mutated promoter; however, in this case, we had to mutate two putative CRE sites to abolished *gpd1* induction upon peroxides. Moreover, cells carrying this mutated promoter do not display any sensitivity defect upon oxidative or osmotic stress. The induction of *gpd1* upon H_2O_2 was restored in the presence of the G4-Atf1 chimera, and this transcription was again stress-dependent.

With these two mutated promoters we conclude that binding of the G4-Atf1 chimera is not sufficient to induce a basal activation of gene expression, thus, we need to impose stress to induce transcription. Upon stress Sty1 translocates into the nucleus. We can speculate that Sty1 may play a role phosphorylating Atf1 or recruiting some components of SAGA complex or RNA Pol II, necessary to induce transcription. Again, recruitment of the TF does not seem to be an issue in CESR activation.

The mechanisms by which Sty1 regulates gene expression are not totally understood. In *S. pombe*, Sty1 has been found recruited to stress promoters [132] and its kinase activity have been shown to be crucial for proper gene induction. In order to dissect the role of Sty1 in transcription initiation we have generated a G4-Sty1 chimera. This chimera is able to complement a *Δsty1* strain and, furthermore, is able to allow induction in *ctt1'.G4BS* and *gpd1'.2G4BS* promoters. That system will permit to dissect the role of Sty1 in gene induction.

Tpx1, the main scavenger of endogenous H_2O_2

Previously in the laboratory, Tpx1 was described as a dual-role peroxiredoxin in fission yeast. Thus, Tpx1 was described as a H_2O_2 sensor in the antioxidant pathway, and as a scavenger of H_2O_2 during

aerobic growth, so cells lacking Tpx1 are not able to grow on solid plates unless they are incubated under anaerobic conditions.

Curiously, we found in the lab that deletion of *trr1* in Δ*tpx1* cells allows them to grow in aerobic conditions. We have already mentioned that Trr1 is involved in Pap1 reduction, so deletion of the *trr1* gene results in a constitutive oxidation and nuclear localization of Pap1, triggering the induction of 80 genes, many of them related to the antioxidant response program. One of the genes up-regulated in ∆*trr1* is *ctt1,* whose transcription is induced 17 fold. Taking these results together we decided to analyze if the constitutive activation of Pap1 pathway was responsible of this suppression phenotype. As expected, Δ*tpx1* Δ*trr1* Δ*pap1* cells were again unable to grow on solid plates under aerobic conditions, suggesting than one or several Pap1-dependent genes could be complementing the aerobic phenotype of ∆*tpx1* cells. Unfortunately, the triple ∆*trr1* ∆*tpx1* ∆*ctt1* mutant is not viable. Alternatively, we checked the effect of over-expressing Ctt1 in Δ*tpx1* cells. In fact, high levels of catalase are sufficient to suppress the aerobic growth defects in Δ*tpx1* cells.

In *S. pombe* there are other three putative peroxide scavengers: the glutathione peroxidase Gpx1, and the peroxiredoxins Pmp20 and BCP. However none of them seem to be important to scavenge endogenous and exogenous H_2O_2. Only the combination of different genes deletions, such as *Δtpx1 Δgpx1 Δpmp20 ΔBCP*, enhances the growth defects of Δ*tpx1* cells. Only Gpx1 has a minor secondary role when extracellular peroxides are added. A strain lacking *ctt1* and *tpx1* show severely impaired growth solid plates even under anaerobic conditions. This result suggests that, in the absence of Tpx1, there is an increase of endogenously generated H_2O_2 which may be detoxified by Ctt1. However, when both enzymes are deleted, cells cannot grow even under anaerobic conditions. These results show that Tpx1 and Ctt1 are the main H_2O_2 scavengers in fission yeast.

Aerobic organisms express a battery of antioxidant activities to counteract the toxic effects of ROS. Our experiments demonstrate that

the peroxiredoxin Tpx1 is the first line of defense to control the H_2O_2 generated due to the aerobic growth, while catalase has a major role controlling high levels of peroxides.

In *E. coli*, the main peroxiredoxin AhpC was demonstrated to act as the principal scavenger of the endogenous H_2O_2 generated inside the cell. When this activity is not present, the high H_2O_2 levels are able to directly induce the OxyR regulon. One of the genes induced by OxyR is the catalase gene. The ability of *E. coli* to grow under aerobic conditions when the activity of AhpC is not present is due to an OxyR-dependent overexpression of catalase [146]. In the case of *S. pombe*, Δ*tpx1* cells are not able to increase the levels of catalase, since catalase expression is dependent on the TF Pap1 and Tpx1 is required for Pap1 activation [147].

Catalase, the essential scavenger of severe oxidative stress

The *S. pombe* genome codes for a single catalase. This enzyme is activated as a consequence of increased levels of peroxides. Therefore, moderate H_2O_2 concentrations activate the Tpx1-Pap1 pathway, which triggers the expression of 80 genes, one of them being *ctt1* [44]. Higher doses of H_2O_2 hyper-oxidize Tpx1, temporarily blocking the Pap1 transcription response, but increasing the activation of the MAPK Sty1 and its TF Atf1. This pathway is able to activate the transcriptional response of over 300 genes, being one of the most induced *ctt1*.

This H_2O_2-dependent induction of catalase highlights the importance of this enzyme for the detoxification of high levels of peroxides. Overexpression of *ctt1* complements oxidative stress sensitivity to peroxides of cells lacking *atf1* or *pap1*, even though these two pathways are able to trigger many genes in response to peroxides.

It could be a good strategy to increase the levels of catalase for the tolerance to oxidative insults. However, having high levels of catalase may be dangerous for the cells due to absence of H_2O_2 required for

signaling processes, and to induction of the iron response, probably by sequestering available iron during the reconstitution of the heme group. Furthermore, the catalytic mechanism of H_2O_2 detoxifications by catalase indicates that low levels of H_2O_2 may induce irreversible oxidation of the enzyme.

It is important to remark the importance of catalase as an iron reservoir, its expression levels seem to modulate the free available iron pool.

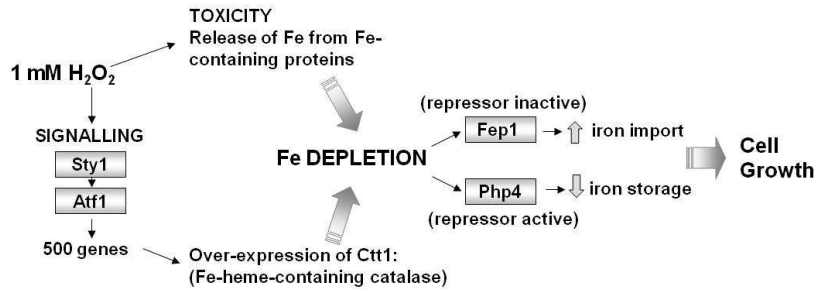

Figure34: Proposed model for a crosstalk between the H_2O_2 toxicity and the activation of the iron regulon in fission yeast. 1 mM H_2O_2 stress causes toxicity partly due to the oxidation and disassembly of iron from Fe-containing proteins. In parallel, those doses of peroxides activate the Sty1/Atf1 pathway, which promotes massive changes in the gene expression program. Over-expression of catalase, a heme containing protein, sequesters most available iron. This iron depletion is sensed by Grx4 which releases Fep1 from promoters to promote iron import and activates Php4 to the block iron storage. This will allow growth resumption.

As shown in **Fig. 34**, we propose that H_2O_2 stress may exert part of this toxicity by decreasing the concentration of available iron. This, in combination with the use of iron to "fill" the newly synthesized catalase may deplete intracellular iron and activate the response to iron depletion. Iron entry would then be required to allow growth resumption after H_2O_2 stress.

Another indication of the critical role of catalase in scavenging high doses of H_2O_2 comes from the fact that cells deleted in the *ctt1* ORF or in the promoter of *ctt1* display high sensitivity to H_2O_2. Moreover, the change of the CRE site to G4BS makes cells sensitive to peroxides too, despite the fact that the basal levels of *ctt1* mRNA from this new promoter

are quite similar to those arising from the wild-type, CRE-containing *ctt1* promoter (see Fig. 21).

CONCLUSIONS

1. Wis4 and Win1 play redundant roles upon oxidative stress.
2. Both Wis4 and Win1 are required for proper tolerance to oxidative and osmotic stress, for Sty1 activation and for transcriptional induction of CESR genes.
3. Pyp1 is the phosphatase responsible to kept Sty1 dephosphorylated under basal conditions.
4. Constitutive activation of Sty1 in cells deleted in *pyp1* is Wis1- and Sty1- dependent.
5. The constitutive activation of Sty1 in Δ*pyp1* cells is maintained when we deleted both MAPKKKs
6. Mkh1, the cell integrity kinase, is not involved in the hyperactivation of Sty1 in the triple mutant Δ*wis4* Δ*win1* Δ*pyp1.*
7. Atf1 and Sty1 are necessary to allow growth under a variety of stress conditions
8. DNA-binding domain of the Atf1 bZIP is able to recover the sensitivity on H_2O_2 plates
9. Wild-type expression of *ctt1* and *srx1* genes is sufficient to restore tolerance to oxidative stress, but not to osmotic stress.
10. Phosphorylation of Atf1 by Sty1 does not promote the stabilization of the TF.
11. Constitutively bound G4-Atf1 triggers the expression of CESR genes in a stress-dependent manner.
12. While Tpx1 is required for basal detoxification of peroxides, catalase is better suited for scavenging high doses of H_2O_2.
13. Upon oxidative stress, cells suffer an iron starvation response as a consequence of oxidative damage and catalase expression.
14. Growth resumption after oxidative stress requires iron import.

MATERIALS AND METHODS

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

1. STRAINS

Strains origins from published results are described in the same papers. The rest of strains and plasmids are described in the tables above.

Table I: Strains from Results 1

Name	Genotype	Origin
972	*h⁻*	[148]
AV18	*h⁻ sty1::kanMX6*	[149]
EP37	*h⁻ wis4::kanMX6*	This work
EP39	*h⁺ win1::kanMX6*	This work
EP42	*h⁻ sty1:HA::kanMX6*	This work
EP43	*h⁺ wis4::natMX6 win1::kanMX6*	This work
EP44	*h⁻ wis1::kanMX6*	This work
EP45	*h⁻ pyp1::natMX6 sty1::kanMX6*	This work
EP48	*h⁺ pyp1::natMX6*	This work
EP49	*h⁻ sty1:HA::kanMX6 pyp1::natMX6*	This work
EP62	*h⁺ pyp1::natMX6 wis4::kanMX6*	This work
EP63	*h⁻ pyp1::natMX6 win1::kanMX6*	This work
EP67	*h⁺ wis1::kanMX6 pyp1::natMX6*	This work
EP70	*h⁺ leu1-32 wis4:.kanMX6*	This work
EP72	*h⁻ mkh1::bleMX6*	This work
EP74	*h⁹⁰ wis4:.kanMX6 win1:.natMX6 pyp1::hphMX6 mkh1::bleMX6*	This work
EP98	*h⁻ ptc2::kanMX6*	This work
EP99	*h⁻ ptc3::kanMX6*	This work
EP103	*h⁺ pyp2::natMX6*	This work
EP110	*h⁻ hrp3::kanMX6*	This work
EP127	*h⁇ ptc4::kanMX6*	This work
EP140	*h⁻ leu1-32 ura4-D18 rds1::natMX6*	This work
EP180	*h⁻ tor1::natMX6*	This work
EP191	*h⁇wis4::kanMX6 win1::natMX6 pyp1::hphMX6 tor1::bleMX6*	This work

NG1	*h⁻ fep1::kanMX6*	This work
IV69	*h⁻ hrp1::kanMX6*	This work

Table II: Strains from Results 2

Name	Genotype	Origin
972	*h⁻*	[150]
AV18	*h⁻ sty1::kanMX6*	[149]
MS98	*h⁻ atf1::natMX6*	This work
IV59	*h⁻ atf1::natMX6 sty1::ura4*	This work
CH1364	*h⁻ leu1-32 ura5-294 lys7-2*	C. Hoffman
EP142	*h⁻ leu1-32 ura5-294 lys7-2 ctt1'::ura5+ lys7+*	This work
EP143	*h⁻ leu1-32 ura5-294 lys7-2 gpd1'::ura5+ lys7+*	This work
EP184	*h⁺ leu1-32 ctt1'.CRE-to-G4BS*	This work
EP185	*h⁺ leu1-32 gpd1'.CRE-to-G4BS*	This work
EP186	*h⁺ leu1-32 ctt1'.CRE-to-G4BS sty1::natMX6*	This work
EP187	*h⁺ leu1-32 ctt1'.CRE-to-G4BS atf1::natMX6*	This work
EP193	*h⁻ leu1-32 atf1::natMX6*	This work
EP199	*h⁻ gpd1::kanMX6*	This work
EP201	*h⁻ leu1-32 atf1::natMX6 sty1::HA::leu1*	This work
EP203	*h⁻ leu1-32 atf1::natMX6 sty1::HA-atf1::leu1+*	This work
EP203.ΔbZIP	*h⁻ leu1-32 atf1::natMX6 sty1::HA-atf1.ΔbZIP::leu1+*	This work
EP203.bZIP	*h⁻ leu1-32 atf1::natMX6 sty1::HA-atf1.bZIP::leu1+*	This work
EP203.bZIP.S438A	*h⁻ leu1-32 atf1::natMX6 sty1::HA-atf1.bZIP.S438A::leu1+*	This work
EP203.S438A	*h⁻ leu1-32 atf1::natMX6 sty1::HA-atf1.S438A::leu1+*	This work
EP203.10D	*h⁻ leu1-32 atf1::natMX6 sty1::HA-atf1.10D::leu1+*	This work
EP203.10M	*h⁻ leu1-32 atf1::natMX6 sty1::HA-atf1.10M::leu1+*	This work
EP206	*h⁻ leu1-32 atf1::natMX6 sty1::G4-FLAG-HA-atf1::leu1+*	This work
EP206.ΔbZIP	*h⁻ leu1-32 atf1::natMX6 sty1::G4-FLAG-HA-atf1.ΔbZIP::leu1+*	This work
EP207	*h⁻ leu1-32 atf1::natMX6 sty1::HA-G4-FLAG::leu1+*	This work
EP212	*h⁺ leu1-32 ctt1'.CRE-to-G4BS sty1::G4-FLAG-HA-atf1::leu1+*	This work
EP212.ΔbZIP	*h⁺ leu1-32 ctt1'.CRE-to-G4BS sty1::G4-FLAG-HA-atf1.ΔbZIP::leu1+*	This work

EP213	*h+ leu1-32 ctt1'.CRE-to-G4BS sty1::HA-G4-FLAG::leu1+*	This work
EP215	*h+ leu1-32 ctt1'.CRE-to-G4BS sty1::G4-FLAG-HA-sty1::leu1+*	This work
EP252	*h- leu1-32 gpd1'.CRE-to-G4BS*	This work
EP255	*h+leu1-32 gpd1'.CRE-to-G4BS*	This work
EP258	*h- leu1-32 gpd1'.CRE-to-G4BS sty1::natMX6*	This work
EP259	*h- leu1-32 gpd1'.CRE-to-G4BS atf1::natMX6*	This work
EP260	*h- leu1-32 ura4-D18 pap1::ura4 atf1::natMX6 sty1::HA:.leu1+*	This work
EP261	*h- leu1-32 ura4-D18 pap1::ura4 atf1::natMX6 sty1::HA:.leu1+*	This work
EP261.ΔbZIP	*h- leu1-32 pap1:.ura4 atf1::natMX6 sty1::HA-atf1.ΔbZIP::leu1+*	This work
EP261.bZIP	*h- leu1-32 pap1:.ura4 atf1::natMX6 sty1::HA-atf1.bZIP::leu1+*	This work
EP262	*h- leu1-32 gpd1'.CRE-to-G4BS sty1::HA-G4-FLAG::leu1+*	This work
EP264	*h- leu1-32 gpd1'.CRE-to-G4BS sty1::G4-FLAG-Sty1::leu1+*	This work
EP271	*h- leu1-32 gpd1'.CRE-to-G4BS sty1::natMX6 sty1::HA-G4-FLAG-Sty1::leu1+*	This work
EP272	*h- leu1-32 gpd1'.CRE-to-G4BS sty1::natMX6 sty1::G4-HÁ-FLAG-Sty1::leu1+*	This work
EP303	*h- leu1-32 atf1::natMX6 sty1::ura4 sty1::HA-atf1.10D::leu1+*	This work
EP307	*h- leu1-32 gpd1'.CRE-to-G4BS sty1::G4-HÁ-FLAG-Atf1::leu1+*	This work
EP312	*h+ leu1-32 sty1::kanMX6 psty1'-HA::leu1*	This work
EP313	*h+ leu1-32 sty1::kanMX6 psty1'-HA-Sty1::leu1*	This work
EP314	*h+ leu1-32 sty1::kanMX6 psty1'-G4-FLAG-HA-Sty1::leu1*	This work
EP314.K49R	*h+ leu1-32 sty1::kanMX6 psty1'-G4-FLAG-HA-Sty1.K49R::leu1*	This work
EP314.T97A	*h+ leu1-32 sty1::kanMX6 psty1'-G4-FLAG-HA-Sty1.T97A::leu1*	This work
EP315	*h+ leu1-32 sty1::kanMX6 psty1'-HA-G4-FLAG-Sty1::leu1*	This work
EP316	*h+ leu1-32 sty1::kanMX6 psty1'-G4-FLAG-Sty1::leu1*	This work

Table III: Strains from Results 3

Name	Genotype	Origin
972	*h-*	
EP197	*h- leu1-32 ura4-D18 ctt1::natMX6*	This work

EP198	*h⁻ ctt1::natMX6*	This work
EP231	*h⁻ leu1-32 ura4-D18 ctt1:.natMX6 sty1::ctt1::leu1+*	This work
NG40	*h⁻ php4::kanMX6*	This work
NG123	*h⁻ php4::HA::kanMX6*	This work
NG129	*h⁻ rbp1::HA::kanMX6*	This work
NG131	*h⁻ php4::HA::kanMX6 grx4::natMX6*	This work
NG135	*h⁻ rbp1::HA::kanMX6 grx4::natMX6*	This work

2. PLASMIDS

All plasmids are expressed under a constitutive promoter, the *sty1* promoter, and integrated at *leu1* locus.

Name	Genotype	Origin
p386'	*pHA*	[151]
p393'	*pHA-Sty1*	This work
p404'	*pG4-FLAG-Sty1*	This work
p409'	*pG4-FLAG-HA-Sty1*	This work
p411'	*pG4-FLAG-HA-Atf1*	This work [113]
p411.ΔbZIP'	*pG4-FLAG-HA-Atf1.ΔbZIP*	[113]
p413'	*pG4-FLAG-HA*	[113]
p414'	*pHA-G4-FLAG-Sty1*	This work
p414.K49R'	*pHA-G4-FLAG-Sty1.K49R*	This work
p414.T97A'	*pHA-G4-FLAG-Sty1.T97A*	This work
p428'	*pHA-Atf1*	[113]
p428.ΔbZIP'	*pHA-Atf1.ΔbZIP*	This work
p428.bZIP'	*pHA-Atf1.bZIP*	[113]
p428.bZIP.S438A'	*pHA-Atf1.bZIP.S438A*	This work
p428.S438A'	*pHA-Atf1.S438A*	This work
p428.10D'	*pHA-Atf1.10D*	This work
p428.10M'	*pHA-Atf1.10M*	This work

3. EXPERIMENTAL PROCEDURES

3.1. Growth conditions

Strains were grown in standard media (minimal media (MM) or rich media containing 0.25g/l of lysine, uracil, leucine, and histidine (YE5S) as described [152]. Anaerobic liquid cultures were grown in flasks filled to the top with medium at 30ºC without shaking. When indicated, 0.02 mg ml^{-1} thiamine was added to MM cultures to block gene expression from the *nmt* (no message in thiamine) promoter.

3.2. 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 media, and 10 to 10^5 cells per dot were spotted onto minimal or rich medium plates containing (or not) a stressor. Plates were incubated at 30ºC for 3-4 days.

To mesure cellular growth on liquid culture, we used an assay based on automatic measurements of optical densities for small (100 µl) cell cultures. Cells were grown to an OD600 of 0.5 under continuous shaking in Erlenmeyer flasks and then diluted to and OD600 of 0.1. 100 µl of the diluted cultures (treated or not with the indicated amounts of stressors) were placed into a 96-well non-coated polystyrene microplate covered with an adhesive plate seal. APower Wave microplate scanning spectrophotometer (Bio-Tek) was used to obtain the growth curves. The OD600 was automatically recorded using Gen5 software. The software was set as follows: OD was measured at 600 nm, incubation temperature was kept at 30ºc, the microplate was subjected to a continuous shaking and the readings were done every 10 min during 48 hours.

3.3. Preparation of *S. pombe* trichloroacetic acid (TCA) extracts and immunoblot analysis

S. pombe cultures (5 ml) at an OD_{600} of 0.5 were pelleted just after the addition of 10% TCA (from a 100% stock) to freeze the thiol redox status. Allow acidification for a minute before harvesting. TCA has to be added without altering the growing conditions of the culture.

Preparation of cell lysates must be performed at 4ºC. Harvest cells by centrifugation at 2,000 g for 3 min at 4ºC. Collect cells into a 1.5 ml eppendorf tube with 1 ml of 20% TCA. Centrifuge at 20,000 g for 30 seconds and take out supernatants. Resuspend pellets in 100 µl of 12.5% TCA. Fill with glass beads up to 85-90% of the volume to allow proper disruption. To disrupt cells, vortex at maximum speed for 5 min in a vortex for microcentrifuge tubes. Pierce two hoes, one in the bottom and the other in the top of each Eppendorf, with a flame-heated needle, and place each tube on the top of another 1.5 ml tube. Centrifuge to separate lysate from the beads at 850 g for 30 sec at 4ºC. Centrifuge the cell lysates at 20,000 g for 20 min at 4ºC. Take out supernatants, and wash pellets with 1 ml of chilled acetone (-20ºC), with the help of the blunt side of a calibrated plastic inoculation loop. Centrifuge at 20,000 g for 5 min at 4ºC. Take out supernatants and dry pellets by placing them for 10 min in an oven pre-heated at 55ºC. Pellets were resuspend 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 SDS-PAGE and immunodetected with anti-Atf1, anti-p38-P (9215l, Cell signaling), anti-Sty1, anti-HA or anti-FLAG (F7425, Sigma). As a loading control we used monoclonal anti-tubulin (Tub2, Sigma) or anti-Sty1.

3.4. RNA analysis

Cells, grown in minimal or rich media to a final OD_{600} of 0.5, were left either untreated or treated for the indicated times and concentrations of H_2O_2. Yeast culture (40 ml) were then centrifuged at 500 g for 3 min and washed with H_2O. Each sample was then resuspended in 0.4 ml of AE buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA pH 8.0). SDS was then added to a final concentration of 1%, and protein and DNA extracted by adding 0.4 ml of a mixture of acidic phenol-chloroform, and incubation at 65ºC for 5 min. Samples were cooled down in ice, and the aqueous phase was separated by centrifugation at 10,000g for 2 min at 4ºC. After chloroform extraction, RNA was precipitated with 100% ethanol plus 0.1 M sodium acetate pH 5.3 and incubation at -20ºC for 1 hour. Pellets were resuspended in DEPC water after washing them with 70% ethanol. RNA concentration was determined at OD_{260} with a nanodrop system and equal amounts (10 µg) were loaded in a formaldehyde agarose gels and transferred to GeneScreen Plus membranes. Hybridization and washes were performed as recommended by the facturer. Membranes were hybridized with [α-^{32}P] dCTP labeled probes containing the open reading frames of the genes indicated in the figure legends. Ribosomal RNA is used as a loading control.

3.5. Protein extraction and immunoprecipitation

Sty1 was isolated by affinity purification followed by mass spectrometry. Total protein extracts of three different strains (972-non tag, EP42, sty1-HA and EP49 sty1-HA Δpyp1) were prepared from 5 liters of growth cultures. Cells were frozen with liquid nitrogen and then broken manually in a mortar. Cell lysates were resuspended in 10 ml of NET-N buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethyl sulphonyl fluoride (PMSF), 5 µg ml^{-1} aprotinin, protease inhibitor cocktail (Sigma, used as described by

manufacturer), 2 mM sodium fluoride (NaF), 0.2 mM sodium orthovanadate (Na_3VO_4), 1 mM β-glycerolphosphate) and centrifuged 40' at 14000 r.p.m. in a Beckman centrifuge. Protein concentration was quantified by Bradford. 230 mg of total protein of each strain were precleared to allow unspecific binding by incubation 1 hour at 4ºC with protein G-sepharose. Precleared supernatants were incubated 4 hours at 4ºC with protein G-sepharose crosslinked to HA antibody. Immunoprecipitates were washed 4 times in Bio-Rad Poly-prep chromatography columns with 5 ml of NET-N buffer, and eluted from columns with 5 washes of 1 ml glycine pH 2. pH of eluted fractions was neutralized with 1 M Tris-HCl pH 8.8. The presence of Sty1-HA in eluates was checked by Western Blot and 1/5 of the selected eluate was loaded on a 8% SDS-PAGE followed by silver staining to compare the specificity of purification in the three strains. The rest of the sample was dialized O/N with 20 mM NH4HCO3 buffer using Spectra/Por dialysis membranes (Spectrum laboratories), and then lyophilized. Samples were send at the Proteomic Facility of the Univesitat Pompeu Fabra to perform the dimethyl labeling.

3.6. Silver staining

Proteins in gel are fixed in 40 ml EtOH, 10 ml of acetic acid and 50 ml Milli-Q water for 30 min. Then proteins are sensitized in 30% EtOH, and 0.2% sodium thiosulphate in Milli-Q water. After sensitizing, the gel is washed 3 x 5 min with Milli-Q water. The silver reaction is performed with 0.25% of silver nitrate in Milli-Q water. After silver staining, the gel is washed 2 x 1 min in Milli-Q water, and proteins are developed in 2.5% sodium carbonate and freshly added 0.007% formaldehyde in Milli-Q water. Developing is stopped by incubating the gel during 10 min in 1.46% EDTA. Finally, the gel is washed 3 x 5 min with Milli-Q water.

3.7. Quantification of proteins by dimethyl labeling

A fraction of the two samples subjected to the ICAT experiments was digested using Sequencing Grade trypsin (Promega, Madison, WI, USA), in 25 mM TEAB. Tryptic peptides were labeled with either normal or deuterium labeled formaldehyde, respectively [146, 153, 154]. A modification was added to the dimethyl labeling protocol [153, 154]: the reaction mixture was not acidified prior to mixing, but instead 8 µl of 1% ammonia solution was added. This modification allowed direct sample loading on a strong anion exchange columns. The mixed, now differentially labeled peptides, were split into two portions, 1/5 for testing of the labeling and 4/5 as to be used for a more comprehensive analysis. The small portion was acidified, and SDS was removed by a cation exchange (AB Sciex, Foster City, CA, USA) step according to the manufacturer's instructions. The SDS depleted dimethyl labeled peptide mixture was hereafter desalted on a home-made Empore C_{18} column (3M, St. Paul) [155] prior to analysis by LC-MS/MS. The larger portion (4/5) was fractionated by strong anion exchange (Empore anion-SR, 3M, St. Paul) as described by Wisniewski *et al* [156]. In accordance with this protocol, the peptides were eluted into six pH fractions (11, 8, 6, 5, 4 and 3). Each fraction was purified on home-made Empore C_{18} columns prior to analysis by LC-MS/MS.

3.8. Liquid chromatography and mass spectrometry of dimethyl labelled peptides

Peptides were analysed by LC-MS/MS using an EasyLC (Thermo Scientific, Odense, Denmark) coupled to an Orbitrap Velos (Thermo Scientific, Bremen, Germany). Peptides were separated by reversed phase chromatography using a 15 cm column with an inner diameter of 100 µm, packed with 5 µm C18 particles (Nikkyo Technos Co., Ltd. Japan). The gradients were generated at 500 nl / min, increasing from

97% buffer A / 3% buffer B (buffer A: 0.1% formic acid, buffer B: 80% acetonitrile, 0.1% formic acid) to 85% buffer A / 15% buffer B in 4 min, followed by a less steep increase to 55% buffer A / 45% buffer B in 90 min. After the gradient, the column was washed for 11 min with 10% buffer A / 90% buffer B. Peptides were loaded directly onto the analytical column at 1.5-2 µl / min using a wash-volume of 4 to 5 times injection volume. The Orbitrap Velos was operated in CID mode with a data acquisition cycle composed of a full scan (1 micro scan at 60,000 resolution) in the Orbitrap (AGC target $1 \cdot 10^6$) followed by fragmentation of up to 10 of the most intense multiply charged ions in the linear trap (MSn AGC target $1 \cdot 10^4$). Dynamic exclusion for the selected ions was set to 60 seconds. A single ion was used as lock mass.

3.9. Quantification of proteins by dimethyl labeling: data analysis

Proteome Discoverer software suite (v1.4, Thermo Fisher Scientific) and the Mascot search engine (v2.3.1, Matrix Science [157]) were used for peptide identification and quantification. The data were searched against an in-house generated database containing all *S. pombe* entries from Swissprot database (release January, 2013) and a list of common contaminants (around 599 entries in total). A precursor ion mass tolerance of 7 ppm at the MS1 level was used, and up to three miss-cleavages for trypsin were allowed. The fragment ion mass tolerance was set to 0.5 Da. Oxidation of methionine, protein acetylation at the N-terminal and all dimethyl forms (light, medium and heavy) of the N-terminal and lysine residues were set as variable modifications; whereas carbamidomethylation on cysteines was set as a fix modification. False discovery rate (FDR) in peptide identification was evaluated by using a decoy database and it was set to a maximum of 5%.

3.10. H_2O_2 scavenging by whole cells

Ten milliliters of exponential growing cell cultures (OD_{600} 0.5) in MM were washed twice with equal volumes of PBS (phosphate buffer saline; 50 mM phosphate buffer containing 0.9% sodium chloride, pH 6.8) and were resuspended with 10 ml into PBS pH 6.8 containing 0.75% of glucose. After 30 min of incubation, cells were treated with different H_2O_2 concentrations. At the indicated times, an aliquot of 1 ml of cell culture was taken from the culture and centrifuged (1 min 13200 r.p.m.) to separate cells from the media, and supernatants were stored on ice until sampling was over. The remaining H_2O_2 in the supernatants was measured with the Amplex red/horseradish peroxidase method, as described [146] with some minor modifications. Briefly, 50 µl of the supernatants, diluted in PBS when necessary, were mixed with 25 µl of 0.02mg ml-1 horseradish peroxidase (HRP) (Sigma) and 25 µl of 200 µM Amplex Red (AR, Molecular Probes) in a 96-well black flat bottom plate (stock solutions of AR and HRP were prepared as follows: 200 µM AR in 50 mM buffer phosphate, pH 7.8, from 10 mM AR in DMSO; and 0.02 mg ml^{-1} HRP in 50 mM buffer phosphate, pH 7.8, from 1.7 mg ml^{-1} HRP in water). Fluorescence was immediately measured at $ƛ_{ex}$ 530 nm in an infinite 200 multimode reader (Tecan Group). A blank was obtained with untreated samples, and this value was subtracted from the rest of measured fluorescence values. Relative fluorescence values were calculated using the maximum fluorescence value for each condition. At the low concentrations of H_2O_2 (100 µM) used in this assay, a decay of peroxides in the absence of cells was detected during the course of the experiments, and substracted from the scavenging values obtained with whole cells.

3.11. H_2O_2 scavenging by cell extracts

Cell cultures were grown to an OD_{600} 0.5. Cells were pelleted, and pellets were washed twice with PBS buffer and were resuspended in 250 µl of NET-N buffer. Cells were broken with glass beads in a BioSpec Mini-BeadBeater 16 Ring Rack. Lysates where then centrifuged to remove cell debris. The protein concentration was determined using the Bradford protein assay (Bio-Rad). Fifty microlitres of protein extracts at a concentration of 0.25 or 1 µg μl^{-1}, as indicated, where then incubated with 2 µM H2O2. To inhibit the activity of catalase by azide as previously reported [158], extracts where pretreated with 1 mM azide during 5 min at room temperature prior to the addition of H2O2. To promote Tpx1 peroxide scavenging, we added 20 ng of recombinant Trx1 with or without 10 ng of recombinant Trr1 [47], and 0.25 mM NADPH (Sigma), as indicated. To inhibit thioredoxin reductase form protein extracts and therefore block Tpx1 recycling, we treated the extracts with 30 µM DNCB (1-Chloro-2,4-dinitrobenzene, Sigma), as reported [159], during 10 min at room temperature prior to the addition of H_2O_2. At the indicated times, the remaining H_2O_2 concentrations were measured with the AR/HRP method, exactly as described above.

APPENDIX

1. Binding of the transcription factor Atf1 to promoters serves as a barrier to phase nucleosome arrays and avoid cryptic transcription.

García P., Paulo E., Gao J., Wayne-P W., Ayté J., Lowy E., Hidalgo E.
Nucleic acid research 2014 Jul. Vol. 42, No. 16 **10351–10359**

García P, Paulo E, Gao J, Wahls WP, Ayté J, Lowy E, Hidalgo E. Binding of the transcription factor Atf1 to promoters serves as a barrier to phase nucleosome arrays and avoid cryptic transcription. Nucleic Acids Res. 2014;42(16):10351-9. doi: 10.1093/nar/gku704.

2. Modification of $tRNA^{Lys}_{UUU}$ by Elongator is essential for efficient translation of stress mRNAS

Fernández-Vázquer J., Vargas-Pérez I., Sansó M., Buhne K., Carmona M., Paulo E., Hermand D., Rodríguez-Gabriel M., Ayté J., Leidel S., Hidalgo E. PLoS Genet. 2013 Jul; 9 (7):e1003647.

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3. Thiol-based H_2O_2 signaling in microbial systems

Boronat S., Domènech A., Paulo E., Calvo I., García-Santamarina S., García P., Encinar del Dedo J., Barcons A., Serrano E., Carmona M., Hidalgo E. Redox biology 2014: 395-399

Boronat S, Domènech A, Paulo E, Calvo IA, García-Santamarina S, García P, Encinar Del Dedo J, Barcons A, Serrano E, Carmona M, Hidalgo E. Thiol-based H2O2 signalling in microbial systems. Redox Biol. 2014 Feb 3;2:395-9. doi:10.1016/j.redox.2014.01.015.

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