REGULATION OF TRANSCRIPTIONAL ACTIVATION

IN RESPONSE TO HEAT STRESS AND OSMOSTRESS

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SUMMARY

All kind of cells are exposed to variations in the environment, such as changes in temperature or osmolarity, which can be harmful or even lethal. To survive to such adverse extracellular conditions, cells are able to generate a set of adaptive responses, which include the regulation of gene expression.

In *Saccharomyces cerevisiae*, increases in temperature lead to deleterious effects on the internal organization of the cell. In response to heat stress, there is a rapid, massive and transient induction of gene expression, mainly controlled by Hsf1 and the general stress-responsive Msn2/4 transcription factors. In this study, by using a genome-wide genetic screen, we identified the network of essential activities required for cell adaptation to heat stress. Moreover, we found that the Rpd3 histone deacetylase (HDAC) complex is an essential component for adaptation and survival to heat stress and it is required for proper regulation of gene expression. Specifically, Rpd3 is needed for activation of the Msn2/4-dependent genes in response to heat stress. Moreover, we found that the large, but not the small Rpd3 complex mediates cell adaptation. Overall, our data provide a description of the physiological response of cells in response to a heat shock and suggest that the large Rpd3L HDAC complex is important for regulating transcriptional activation in a promoter-dependent manner in response to heat stress.

Increases in the extracellular osmolarity activate the Hog1 stress-activated protein kinase (SAPK), which is essential for the induction of diverse osmoadaptive responses, such as regulation of gene expression. At least five transcription factors have been shown to be controlled by Hog1, although recent data suggest that other transcription factors might be involved in the regulation of osmoresponsive genes under the control of the SAPK. Here we show that the Rtg1 and Rtg3 transcription factors regulate the expression of a set of genes upon osmostress in a Hog1-dependent manner. In response to osmostress, Hog1 is required for the nuclear accumulation of the Rtg1/3 transcription complex. Once in the nucleus, Hog1 is recruited at promoters and its activity is required for the binding of Rtg1/3 to chromatin. Moreover, Rtg3 phosphorylation by Hog1 is an important step for proper transcriptional activation. Taken together, our results suggest a new role for the Hog1 SAPK, in controlling the Rtg1/3 transcription complex.

Totes les cèl·lules estan exposades a canvis en el medi ambient, com canvis en la temperatura o l'osmolaritat, que poden ser perjudicials o inclús letals. Per tal de sobreviure a condicions ambientals adverses, les cèl·lules son capaces de generar un conjunt de respostes adaptatives, incloent la regulació de l'expressió gènica.

A *Saccharomyces cerevisiae*, un increment en la temperatura comporta diversos efectes deleteris en l'organització interna de la cèl·lula. En resposta a un estrés tèrmic, es produeix una inducció ràpida, massiva i transitòria d'expressió gènica, que es controla principalment pels factors de transcripció Hsf1 i Msn2/4. En aquest estudi, fent servir un crivatge genètic a gran escala, hem identificat el conjunt d'activitats que es requereixen per a l'adaptació cel·lular a l'estrés tèrmic. A més, hem trobat que el complex de desacetilació d'histones de Rpd3 és un component essencial per a l'adaptació i la supervivència a l'estrés tèrmic, i que es requereix per a l'adequada regulació de l'expressió gènica. Concretament, Rpd3 es necessita per a l'activació dels gens depenents de Msn2/4 en resposta a estrés tèrmic. A més, hem trobat que és el complex gran de Rpd3, però no el petit, el qui media l'adaptació cel·lular. En conjunt, les nostres dades aporten una descripció de la resposta fisiològica de les cèl·lules davant d'un estrés tèrmic i suggereixen que en resposta a l'estrés tèrmic, el complex gran de Rpd3 és important per a regular l'activació transcripcional d'una manera depenent de promotor.

Un increment en l'osmolaritat externa activa la quinasa activada per estrés (SAPK) Hog1, que és essencial per induir diverses respostes adaptatives, com la regulació de l'expressió gènica. S'ha demostrat que Hog1 controla al menys cinc factors de transcripció, malgrat estudis recents suggereixen que altres factors de transcripció poden estar involucrats en la regulació de gens de resposta a estrés osmòtic sota el control de Hog1. Aquí ensenyem que els factors de transcripció Rtg1 i Rtg3 regulen l'expressió d'un conjunt de gens en resposta a estrés osmòtic, d'una manera depenent de Hog1. En resposta a estrés osmòtic, Hog1 es requereix per a l'acumulació nuclear del complex de transcripció de Rtg1/3. Un cop al nucli, Hog1 es recluta als promotors i la seva activitat es requereix per a la unió de Rtg1/3 a la cromatina. A més, la fosforilació de Rtg3 per Hog1 és un pas important per a l'adequada activació transcripcional. En conjunt, el resultats suggereixen un nou paper per a la quinasa d'estrés Hog1 en el control del complex de transcripció Rtg1/3.

PROLOGUE

When environmental conditions change abruptly, cells must rapidly generate a set of adaptive responses to adapt to the new conditions. A crucial aspect of the adaptive responses to stress is the change of the gene expression program, usually resulting in the immediate down-regulation of the house-keeping genes and the rapid induction of crucial stress-responsive genes. Along the years, multiple efforts have been focused to elucidate the important events occurring during the transcription cycle of eukaryotes, for the rapid and specific activation of gene expression in response to external stimuli. It is becoming increasingly apparent that, in addition to the regulated recruitment of the transcriptional machinery to the genes, regulation of chromatin plays a crucial role for the control of gene expression. On the other hand, it is well known the role of signaling pathways in regulating different specific transcriptional activators in front of a specific stimulus.

During the last decade, the study of chromatin regulation has been a predominant field of research. The diverse histone covalent modifications and nucleosome positioning in chromatin have been widely studied at a genome-wide level, usually using the budding yeast *S. cerevisiae* as a model. Evidence show that any given histone modification may have a different effect on transcription depending on the specific environmental conditions and chromatin context. Elucidating the role of chromatin modifiers and remodelers involved in regulation of gene expression in response to stress should help to understand how eukaryotic cells rapidly induce and fine-tune transcription in response to adverse conditions. In yeast, heat responsive genes are very fast responders, but the mechanisms to achieve such a rapid and efficient response are not well known. In this work, from a genetic screen used to identify the essential genes for survival in heat stress, we identified the Rpd3 HDAC complex, as a key player in the regulation of gene expression and cell adaptation to heat stress.

Another field of intense research during the last decade has been focused in the role of mitogen-activated protein kinases (MAPKs) in the regulation of gene expression. In *S. cerevisiae*, increases in the external osmolarity activate the stress-activated Hog1 MAPK, the functional homolog of the mammalian JNK and p38 MAPKs. It has been shown that fundamental mechanisms to regulate transcription have been preserved from yeast to humans, and Hog1 has served as a model for the study of how MAPKs control gene expression. At initiation of transcription, active Hog1 targets and regulates several specific transcription factors, using distinct mechanisms of control. In this study, we describe a new mechanism by which Hog1 regulates transcriptional activation, through the control of the Rtg1/3

transcription factors. Altogether, the results presented offer new insights in the regulation of transcriptional activation in response to stress.

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INTRODUCTION

1.- TRANSCRIPTION AND CHROMATIN IN YEAST

1.1.- The eukaryotic transcription cycle

Transcription is a complex process responsible for the generation of a mature messenger RNA (mRNA) molecule. Transcription of eukaryotic genes can be performed by three different DNA-dependent RNA polymerases: RNA polymerase I, which exclusively transcribes ribosomal RNA genes, RNA polymerase II, used to transcribe protein-coding genes, and RNA polymerase III, which is responsible for the transcription of tRNA genes, 5S RNA genes and snRNA genes.

The RNA Polymerase II (Pol II) is a large multisubunit enzyme with a mass of about 0.5 MDa. A unique feature of the RNA Pol II is the C-terminal domain (CTD) of its largest subunit, which is composed of a highly conserved, tandemly repeated heptapeptide sequence (YSPTSPS). The RNA Pol II CTD undergoes extensive phosphorylation and dephosphorylation events during the transcription cycle, which are important for the recruitment of various factors that control the different stages of transcription (Buratowski, 2003;Egloff and Murphy, 2008). RNA Pol II is recruited to the promoters in an unphosphorylated form. During initiation of transcription, the RNA Pol II CTD is predominantly phosphorylated at Ser 5 and Ser 7, whereas during transcription elongation through the coding regions, Ser5 and Ser7 are dephosphorylated and Ser 2 becomes the predominant phosphorylated residue (Chapman *et al.*, 2007;Egloff *et al.*, 2008) (see below).

The transcription cycle consists of distinct steps, which can be key regulatory stages of transcription (Fuda *et al.*, 2009):

Chromatin opening: the transcription cycle begins with RNA Pol II gaining access to the promoters. At many genes, promoter regions are covered by nucleosomes, thus inaccessible for the Pol II machinery. For these genes, recruitment of chromatin remodelers is required to open chromatin structure and make promoters accessible (see next sections of introduction).

PIC formation: a key regulatory step for transcription is the preinitiation complex (PIC) assembly at the promoters. The PIC includes the RNA Pol II and the general transcription factors (GTFs) TFIID, TFIIB, TFIIE, TFIIF, TFII and TFIIH, as well as several additional cofactors (Orphanides *et al.*, 1996). Several gene-specific activators have a role in promoting the formation of the PIC.

Initiation: after the PIC is assembled at the promoters, the DNA is unwound at the transcription start site (TSS) and an open complex between Pol II and the DNA template is formed. Then, Pol II initiates transcription with the formation of the first RNA phosphodiester bond (Orphanides *et al*., 1996). This is accompanied by phosphorylation at Ser5 of the Pol II CTD, which signals the recruitment of mRNA capping enzyme, and other factors required for initiation of transcription (Egloff and Murphy, 2008).

Promoter clearance: at this step the PIC is partially disassembled. A subset of GTFs remains at the promoter, serving as a scaffold for the formation of the next transcription initiation complex, and Pol II enters into the coding region forming the transcription elongating complex (TEC). Of all the GTFs, only TFIIF can be found at the TEC (Yudkovsky *et al*., 2000).

Escape from pausing: At certain genes of higher eukaryotic organisms, such as *Drosophila* or mammals, Pol II gets paused after the synthesis of approximately 20-50 bases downstream of the TSS (Rougvie and Lis, 1988;Zobeck *et al.*, 2010). Pol II paused at early elongation is thought to allow a rapid induction of target genes when appropriate environmental or developmental signal is encountered. When the blockage of Pol II is overcome, the polymerase enters into productive transcription elongation (Levine, 2011).

Productive elongation: whereas the polymerase travels through the coding region, phosphorylation of the CTD at Ser2 signals the recruitment of factors required for chromatin remodeling, mRNA processing, termination and mRNA export (Egloff and Murphy, 2008). During transcription elongation, chromatin modifications ahead of elongating Pol II are crucial for opening the chromatin to allow the efficient passage of the polymerase, whereas other chromatin modifications are essential behind the passage of Pol II, to re-establish a more repressive chromatin environment and prevent cryptic transcription initiation (Akey and Luger, 2003;Workman, 2006).

Termination and recycling: after the Pol II complex transcribes the genes, it is removed from the DNA, and the RNA is released to be further processed and exported to the cytoplasm. Finally, the free polymerase can reinitiate transcription. Indeed, once the promoter is cleared, the next round of transcription can be reinitiated (Cramer, 2006;Zawel *et al.*, 1995).

For the regulation of protein-coding gene expression, besides the RNA Pol II and the GTFs, thousands of activators, repressors and co-regulators dictate when, where and at what level genes are transcribed. Thus, transcription is a highly regulated multistep process and,

following a specific stimulus, gene-specific regulators can act at different steps of the cycle, resulting in the regulated induction or repression of a specific gene.

1.2- Chromatin remodeling

Chromatin is the state in which DNA is packaged within the eukaryotic cell. The basic unit of chromatin is the nucleosome, composed of an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped (Figure 1). The packaging of DNA into chromatin provides a natural barrier for the factors to access the DNA template. Thus, chromatin structure has an inhibitory effect in almost all DNA-related metabolic processes, including transcription, recombination, DNA repair, replication, and others. In order to overcome this chromatin inhibitory effect and allow factors to access the DNA sequences, the eukaryotic cells have mechanisms that modulate nucleosome position and structure (Bell *et al.*, 2011).

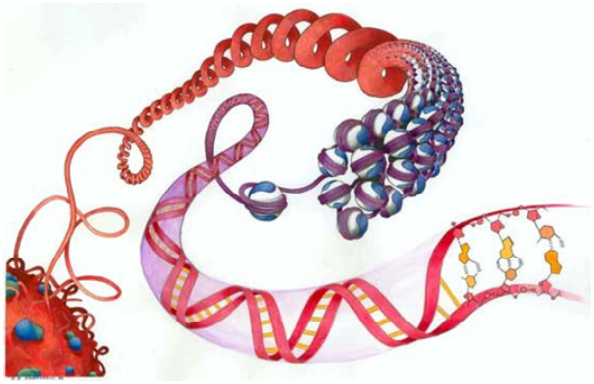

Figure 1. DNA is packaged into chromatin.
DNA is compacted into chromatin in order to fit inside the nuclei of eukaryotic cells. The fundamental unit of chromatin is the nucleosome, composed of 147 bp of DNA wrapped around an octamer of the 4 core histones. In turn, arrays of nucleosomes are packaged to make a higher level of compaction (www.ecrc.ed.ac.uk).

The ATP-dependent chromatin remodeling complexes utilize ATP hydrolysis to reorganize nucleosomal arrays in a non-covalent manner. In yeast, these protein complexes are divided into families by homology of their protein subunits: SWI/SNF family (SWI/SNF and RCS), ISWI family (ISWI1 and ISWI2), CHD family (Chd1) and INO80 family (INO80 and SWR1) (Gangaraju and Bartholomew, 2007;Smith and Peterson, 2005). Importantly, these remodeling complexes are often recruited at chromatin by domains that recognize histone posttranslational modifications (see below).

It is known that nucleosomes are highly dynamic during the process of transcription. Nucleosomes are evicted or displaced to some extent both at promoter and coding regions of active genes (Cairns, 2009;Jiang and Pugh, 2009). As described before, during elongation, displacement of nucleosomes is essential to permit the passage of the RNA pol II machinery along the gene (Bernstein *et al.*, 2004;Workman, 2006). Moreover, nucleosomes are reassembled behind the elongating Pol II as the genes turn off with the help of histone chaperones, also known as nucleosome assembly proteins (Adkins and Tyler, 2006;Klopf *et al.*, 2009). The nucleosome reassembly behind passing polymerase at coding regions is critical to hide cryptic sites of initiation at the ORFs and maintain the fidelity of transcription initiation (Akey and Luger, 2003).

1.3.- Histone modifications

The core histones H3, H4, H2A and H2B are predominantly globular except for their N-terminal tails, which are unstructured. Mainly the N-terminal tails, yet as well the histone globular cores, are subject to a variety of covalent posttranslational modifications including acetylation, phosphorylation, methylation, ubiquitylation, ADP-ribosylation, and glycosylation (Kouzarides, 2007;Vaquero et al., 2003) (Figure 2). There are over 60 different residues on histones where modifications have been detected either by specific antibodies or mass spectrometry. However, this represents a huge underestimate of the number of modifications that can take place on histones. Extra complexity comes partly from the fact that methylation at lysines or arginines may be one of three different forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines. This vast array of modifications gives enormous potential for functional responses (Kouzarides, 2007).

It is well known that histone modifications are involved in regulating the remodeling of chromatin structure. There are at least two characterized mechanisms for the function of modifications. The first is the disruption of contacts between nucleosomes in order to ‘‘unravel’’ chromatin. With the exception of methylation, histone modifications result in a change of the net charge of nucleosomes, which could relax inter- or intranucleosomal DNA-histone interactions (Kouzarides, 2007). The second function is the recruitment of non-histone proteins (reviewed in (Seet et al., 2006)). Individual histone modifications or modification patterns can be read by other proteins or complexes such as remodellers that further modify chromatin structure and dynamics (Jenuwein and Allis, 2001;Strahl and Allis, 2000;Turner, 2000).

For the purpose of transcriptional regulation, modifications can be divided into those that correlate with activation and those that correlate with repression (Li *et al.*, 2007a). For instance, histone acteylation has been generally associated with activation (Kurdistani and Grunstein, 2003;Shahbazian *et al.*, 2007). This can be attributed to the fact that acteylation neutralizes the basic charge of histone lysines and may lead to loss of DNA-histone bounds. Moreover, acetylation leads to the formation of a different chromatin surface that may be recognized by chromatin remodelers that act as activators (Kouzarides, 2007). Indeed, there is a strong association between histone acteylation and a subsequent loss of histones (Erkina and Erkine, 2006;Reinke and Horz, 2003;Zhao *et al.*, 2005). Histone acetylation is produced by the action of HAT (Histone Acetyl Transferase) - containg complexes, such as SAGA, ADA, NuA3, NuA4 and others (Brown *et al.*, 2000;Howe *et al.*, 1999)

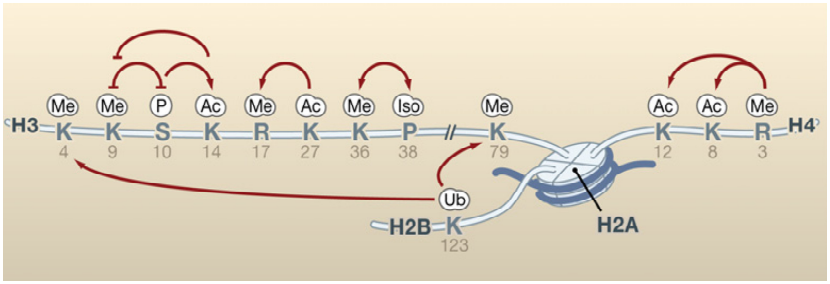

Figure 2. Posttranslational histone modifications.
Residues of histone cores and N-terminal tails may suffer covalent posttranslational modifications, which are usually involved in regulating chromatin structure and gene expression. Communication between histone modifications, also referred to as "crosstalk", occurs either between modification on the same or on different histones. Examples of positive influence of one modification over an other is shown (Kouzarides, 2007).

Since acetylation is a reversible modification, histone deacetylase (HDAC)-containg complexes catalyze the deacetylation reaction. Correspondingly, histone deacetylation has been generally associated with repression of transcription. Indeed, genome-wide deacetylation has been found in heterochromatin and silenced genes (Imai *et al.*, 2000;Johnson *et al.*, 2009;Ng and Bird, 2000;Shahbazian and Grunstein, 2007). However, several studies demonstrated that deacetylases may also have a positive role in transcription initiation (Bernstein *et al.*, 2000;Kurdistani and Grunstein, 2003;Robyr *et al.*, 2002;Shahbazian *et al.*, 2007). For instance, the yeast histone deacetylase Hos2 is essential for inducing the expression of the genes *INO1* and *GAL1* (Wang et al., 2002). Moreover, activation of *GAL4* gene is associated with a decrease of histone 4 acetylation (Deckert and Struhl, 2001). Furthermore, the yeast Rpd3 histone deacetylase has also been reported to be required for gene activation (see below). Therefore, although traditionally histone deacetylation has been associated with

repression of transcription, it is increasing the number of examples in which there is the opposite effect. Actually, it is becoming likely that any given histone modification has the potential to activate or repress under different conditions and different chromatin context.

1.4.- The Rpd3 HDAC complex

There are 10 known HDACs in *Sacharomyces cerevisiae* classified in three families, based on their homology with mammals. The class I HDACs includes Rpd3, Hos1 and Hos2; class II HDACs includes Hda1 and Hos3, and class III HDACs includes Sir2 (also known as Sirtuin 2) and four Hst (homolog of Sirtuin 2) proteins.

The Rpd3 histone deacetylase was isolated genetically in a screen for mutants with reduced potassium dependency (rpd phenotype mutants) in a potassium transporter gene deletion background (Vidal et al., 1990). Early studies demonstrated that Rpd3 was required to achieve maximum positive and negative transcriptional states, thus implicating it in transcriptional regulation (Vidal and Gaber, 1991;Vidal et al., 1991). A later genome-wide analysis of histone deacetylase function in yeast showed that Rpd3 regulates the transcription of a wide range of genes by deacetylating mainly histones H3 and H4, and deletion of the deacetylase leads to both upregulation and downregulation of large sets of genes (Bernstein et al., 2000;Robyr et al., 2002). Rpd3 is recruited to DNA by at least two different mechanisms: one involves direct recruitment to gene promoters through specific DNA binding proteins, and the other involves genome-wide recruitment within coding and intergenic regions, independent of a sequence-specific protein (Kurdistani *et al.*, 2003).

There are two known Rpd3 complexes within *S. cerevisiae*, primarily identified by gel filtration elution profiles (Kasten *et al.*, 1997;Lechner *et al.*, 2000;Rundlett *et al.*, 1996): the small Rpd3S complex (0.6 MDa) and the large Rpd3L complex (1.2 MDa). Both complexes, which are functionally distinct (see below) share a core set of three subunits including Rpd3, Sin3 and Ume1. Additionally, the Rpd3L complex contains the proteins Pho23, Sap30, Sds3, Cti6, Rxt2, Rxt3, Dep1, Ume6, and Ash1, whereas the Rpd3S complex contains Rco1 and Eaf3 (Carrozza *et al.*, 2005a;Carrozza *et al.*, 2005b) (Figure 3).

The role of the Rpd3S complex is well defined and established. It is recruited to coding regions of transcribed genes, where it deacetylates histones behind the elongating polymerase, to help reestablishing a repressive chromatin structure and avoid spurious transcription initiation (Carrozza *et al.*, 2005b;Govind *et al.*, 2010;Keogh *et al.*, 2005). The Rpd3S complex is recruited

through a signaling pathway of the elongating RNA Pol II. The elongating Pol II phosphorylated at Ser2 of the CTD recruits the Set2 histone methyltransferase. Set 2 methylates histone 3 Lysine 36 (H3K36) through the body of the gene as it is transcribed. The H3K36 mark recruits the Rpd3S complex by the Eaf3 chromodomain (methyl lysine-binding domain) (Carrozza et al., 2005b) and the Rco1 plant homeobox domain (PHD) (Li *et al.*, 2007b) (Figure 3).

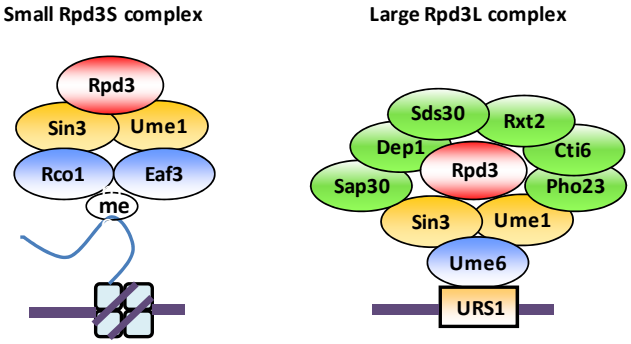

Figure 3. *S. cerevisiae* Rpd3 HDAC complexes.
Rpd3 can form a small Rpd3S complex (0,5MDa) or a large Rpd3L complex (1,2MDa). The Rpd3S complex recognizes the H3K36me mark though the cooperation of Eaf3 and Rco1 subunits, and is involved in repression of spurious transcription initiation at coding regions. The Rpd3L complex is recruited at promoters through DNA sequence specific regulators, such as Ume6, which recognizes the URS1 elements, and is involved in regulating transcription initiation.

Contrary to Rpd3S, the Rpd3L complex has a more promiscuous role in gene expression. Indeed, the majority of the Rpd3-dependent effects on gene expression are due to the Rpd3L complex, which is specifically recruited to promoters and functions in transcription initiation. Early studies showed that on the *INO1* promoter, the sequence-specific transcription repressor Ume6 recruits Rpd3 through interaction with Sin3 for the repression of transcription (Kadosh and Struhl, 1998). Also, Rpd3 is recruited by the repressor Ume6 at promoters of a large set of early meiotic genes for repressing gene expression (Fazzio et al., 2001;Goldmark et al., 2000) (Figure 3). Interestingly, Ume6 is not the only protein that targets the Rpd3L complex at promoters. It has been shown that the Rpd3L complex may be targeted to specific promoters by alternative specific DNA binding proteins that function as repressors, like Ash1 (Carrozza et al., 2005a).

Although many genetic studies have described Rpd3 as a negative regulator of transcription, other reports have demonstrated that Rpd3 is also required for transcription activation of several genes. For instance, it is required for induction of the osmoresponsive genes (de Nadal et al., 2004), as well as for activation of the DNA damage-inducible genes *RNR3* and *HUG1* (Sharma *et al.*, 2007). Although the precise mechanism by which Rpd3

activates transcription of genes induced by osmotic shock and DNA damage is not clear, it is known that Rpd3 is required for recruitment of polymerase at the promoters (de Nadal *et al.*, 2004;Sharma *et al.*, 2007). Remarkaby, induction of the anaerobic *DAN*/*TIR* genes requires Rpd3 dependent histone deacetylation for nucleosome displacement and stable binding of the Upc2 activator at promoters (Sertil *et al*., 2007). Moreover, Rpd3 has been shown to be required for induction the yeast transcriptional regulator *HAP1* gene (Xin *et al*., 2007), and the multidrug resistance efflux pump *PDR5* gene (Borecka-Melkusova et al., 2008). Thus, it seems that the Rpd3L complex can function as an activator of transcription in response to specific situations (see results and discussion).

2.- HEAT STRESS

2.1.- The damaging effects of heat

One of the most common environmental variations that cells suffer is the alteration of extracellular temperature. A sudden increase of external temperature has several deleterious effects on the internal organization of the cell, mainly due to aggregations of proteins and an imbalance of protein homeostasis (Reviewed in (Richter *et al.*, 2010;Riezman, 2004). Therefore, for all living organisms, increase of extracellular temperature represents a challenging problem for survival.

One of the major damages observed in response to heat stress are defects of the cytotoskeleton. These include the aggregation and reorganization of cytoskeleton filaments into stress fibers and, in the case of a sever heat stress, the collapse of cytoskeleton fiber networks. In addition to the cytoskeleton effects, organelles like Golgi and endoplasmic reticulum become fragmented and disassemble. Moreover, the number and integrity of mithochondria and lysosomes decrease, leading to a dramatic loss of ATP levels. The nucleoli, sites of ribosome assembly, swell and large granular depositions consisting of aggregating RNA and ribosomal proteins are formed. Finally, cellular membranes are as well severely affected by heat stress, due to aggregation of membrane proteins and an increase of membrane fluidity. The higher permeability of the membrane leads to a drop of cytosolic pH and changes in ion homeostasis (Richter *et al.*, 2010;Riezman, 2004). Together, all these effects stop growth and lead to a cell cycle arrest. Depending on the duration and severity of the heat stress, the accumulation of defects can result in cell death (Riezman, 2004). Consequently, the ability to respond rapidly to increases of extracellular temperature is essential for cell viability.

There are at least two specific mechanisms to sense temperature changes. On one hand, heat-induced accumulation of denatured proteins results in the activation of the heat shock transcription factor 1 (Hsf1), which controls the Heat Shock Response (see below) (Franzmann *et al.*, 2008;Nollen and Morimoto, 2002). On the other hand, temperature can be sensed directly through primary thermosensory structures such as DNA, RNA, proteins and lipids that either have a direct effect or lead to the activation of signal transduction pathways (Klinkert and Narberhaus, 2009;Nadeau and Landry, 2007). It is known that in response to heat stress, cells undergo a huge remodeling of the transcriptional program. Genome-wide expression analysis to explore how *S. cerevisiae* remodels gene expression in response to environmental changes show that upon a heat shock, the expression of approximately 15% of the genes rapidly change more than two fold (Causton et al., 2001;Gasch et al., 2000). In budding yeast, the remodeling of the gene expression program in response to heat stress mainly involves two transcription factors, the Hsf1 and the partially redundant transcription factors Msn2 and Msn4 (Estruch, 2000;Riezman, 2004) (see below). Moreover, the cell integrity pathway responds to heat shock by activating the MAPK Mpk1, which regulates the MADS-box Rlm1 transcription factor (Jung and Levin, 1999;Jung *et al.*, 2002;Nadeau *et al.*, 2007).

2.2.-The Heat Shock Response: Heat Shock Factor 1 (Hsf1)

The Heat Shock Response is a protective response to high temperature, broadly conserved from yeast to humans, and is characterized by a massive, rapid and transient induction of gene expression. The genes induced in the Heat Shock Response mostly encode for Heat Shock Proteins (Hsp), which are mainly molecular chaperones that relieve the defects of wrong folding and prevent protein aggregation to keep protein homeostasis. Moreover, they also function in a wide range of cellular processes, such as ubiquitination and proteolysis, vesicular transport, maintenance of the cell wall, carbohydrate metabolism and others (Richter et al., 2010;Riezman, 2004).

The Heat Shock Response is not only invoked in response to increases of temperature but also in response to other stresses, such as oxidative stress, heavy metals or ethanol (Courgeon *et al.*, 1984;Michel and Starka, 1986). In all eukaryotic cells, regulation of gene expression in response to heat stress and other stress conditions is controlled by Heat Shock Factors (Hsfs). In higher eukaryotes, such as mammals or plants, there are generally several forms of Hsfs, among which the Hsf1 is the master regulator of the heat shock response. However, in the case of lower organisms, such as yeast or *Drosophila*, there is only a single and

essential *HSF* gene, which encodes for *Hsf1* (Akerfelt *et al.*, 2010;Morimoto, 1998;Pirkkala *et al.*, 2001).

Heat Shock Factor 1 (HSF1)

Hsf1 is highly conserved in its overall fundamental structure from yeast to humans. It is composed of an N-terminal "winged" helix-turn-helix DNA binding domain followed by two hydrophobic repeat domains (HR-A and HR-B) and by a loosely defined regulatory domain. An additional hydrophobic repeat, HR-C, is localized further downstream and followed by a transactivating domain (Hashikawa and Sakurai, 2004;Hashikawa *et al.*, 2007;Vuister *et al.*, 1994a;Vuister *et al.*, 1994b). *S. cerevisiae* Hsf1 contains an additional N-terminal activation domain with respect to other organisms (Sorger and Pelham, 1988). The two activation domains differ in their relative activation potentials and functionalities during the time course of heat stress. Whereas the N-terminal activation domain mediates a transient response to elevated temperatures, the C-terminal activation domain is required to mediate both a transient and a sustained response (Nieto-Sotelo et al., 1990;Sorger and Pelham, 1988).

Hsf1 binds to gene promoters containing Heat Shock Elements (HSE). The typical HSE is composed of three or more sequential inverted repeats of the pentanucleotide motif nGAAn. In natural promoters this consensus sequence is rarely preserved. Currently, HSEs are separated into three groups: perfect, gapped and stepped HSEs. A perfect HSE has all three inverted repeats in a contiguous array (Xiao et al., 1991). Gapped HSEs have two consecutive inverted sequences, with the third sequence separated by 5 bp (Santoro et al., 1998). Stepped HSEs have 5-bp gaps separating all three modules (Yamamoto et al., 2005). In yeast, it has been described that stepped HSEs, especially with deviation from consensus, are bound by Hsf1 in an inducible manner (Erkine et al., 1999;Hahn et al., 2004), and require the cooperative action of other activators (Amoros and Estruch, 2001;Hahn *et al.*, 2004;Uffenbeck and Krebs, 2006), while perfect and stepped HSEs without mismatches are usually constitutively occupied by Hsf1 (Gross *et al.*, 1990;Hahn *et al.*, 2004). Approximately 3% of the yeast genome genes are direct targets of Hsf1, containing different HSE-composition at their promoters (Hahn et al., 2004). Finally, Hsf1 may bind to other specific sequences (Sakurai and Takemori, 2007;Yamamoto et al., 2005).

In some eukaryotic organisms such as mammals or *Drosophila*, trimerization of Hsf1 is a prerequisite for binding to HSEs. In these organisms, under physiological conditions, Hsf proteins are found as inactive monomeric forms in the cytoplasm. Then, in response to heat

stress they trimerize, enter into the nucleus and bind to HSEs (Akerfelt *et al.*, 2010). However, in *S. cerevisiae*, Hsf1 constitutively forms a trimer, although some reports suggest that perharps some form of regulated monomer-trimer transition functions as well (Erkina *et al.*, 2006;Erkine *et al.*, 1999). Another remarkable difference between yeast and other eukaryotic organisms is that the yeast Hsf1 has the capability to bind the HSEs in the absence of heat shock (Jakobsen and Pelham, 1988). However, the binding is not constitutive but rather increases with the degree of the temperature stress, depending on the HSE-composition at the promoters, as described above. Moreover, it is known that Hsf1 undergoes post-translational modifications, such as phosphorylation or SUMOylation (Guettouche *et al.*, 2005;Hietakangas *et al.*, 2003;Hilgarth *et al.*, 2004). Such modifications regulate HSF1 DNA binding (Anckar et al., 2006) and transcriptional activity (Hong et al., 2001), although their role is not well elucidated.

As mentioned before, it is believed that the disturbance of protein homeostasis leads to the activation of the Heat shock Response. Indeed, a model for the regulation of Hsf proteins by chaperones (known as the *chaperone tritation model*) has been proposed by Voellny and collaborators (Voellmy and Boellmann, 2007). This model explains the inactivation of heat shock transcription factors by unemployed chaperones, and their dramatic activation if chaperones are busy due to the presence of unfolded proteins. After the heat shock, once the cell returns to normal function, the excess of free chaperones leads again to the downregulation of the transcriptional regulator. Interestingly, additional mechanisms of Hsf1 regulation upon heat shock are becoming known for mammalian cells, such as a translation elongation factor, eEF1A, and non-coding RNAs, such as HSR1, which act in tandem to activate Hsfs during heat stress (Shamovsky *et al.*, 2006).

2.3.- The General Stress Response: Msn2/4 transcription factors

In *S. cerevisiae,* the remodeling of gene expression in response to heat stress also involves the General Stress Response (GSR) or Environmental Stress Response (ESR) (Figure 4). The GSR includes a large fraction of genes which are induced or repressed in common in response to a wide variety of stress environmental conditions, such as heat stress, oxidative stress, nutrient starvation or osmotic stress (Gasch, 2007;Gasch and Werner-Washburne, 2002). Genome-wide expression assays performed in yeast under different kind of stresses have shown that the GSR consists of approximately 600 repressed genes, that mainly include ribosomal protein genes and protein synthesis related genes, and 300 induced genes, that

codify for products mainly involved in antioxidant and carbohydrate metabolism, protein folding, cell signaling and other functions (Causton *et al.*, 2001;Gasch *et al.*, 2000).

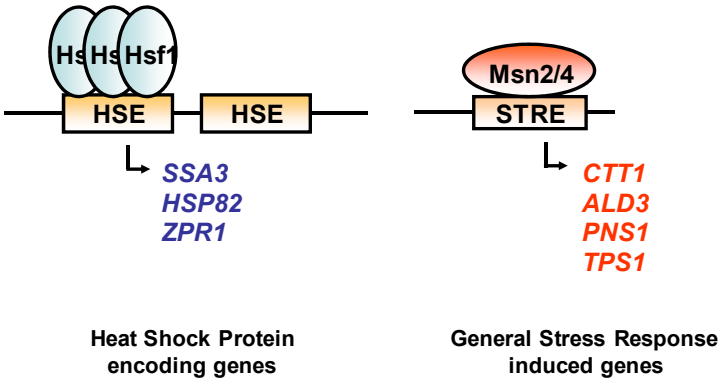

Figure 4. In *S. cerevisiae*, remodeling of gene expression upon heat stress involves different transcription factors.
When cells are exposed to heat stress, a rapid induction of gene expression is produced, known as the Heat Shock Response (HSR). The HSR is mainly controlled by Hsf1, which mainly induces genes that encode for chaperones, known as Heat Shock Proteins. Moreover, in response to a heat stress, the Msn2/4 transcription factors mediate induction of general stress-responsive genes.

The induction of GSR-dependent genes is controlled by the partially redundant transcription factors Msn2 and Msn4. Different signal transduction pathways can activate the Msn2/4 activators in response to different stresses. Examples of these pathways are the High Osmolarity Glycerol (HOG) pathway in response to osmotic shock (O'Rourke and Herskowitz, 2004;Schuller *et al.*, 1994) (see below), the MEC pathway (S-phase DNA damage check point) in response to DNA damage (Gasch *et al.*, 2001), and the Protein Kinase A (PKA) and Target of Rapamycin (TOR) pathways upon stress relief and glucose starvation (Beck and Hall, 1999;Zurita-Martinez and Cardenas, 2005). It is known that the PKA and TOR pathways are also involved in the regulation of Msn2/4 transcription factors in response to heat shock, although the upstream mechanisms that regulate the activators in these conditions are not defined (Beck *et al.*, 1999;Santhanam *et al.*, 2004).

Msn2/4 transcription factors

Msn2 and Msn4 are Cys2His2 Zinc finger-type transcription factors that recognize and bind the consensus Stress Response Element (STRE) sequence both *in vivo* and *in vitro* (Gorner *et al.*, 1998;Martinez-Pastor *et al.*, 1996). The STRE element is characterized by the core sequence CCCCT in either orientations and it is usually found in two or more copies at Msn2/4 target promoters (Moskvina *et al.*, 1998;Treger *et al.*, 1998). However, Msn2/4 may also bind

upstream of genes with other consensus sites in concert with other transcription factors (Ni *et al.*, 2009).

Although often considered to be functionally redundant in part because they activate gene expression through a common site, Msn2 and Msn4 are differentially regulated and may play distinct roles under different environmental conditions (Garreau *et al.*, 2000;Gasch *et al.*, 2002). So far, oscillatory nuclear transport of Msn2/4 has been considered as the primary regulatory step for Msn2/4 activity. Under physiological conditions, Msn2/4 transcription factors localize in the cytoplasm and rapidly accumulate in the nucleus upon stress (Gorner *et al.*, 1998;Jacquet *et al.*, 2003). PKA-dependent phosphorylation of the nuclear localization signal (NLS) of Msn2 has been demonstrated to inhibit nuclear import of Msn2 (Gorner *et al.*, 2002). On the other hand, PP2A phosphatase has been shown to inhibit nuclear export of Msn2 upon inactivation of TOR (Beck *et al.*, 1999;Santhanam *et al.*, 2004). In addition, Msn2/4 activity can also be regulated at the level of DNA binding (Hirata *et al.*, 2003), transactivation (Boy-Marcotte *et al.*, 2006), and degradation associated with transcription initiation (Durchschlag *et al.*, 2004;Lallet *et al.*, 2006;Lallet *et al.*, 2004).

The relative contribution of Msn2/4 to gene induction upon heat stress has been analyzed in several studies (Amoros *et al.*, 2001;Boy-Marcotte *et al.*, 1999). Msn2/4 transcription factors bind STRE sequences of a large array of genes partially overlapping the Hsf1-regulated genes (Hahn *et al.*, 2004). Indeed, it has been shown that whereas some heat shock genes (such as *SSA3, HSP82,* or *ZPR1*) are exclusively controlled by Hsf1, other heat shock genes (such as *HSP12, HSP104* or *HSP26*) are co-regulated by both Msn2/4 and Hsf1 transcription factors in response to heat shock. On the other hand, there are as well heat-responsive genes containing only STRE sequences at their promoters, exclusively controlled by the Msn2/4 transcription factors (such as *CTT1* and *ALD3*) (Amoros *et al.*, 2001;Boy-Marcotte *et al.*, 1999;Grably *et al.*, 2002).

2.4.- Regulation of chromatin in response to heat stress

Remodeling of chromatin structure is essential for the efficient regulation of transcription in response to stress (Jiang *et al.*, 2009;Shivaswamy *et al.*, 2008;Uffenbeck *et al.*, 2006). Indeed, the heat shock genes represent an excellent model to investigate chromatin remodeling events since, upon heat shock, these genes undergo an extensive and rapid nucleosome rearrangement at promoters and coding regions (Erkina *et al.*, 2008;Erkine and

Gross, 2003;Gross *et al.*, 1993;Petesch and Lis, 2008;Zhao *et al.*, 2005). However, the extent of histone displacement differs between different heat shock genes (Erkina *et al.*, 2006).

Several works during the last decade have analyzed the recruitment and function of chromatin remodelers at heat stress-responsive genes (Jiang *et al.*, 2009;Zanton and Pugh, 2006a). For example, it is known that the ATP-dependent chromatin remodeling SWI/SNF complex is directly recruited to heat shock gene promoters during heat induction and is required for histone displacement, Pol II recruitment and Hsf1 binding (Erkina *et al.*, 2010;Shivaswamy and Iyer, 2008;Zhao *et al.*, 2005). However, requirement of SWI/SNF complex is different for different heat-responsive genes. At Hsf1-dependent genes, such as *HSP82* or *SSA4*, the SWI/SNF action is not as critical as at genes redundantly controlled by Hsf1 and Msn2/4, such as *HSP12* (Erkina *et al.*, 2008). Moreover, recent studies showed a functional interplay between the chromatin remodeling complexes RSC, SWI/SNF and ISWI for nucleosome displacement and Pol II recruitment at the heat-induced promoters (Erkina *et al.*, 2010). However, again there are different contributions of these remodelers at Hsf1 or Msn2/4 dependent genes (Erkina *et al.*, 2010).

It is known that Hsf1 recruitment and activity at heat responsive promoters is required for histone displacement and Pol II loading (Erkina *et al.*, 2006). Moreover, chromatin remodeling at promoters is necessary for enhanced Hsf1 binding during stress (Erkina *et al.*, 2008;Erkina *et al.*, 2010). While the Hsf1 system is actively involved in chromatin remodeling events at gene promoters, the role of Msn2/4 in these processes is poorly understood. However, at promoters with HSEs and STREs, such as *HSP12* promoter, Msn2/4 transcription factors are required for chromatin remodeling and binding of Hsf1 (Erkina *et al.*, 2008).

Besides nucleosome remodeling, post-translational modifications of histones have also been coupled to heat-dependent changes in gene expression. Similarly to other genes, a mild and transient increase in histone acetylation is detected at *HSP82* promoter, prior to major nucleosome displacement upon heat shock (Zhao *et al.*, 2005). Interestingly, Erkina and Erkine demonstrated that the degree and kinetics of H3 acetylation differ drastically between different heat-responsive promoters, although the extend of chromatin acetylation often correlates with the extend of histone displacement (Erkina *et al.*, 2006). Therefore, histone acetylation has been associated with histone displacement and transcription activation of heat shock genes (Erkina *et al.*, 2006;Zhao *et al.*, 2005). Correspondingly, the Rpd3 histone deacetylase complex plays a repressive role at promoters and ORFs of Hsf1-dependent genes (Kremer and Gross, 2009). However, de Nadal *et al*. (2004) showed that Rpd3 is required for

activation of gene expression in response to heat stress (de Nadal *et al.*, 2004). Moreover, it has been reported that Rpd3 deacetylase modulates gene expression in response to heat stress and other environmental stresses, playing a role both in activation and repression of transcription (Alejandro-Osorio *et al.*, 2009). In the first part of this thesis, we analyze and discuss the role of the Rpd3 HDAC complex in regulation of transcription activation in response to heat stress.

3.- OSMOSTRESS

3.1.- Hyperosmotic shock

Another common type of stress at which yeast cells are exposed is the osmostress. In yeast, cytoplasmic water activity is of special importance. Water activity is defined as the chemical potential of free water in solution. To maintain an adequate cell volume and the appropriate conditions for biochemical reactions, the water activity of the cytoplasm and its organelles has to be lower than that of the surrounding media (Blomberg and Adler, 1992;Wood, 1999;Wood, 2006). Two different situations with respect to the external osmolarity can alter cell water activity: a decrease of external osmolarity (hypoosmotic shock) and an increase of external osmolarity (hyperosmotic shock). Upon a hypoosmotic shock, cells suffer a rapid water influx, cell swelling and increase in cell membrane turgor (Hohmann *et al.*, 2007). Contrary, in the case of hyperosmotic shock (in the literature, often synonymous with osmostress), there is a rapid cellular water outflow, which leads to cell shrinking. The water outflow results in an increased concentration of biomolecules and ions in the cell, which usually leads to an arrest of cellular activity (de Nadal *et al.*, 2002;Hohmann, 2002;Hohmann *et al.*, 2007).

In the yeast's natural environment, osmolarity can change widely and rapidly, which supposes a threat to the cell survival. For this reason, yeast cells have developed mechanisms to adapt and ensure survival in front of osmotic changes. Since passive flow of water occurs very fast, the survival mechanisms need to be activated within the first seconds after a sudden osmotic shift (Blomberg *et al.*, 1992). Adaptation to a hyperosmotic shock is an active process that comprises two distinct phases: first, detection of the external hyperosmolarity and second, development of appropriate responses to maintain optimal cellular activity. In *S. cerevisiae*, production and accumulation of chemically inert osmolytes, such as glycerol, allows the cells to increase the internal osmolarity and plays a central role in the process of

osmoadaptation. Therefore, yeast cells can be metabolically active and proliferate over a range of external water activity (de Nadal *et al.*, 2002;Hohmann, 2002;Hohmann *et al.*, 2007).

3.2.- Signaling pathways involved in osmoadaptation

In response to osmostress, *S. cerevisiae* activates different signaling pathways. By far, the best characterized system is the High Osmolarity Glycerol (HOG) response pathway. The HOG pathway is a stress-activated protein kinase (SAPK) cascade. SAPKs are members of the mitogen-activated protein kinases (MAPK) family and are key stress-signaling molecules in eukaryotic cells. Cells defective for this pathway or unable to activate it cannot survive in high-osmolarity medium (Brewster *et al.*, 1993). Thus, the role of the HOG pathway is to orchestrate a physiologically significant part of the yeast cell response to high osmolarity (de Nadal *et al.*, 2002;Hohmann, 2002;Hohmann *et al.*, 2007) (see below).

In addition to the HOG pathway, the cAMP-dependent Protein Kinase A (PKA) pathway affects gene expression upon hyperosmotic shock (Martinez-Pastor *et al.*, 1996;Norbeck and Blomberg, 2000). However, this pathway mediates a general stress response observed under different stress conditions, such as heat shock, nutrient starvation, high ethanol levels, oxidative stress and osmotic stress (Marchler *et al.*, 1993;Ruis and Schuller, 1995). Therefore, PKA does probably not respond directly to osmotic changes. In fact, it is not fully understood how the activity of PKA is regulated by stress. On the other hand, another signaling molecule has been involved in mediating osmoadaptation. The Sch9 protein kinase, which is a central regulator of nutrient responsive growth and longevity (Pascual-Ahuir and Proft, 2007;Smets *et al.*, 2010), contributes to induced gene expression together with the HOG pathway (Pascual-Ahuir and Proft, 2007). Finally, it has also been observed that osmostress stimulates production of phosphatidylinositol-3,5-bisphosphate, a molecule that might be a new type of phosphoinositide second messenger in an osmotic signaling system (Dove et al., 1997).

3.3.- The High Osmolarity Glicerol (HOG) pathway

The HOG pathway is the best-understood osmoresponsive signaling pathway in eukaryotes and one of the best-characterized MAPK cascades. It specifically responds to increases in extracellular osmolarity and is required for cell survival under these conditions. Activation of this pathway results in the initiation of a set of osmoadaptive responses, which include regulation of gene expression, control of cell cycle progression, regulation of protein

synthesis and control of metabolism (de Nadal *et al.*, 2002;Hohmann, 2002;Hohmann *et al.*, 2007). The HOG pathway has its equivalent system in mammalian cells, the p38 and the c-Jun N terminal kinase (JNK) pathways. The high conservation of function between HOG pathway and p38 pathway is illustrated by the fact that the corresponding human genes complement the yeast mutants in the yeast HOG pathway (Galcheva-Gargova *et al.*, 1994;Han *et al.*, 1994;Sheikh-Hamad and Gustin, 2004;Takekawa *et al.*, 1997).

3.3.1.-Components and organization

The MAPK pathways are organized in modules containing at least three types of protein kinases, which transmit signals by sequential phosphorylation events in a hierarchical way. The broad outline of the HOG pathway is fairly well described. It consists of five protein kinases: three MAPKKKs, which are Ssk2, Ssk22 (Maeda *et al.*, 1995) and Ste11 (Posas and Saito, 1997) activate a single downstream MAPKK, Pbs2, which is the specific activator of the MAPK Hog1 (Brewster *et al.*, 1993;Hohmann, 2002;Hohmann, 2009) (Figure 5).

The HOG pathway is activated by two independent upstream sensing mechanisms that lead to the activation of either the Ssk2 and Ssk22 or alternatively the Ste11 MAPKKKs. The first mechanism involves a “two component” osmosensor, composed of the Sln1-Ypd1-Ssk1 proteins. The transmembrane protein Sln1, homolog of bacterial two-component signal transducers, acts as the first two-component system, and the Ypd-Ssk1 pair functions as the second two-component system. Using a phospho-relay mechanism involving the Ypd1 and Ssk1 proteins, Sln1 is able to control the activity of Ssk1, which in turns interacts with and regulates the Ssk2 and Ssk22 MAPKKKs and subsequently the Pbs2 MAPKK activation (Posas *et al.*, 1996) (see figure 5). At normal osmolarity, the osmosensor Sln1 is active and the HOG pathway is downregulated. In a high osmolarity environment, Sln1 switches off resulting in activation of the HOG pathway. Genetic disruption of the *SLN1* gene is lethal, due to constitutively activation of the HOG pathway (Maeda *et al.*, 1994).

The second mechanism involves the transmembrane protein Sho1 and the putative osmosensors Hkr1 and Msb2 mucin-like proteins, as well as Opy2 (de Nadal *et al.*, 2007;Maeda *et al.*, 1995;Posas *et al.*, 1998;Tatebayashi *et al.*, 2007). Sho1 activation involves a rapid and transient formation of a protein complex at the cell surface, specifically at places of cell growth (Raitt *et al.*, 2000;Reiser *et al.*, 2000). First, Sho1 recruits Pbs2 to the cell surface. In addition, Cdc42 binds to both Ste20 and the Ste11-Ste50 complex. Consequently, Cdc42 brings activated

Ste20 to its substrate Ste11 MAPKKK (Tatebayashi *et al.*, 2006;Truckses *et al.*, 2006). Once activated, Ste11 activates Pbs2, which in turn, activates the Hog1 MAPK (Posas *et al.*, 1997).

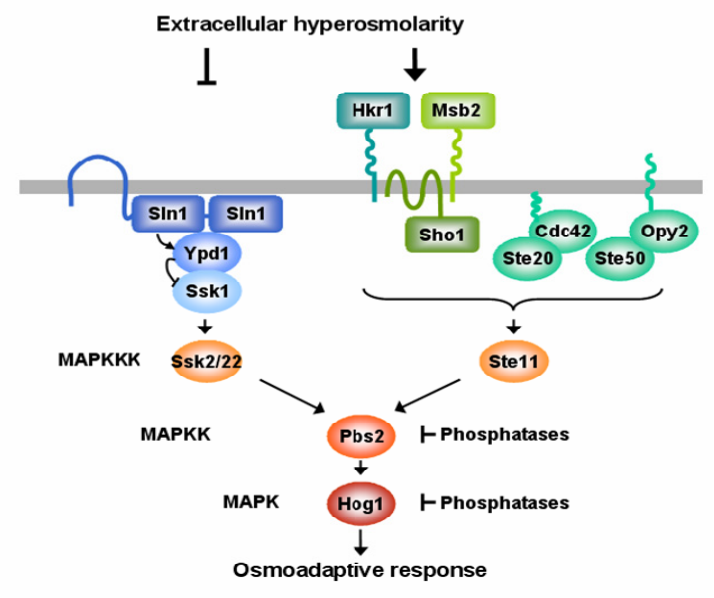

Figure 5. Schematic diagram of the yeast HOG pathway.
Two major independent upstream osmosensing mechanisms lead to the activation of specific MAPKKKs and converge on a common MAPKK, Pbs2. Under osmostress, Pbs2 activates the MAPK Hog1, which induces a set of osmoadaptive responses (de Nadal and Posas 2010).

Although the presence of two branches suggests redundant functions, it is unlikely that cells maintain two different complex pathways to activate Pbs2. It has been speculated that the two branches may interpret osmotic changes via different physical stimuli. Because components of the Sho1 branch are localized or recruited to places of active cell growth (Raitt *et al.*, 2000;Reiser *et al.*, 2000), the Sho1 branch would be involved in the sensing of osmotic changes during cell growth and expansion, while the Sln1 branch would be implicated in sensing osmotic changes in the environment (Hohmann, 2002;Hohmann, 2009).

3.3.2.- Signaling through the HOG pathway

Activation of the two upstream osmosensing branches converges on Pbs2 MAPKK phosphorylation and subsequent activation of Hog1 in the cytoplasm (Brewster *et al.*, 1993). While under normal conditions Hog1 appears to be distributed between the cytosol and the nucleus, Hog1 phosphorylation causes a rapid and marked concentration of the protein in the nucleus (Ferrigno *et al.*, 1998;Reiser *et al.*, 1999). Nuclear accumulation of Hog1 suggests that an important part of Hog1 actions might be located in the nucleus (see below). However, a

portion of activated Hog1 remains in the cytosol, where it is clear that also mediates regulatory effects (Bilsland-Marchesan *et al.*, 2000;Proft and Struhl, 2004;Thorsen *et al.*, 2006).

Hog1 phosphorylation and activation is a transient event (Jacoby *et al.*, 1997;Maeda *et al.*, 1994), suggesting that strong downregulatory mechanisms must exist to prevent extended Hog1 activation. Indeed, downregulation of signaling is absolutely required, because sustained activation of Hog1 is lethal (Maeda *et al.*, 1994;Vendrell *et al.*, 2011). In this regard the HOG pathway is controlled by the activity of phosphatases and various feedback systems. Hog1 and Pbs2 activities decrease by direct dephosphorylation through the type 2C serine/threonine phosphatases and the Tyr phopshatases Ptp2 and Ptp3 (Saito and Tatebayashi, 2004). Moreover, closure of the Fps1 glycerol channel has been proposed to act as a feedback mechanism that limits sensor activation in the HOG pathway (Hohmann *et al.*, 2007). In addition, phophorylation of Sho1 upon Hog1 activation seems to be important to decrease signaling though this branch (Hao *et al.*, 2007). Recently, in contrast to the typical assumption that the signal originates in response to the presence of the stimuli, it was reported that Sln1 branch shows high basal signaling that is restricted by a Hog1-mediated feedback mechanism. The high basal signaling of the pathway allows high efficiency of the signaling system, with fast responsive capability and high sensitivity to small variations in extracellular osmotic changes (Macia *et al.*, 2009).

3.3.3.- Physiological roles of Hog1

Once activated, the Hog1 MAPK induces a set of adaptive responses, essential for cell adaptation and survival to osmostress. Adaptive responses involve several aspects of cell biology such as gene expression, cell cycle progression, protein synthesis and metabolic adaptation (de Nadal *et al.*, 2002;Hohmann, 2002;Hohmann *et al.*, 2007).

Regulation of gene expression

One of the main functions of MAPKs in response to stress is the regulation of gene expression. Genome-wide transcription studies in *S. cerevisiae*, revealed that a large number of genes (5-7%) show significant and transient changes in their expression levels after a mild osmotic shock (Gasch *et al.*, 2000;O'Rourke *et al.*, 2004;Posas *et al.*, 2000;Rep *et al.*, 2000). Studies using a *hog1* mutant strain, averaging different stress conditions and different times of stress, showed that approximately two-thirds of the osmostress-induced genes require Hog1 to be transcribed (O'Rourke *et al.*, 2004;Posas *et al.*, 2000). Thus, the Hog1 MAPK plays a key role in gene expression regulation upon osmostress. The Hog1 dependent osmostress-induced

genes are implicated mainly in carbohydrate metabolism, general stress protection, protein biosynthesis, ion homeostasis, signal transduction and aminoacid metabolism (Posas *et al.*, 2000). The mechanisms by which the MAPK regulates transcription upon stress are diverse (see below).

Regulation of cell cycle progression

Progression through the cell cycle is critically dependent on the presence of nutrients and stress stimuli. In response to osmostress, Hog1 mediates a transient cell cycle arrest to permit the full development of adaptive responses before cell cycle progression (Clotet and Posas, 2007). The cell cycle is divided in four phases: G_1-phase, S-phase (DNA synthesis), G_2-phase and M-phase (mitosis). Transitions between G_1/S and G_2/M are strongly regulated in order to provide a successful cell division. This regulation is possible due to various checkpoints that monitor proper completion of each stage of the cell cycle.

In yeast, Cdc28 is the unique CDK (Cyclin Dependent Kinase) that controls cell cycle progression. Cyclins are the proteins responsible for binding and activating Cdc28, and are specific for G_1-phase, S-phase or G_2-phase. Cdc28 activity can be regulated through the synthesis and degradation of cyclins, through association with CDK inhibitors (Sic1 and Far1), and through phosphorylation and dephosphorylation by Swe1 and Mih1 (Dirick *et al.*, 1995;Lew and Reed, 1993;Nasmyth, 1993). Upon osmostress, Hog1 controls G_2 progression by a dual mechanism: the downregulation of cyclin Clb2 levels, and the direct phosphorylation of Hsl1, which regulates Swe1 and the G_2 checkpoint kinase (Cid *et al.*, 2001;Clotet *et al.*, 2006;Shulewitz *et al.*, 1999). Moreover, Hog1 also controls the G_1/S transition, as well through a dual mechanism that involves regulation of cyclin expression and targeting of the cell cycle regulatory protein Sic1 (Escote *et al.*, 2004;Schaber *et al.*, 2010). Finally, it was recently described that Hog1 also modulates a transient delay of S-phase by at least two different mechanisms. On one hand, Hog1 prevents firing of the replication origins by delaying accumulation of the S-phase cyclins. On the other hand, the MAPK prevents S-phase progression by interacting with components of the replication complex and delaying phosphorylation of the DNA polymerase Dpb2 subunit. (Yaakov *et al.*, 2009). The Hog1-dependet delay of replication could be important to allow Hog1 to induce gene expression during replication (Yaakov *et al.*, 2009).

Regulation of protein synthesis

In response to stress, there is a transient decrease in protein synthesis caused by a decrease in amino-acid uptake, repression of ribosomal protein genes and a decrease in translation efficiency (Norbeck and Blomberg, 1998;Uesono and Toh, 2002). Upon osmostress, the HOG pathway is involved in the control of protein translation. In this regard, the yeast Rck2 kinase, which resembles the mammalian CaM kinases, down-regulates protein synthesis after osmotic shock by directly regulating the elongation factor EF-2 in a Hog1-dependent manner (Bilsland-Marchesan *et al.*, 2000;Teige *et al.*, 2001).

An overall reduction of protein synthesis may be compatible with a transient inhibition of cell growth and proliferation caused by osmostress. However, expression of genes encoding important functions for stress adaptation is stimulated and their translation has to be ensured. This suggests that mechanisms exist that allow the preferential translation of subsets of mRNAs under certain conditions, although little is known about the molecular bases underlying this phenomenon (Melamed *et al.*, 2008).

Metabolic adaptation

One of the most important roles of the HOG pathway in osmostress adaptation is the control of glycerol accumulation. Upon osmostress, increased levels of glycerol (and other osmolytes) inside the cell lead to the replacement of excessive inorganic ions and the restoration of intracellular electrolyte homeostasis (Hohmann, 2002;Hohmann, 2009). Hog1 controls glycerol accumulation at several steps. For instance, Hog1 phosphorylates and activates the phosphofructo-2-kinase (PF2K) enzyme, which causes a stimulation of the upper part of glycolisis and increases the rate of glycerol production (Dihazi *et al.*, 2004). Moreover, Hog1 controls the activity of the glycerol export channel Fps1 (Thorsen *et al.*, 2006). In addition, activation of Hog1 leads to the induction of osmolyte-synthesizing genes, such as *GPD1* (encoding glycerol-3-phosphate dehydrogenase 1) and *TPS2* (encoding trehalose-6-phosphate synthatase) (Ruis *et al.*, 1995), which results in the increase of glycerol and trehalose levels, respectively. As well, Hog1 activation leads to the induction of Stl1 active glycerol uptake system, which allows accumulating glycerol from the surrounding media (Rep *et al.*, 2000).

On the other hand, Hog1 mediates a strong increase in expression of genes encoding enzymes involved in redox metabolism and defense from oxidative damage. Examples of such genes include *CTT1* (catalase T), *TRX2* (thioredoxin 2), *TTR1* (glutaredoxin), and acetaldehyde

dehydrogenase encoding genes (Blomberg, 2000;Rep *et al.*, 2000). However, the role of these redox enzymes upon these conditions is not well understood. Finally, it was recently shown that cells lower the intracellular sterol content in response to osmostress conditions, and the Hog1 MAPK is involved in the modulation of ergosterol homeostasis (Montanes *et al.*, 2011).

3.4.- Regulation of transcription by Hog1

The more traditional mechanism by which MAPKs modulate gene expression is the targeting and control of promoter-specific transcription factors (Karin and Hunter, 1995;Kyriakis and Avruch, 2001;Treisman, 1996). However, it is increasingly obvious that MAPKs do not only modulate transcription by targeting transcription factors alone, but may be part of the transcriptional machinery at target genes and modulate different steps of the transcription cycle (de Nadal and Posas, 2010;Martinez-Montanes *et al.*, 2010;Weake and Workman, 2010).

Exposure of yeast cells to high osmolarity results in direct phosphorylation of specific transcription factors by the Hog1 MAPK for stimulation of transcription initiation (de Nadal *et al.*, 2003;Proft *et al.*, 2001). Remarkably, upon osmostress, Hog1 is recruited to the osmoresponsive genes (Alepuz *et al.*, 2001;Pascual-Ahuir *et al.*, 2006;Pokholok *et al.*, 2006;Proft *et al.*, 2006). Once at promoters, Hog1 recruits Pol II, Ubp3 deubiquitinase, and SAGA and Mediator complexes for activation of gene expression (Alepuz *et al.*, 2003;Proft and Struhl, 2002;Sole *et al.*, 2011;Zapater *et al.*, 2007). In addition, histone deacetylation at promoters and chromatin remodeling is mediated by the Rpd3 HDAC complex and the RSC complex, which are recruited by the Hog1 MAPK (de Nadal *et al.*, 2004;Mas *et al.*, 2009). Finally, the role of Hog1 in the transcription cycle regulation is not limited to transcription initiation but rather extends to the process of transcriptional elongation (Mas *et al.*, 2009;Proft *et al.*, 2006) (Figure 7).

3.4.1.- Transcription factors downstream of Hog1

In yeast, five transcription factors that activate transcription have been proposed to be controlled by Hog1: the bZIP protein Sko1 (Proft *et al.*, 2001), the MADS box protein Smp1 (de Nadal *et al.*, 2003), the Hot1 protein (which does not belong to a known family of transcription factors) (Rep *et al.*, 1999), and the redundant zinc finger proteins Msn2 and Msn4 (Schuller *et al.*, 1994) (Figure 6). These factors are unrelated, and the mechanisms by which Hog1 regulates their function differ from one to another. There is a differential contribution in gene

expression of each of these factors, due to different promoter-specificity and different association dynamics to promoters (Capaldi *et al.*, 2008;Ni *et al.*, 2009). Moreover, several other transcription factors may regulate gene expression upon osmostress under the control of Hog1 (Miller *et al.*, 2011).

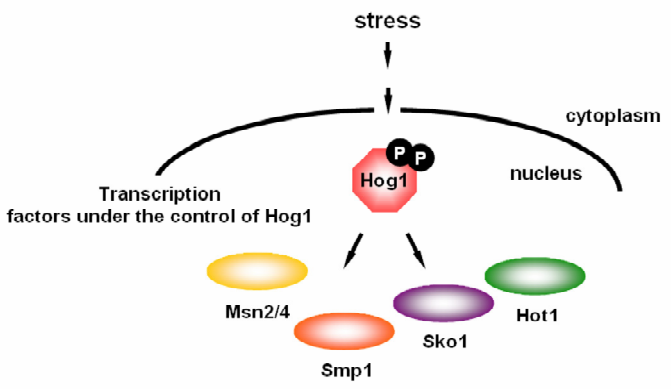

Figure 6. Transcription activators regulated by Hog1 upon osmostress.
Hog1 regulates induction of gene expression by controlling the following transcription factors: the redundant zinc finger proteins Msn2 and Msn4, the MAPD box Smp1, the bZip Sko1 and the Hot1 protein. These factors are unrelated and the mechanisms by which Hog1 regulates their function differ from one to another. Moreover, other transcription factors may regulate induction of osmoresponsive genes under the control of Hog1.

The bZIP protein Sko1

Sko1, also known as Acr1, is a protein belonging to the ATF/CREB family of AP1-related transcription factors (ATF) (Nehlin *et al.*, 1992;Vincent and Struhl, 1992), which in mammalian cells are known as cAMP response element CRE-binding (CREB) proteins (de Cesare D. and Sassone-Corsi, 2000). Such factors possess a leucine zipper (bZIP) domain for dimerization, and an adjacent basic transcription activation domain.

Sko1 inhibits transcription of several genes that are inducible by osmostress by recruiting the general corepressor complex Ssn6-Tup1 (Garcia-Gimeno and Struhl, 2000;Proft and Serrano, 1999). These genes are *GRE2*, involved in ergosterol metabolism, and *HAL1*, involved in ion homeostasis, among others. Release from Ssn6-Tup1 repression in response to osmostress requires direct phosphorylation of Sko1 by the Hog1 MAPK (Proft *et al.*, 2001). Hence, in a *hog1*Δ mutant, expression of these genes is low or undetectable and unresponsive to an osmotic shock. Interestingly, phosphorylation of Sko1 by Hog1 switches Sko1 activity from a repressing to an activating state, which involves recruitment of SWI/SNF and SAGA complexes (Guha *et al.*, 2007;Proft *et al.*, 2002). In addition, PKA phosphorylates Sko1 to

maintain its repressor activity. Thus, Sko1 transcriptional repression is controlled directly by the Hog1 MAPK in response to stress, and this effect is further modulated by an independent signaling mechanism through the PKA pathway (Proft *et al.*, 2001). Moreover, the signaling molecule Sch9 also directly phoshorylates Sko1 and is required for activation of Sko1-dependent genes (Pascual-Ahuir *et al.*, 2007).

The MADS box protein Smp1

Smp1 is a member of the MEF2C family of transcription factors (Yu *et al.*, 1992). It was identified by a genetic screen that isolated genes whose overexpression was able to induce an osmoresponsive reporter gene. It was shown that Smp1 and Hog1 interact and Smp1 is directly phosphorylated by Hog1 on several residues within its transactivation domain (de Nadal *et al.*, 2003). Phosphorylation of Smp1 by the MAPK is important for its function, since a mutant allele unable to be phosphorylated by the MAPK display impaired gene expression upon stress (de Nadal *et al.*, 2003). Furthermore, the same study reported that Hog1 could be playing a role during stationary phase by indirectly controlling Smp1 subcellular localization (de Nadal *et al.*, 2003).

The Hot1 transcription factor

Hot1 (High-Osmolarity-induced Transcription 1) is a transcription factor that was identified in a two-hybrid screening for proteins interacting with Hog1. Hot1 controls a small subset of genes, including *STL1*, which encodes a glycerol proton simporter, and *GPD1* and *GPP2*, involved in glycerol biosynthesis (Rep *et al.*, 1999). This transcription factor is a nuclear protein both under standard growth and osmotic stress conditions. It is always associated to the *GPD1* promoter, although its level on the promoter increases upon stress, in a Hog1-independent manner. The scenario seems to be different in the *STL1* promoter, where Hot1 binds only under osmostress and needs the Hog1 catalytic activity to become associated with the promoter (Alepuz *et al.*, 2001). Hot1 also appears to be bound at the promoters of *CTT1,* which encodes a cytosolic catalase, and *HSP12*, encoding a small heat shock protein, although its contribution to gene induction in these genes is minor (Alepuz *et al.*, 2001;Rep *et al.*, 1999).

Interestingly, the Hog1 kinase itself becomes associated with the stress-responsive promoters, and its recruitment is dependent on the presence of the transcription factor (Alepuz *et al.*, 2001). Moreover, Hot1 is directly phosphorylated by Hog1, although this phosphorylation is not critical for regulation and activation per se (Alepuz *et al.*, 2003). These data suggested that activation of gene expression by Hot1 must be driven by a mechanism

other than phosphorylation of the activator by the MAPK. Interestingly, Pol II binding at the Hot1-dependent promoters depends on Hog1 kinase activity (Alepuz *et al.*, 2003). Therefore, a mechanism for regulation of gene expression was proposed in which Hot1 protein acts as an anchor for the Hog1 MAPK, and Hog1 is the key factor for inducing gene expression, by directly recruiting the Pol II holoenzyme.

Msn2/4 transcription factors

As described before, in response to different stress conditions, the zinc finger-type proteins Msn2 and Msn4 induce the expression of the general stress-responsive genes (Martinez-Pastor *et al.*, 1996;Schmitt and McEntee, 1996). Msn2/4 localize in the cytoplam under standard growth conditions and translocate into the nucleus under stress. Once in the nucleus, they induce the expression of an extensive group of genes, including *CTT1* and *HSP12* (Capaldi *et al.*, 2008;Gasch *et al.*, 2000;Miller *et al.*, 2011). Although the activation of Msn2/4-dependent genes is dependent on the Hog1 MAPK in osmostress, the localization of Msn2/4 is unaffected in a *hog1*Δ mutant (Gorner *et al.*, 1998). Notably, Hog1 itself becomes associated with the promoters of these genes and this is dependent on both the Hog1 catalytic activity and the presence of the Msn2/4 transcription factors (Alepuz *et al.*, 2001).

3.4.2.- Hog1 is part of the transcription initiation machinery

Recruitment of the MAPK to target promoters is mediated through physical interactions with specific transcription factors that function as anchors to chromatin. As described, recruitment of Hog1 to the *STL1* promoter depends on the activator Hot1, whereas recruitment of the kinase to the *CTT1* promoter depends on the transcription factors Msn2 and Msn4. The appearance of Hog1 at target promoters indicates that Hog1 itself might be part in the activation process (Alepuz *et al.*, 2001). This possibility suggested a new dimension to gene regulation by signaling kinases.

As referring before, phosphorylation of both Smp1 and Sko1 transcription factors by Hog1 in response to osmotress is totally or partially required for transactivation. On the other hand, phosphorylation of Hot1 or Msn2/4 by the MAPK seems not to be essential for gene expression. However, recruitment of the active Hog1 MAPK by Hot1 and Msn2/4 activators is critical for gene expression. The observation that the kinase activity of Hog1 is needed for transcriptional activation but that the phosphorylation of some transcription factors is not an absolute requirement indicates that Hog1 must induce activation of gene expression by a mechanism other than phosphorylation of the activators. In fact, active Hog1 is required for

RNA polymerase II recruitment to osmoresponsive promoters in response to stress (Alepuz *et al.*, 2003). In addition, Hog1 is important to recruit two major transcriptional coactivators, which have been identified as essential for cell viability in osmostress: the SAGA and Mediator complexes. Whereas Mediator is crucial for proper gene expression under both mild and high osmostress conditions, the role of SAGA seems to be dependent on the strength of osmostress (Zapater *et al.*, 2007). Moreover, the Ubp3 ubiquitin protease is also recruited at promoters of osmoresponsive genes by Hog1 and is required to modulate transcription, indicating a clear role in ubiquitination/deubiquitination in gene expression (Sole *et al.*, 2011).

3.4.3.- Chromatin modification and Hog1

The specific association of Hog1 to stress-responsive promoters suggested that the MAPK could be playing a role in chromatin modification. A genetic screen designed to identify mutations that render osmosensitive cells showed that the Rpd3 histone deacetylase (HDAC) complex is essential for adaptation to omostress. Surprisingly, Rpd3 plays a global role for the induction of osmoresponsive genes, and this is dependent on Hog1. Indeed, more than 90% of the genes induced in response to osmostress, that are Hog1-dependent, have a significant reduction in expression levels in an *rpd3* mutant strain. Interestingly, Rpd3 becomes associated with the promoters of osmoresponsive genes in response to osmostress and its recruitment is dependent on Hog1. Moreover, histone deacetylation and binding of Pol II at the osmoresponsive promoters is reduced in an *rpd3* mutant (de Nadal *et al.*, 2004). Therefore, a model was proposed in which, upon osmostress, the specific transcription factors recruit Hog1 at the promoters, and Hog1 chromatin binding facilitates the direct recruitment of the Rpd3 deacetylase complex. Then, binding of the Rpd3 complex to specific promoters leads to histone deacetylation, entry of RNA Polymerase II and induction of gene expression (de Nadal *et al.*, 2004).

3.4.4.- Regulation of transcription elongation

It is described that Hog1 interacts with elongating RNA polymerase II and with general components of the Transcription Elongation Complex (TEC) (Proft *et al.*, 2006). Moreover, Hog1 is recruited to coding regions of the osmoresponive genes and travels with elongating polymerase (Pascual-Ahuir *et al.*, 2006;Pokholok *et al.*, 2006;Proft *et al.*, 2006). Interestingly, the selective association of Hog1 to ORFs is mediated by the 3' UTRs of osmoresponsive genes, although the underlying mechanism is unknown (Proft *et al.*, 2006). Hog1 recruitment at

coding regions is essential for an increased association of RNA Pol II to ORFs, suggesting that it directly affects the process of elongation (Proft *et al.*, 2006).

A major role of Hog1 in regulating transcriptional elongation is the direct recruitment of the chromatin remodeling RSC complex at coding regions of the osmoresponsive genes. *rsc* mutants display reduced Pol II association at ORFs, reduced stress gene expression and enhanced sensitivity to osmostress (Mas *et al.*, 2009). Moreover, Hog1 also recruits the Ubp3 ubiquitin protease at coding regions to modulate transcriptional elongation. Ubp3 mutants show reduced Pol II occupancy at ORFs and are defective in expression of osmoresponsive genes (Sole *et al.*, 2011).

Therefore, Hog1 plays roles in different steps of mRNA biogenesis. An overview of the roles of Hog1 for regulation of transcription is depicted in figure 7.

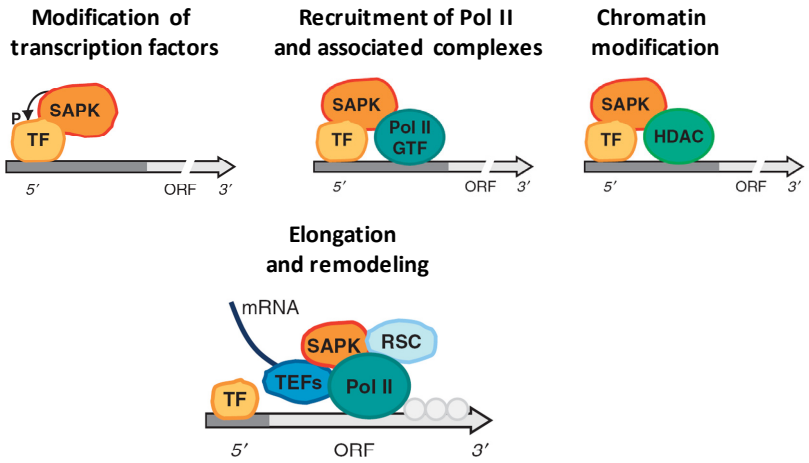

Figure 7. Hog1 regulates different phases of the transcription cycle.
The role of Hog1 in transcription initiation involves several mechanisms, such as modification of transcription factors, recruitment of coactivators and Pol II to osmoresponsive promoters, and targeting chromatin modification. Moreover, Hog1 acts as a stress specific transcription elongation factor and is involved in chromatin remodeling (de Nadal and Posas, 2010).

4.- THE MITHOCONDRIAL RETROGRADE SIGNALING

4.1.- Mitochondria and respiration

Mitochondrion is a semiautonomous, self-reproducing organelle in the cytoplasm of eukaryotic cells that has several copies of circular double-stranded mitochondrial DNA (mtDNA). This organelle is involved in a large number of processes, such as oxidative phosphorylation, signaling, cellular differentiation and cell death. However, its best characterized function is related to the production of energy. In fact, mitochondria have been defined as the *energy powerhouse of the cell* (Zhang and Qi, 2008). Interestingly, the mitochondrion is a highly dynamic organelle that continuously and dynamically change its metabolic activity, biomass and morphology (Chan, 2006;Detmer and Chan, 2007;Zhang and Qi, 2008).

Respiration is the set of catabolic reactions that are located mainly in mithocondria, to convert biochemical energy from nutrients into adenosine triphosphate (ATP). Aerobic respiration is a phenomenon that requires oxygen as a common oxidizing agent or electron acceptor (see below). However, some organisms live in places where oxygen is not always present. Then, instead of using aerobic respiration to obtain energy, organisms use anaerobic respiration or fermentative metabolism. In this regard, in *S. cerevisiae* the mitochondrial respiration is repressed as long as sufficient ATP can be produced by the fermentation of sugars (such as glucose), and is only activated in the presence of non-fermentable carbon sources (such as glycerol or acetate). This phenomenon is known as *glucose repression* or *catabolite repression* (Gancedo, 1998). Aerobic respiration can be broadly divided into three main pathways: glycolisis, occurring in the cytoplasm, tricarboxylic acid (TCA) cycle (also known as Krebs cycle) and electron transport chain, both of them occurring in the mitochondria.

Glycolisis

Glycolisis occurs within the cytosol of the cell, and it is the initial process of most carbohydrate catabolism. It consists of a sequence of reactions that converts one molecule of glucose into two molecules of pyruvate with the concomitant production of a relatively small amount of adenosine triphosphate (ATP). As a result, this process generates, besides ATP and NADH as cellular energy sources, a variety of six- and three-carbon intermediate compounds, which may be removed at various steps in the process for other cellular purposes, such as

anabolic processes. Pyruvate produced during glycolisis may be processed in the Krebs cycle as part of aerobic metabolism.

Because glycolisis does not use oxygen in its chemical process, organisms continue to carry out glycolisis in the absence of oxygen. Then, pyruvate obtained in the glycolisis pathway is not transported into mitochondria and metabolized by cellular respiration. Instead, pyruvate may undergo a process of fermentation. Cells oxidize pyruvate producing either lactic acid (lactic acid fermentation) or ethanol (alcoholic fermentation). The alcoholic fermentation is the one used by yeast cells and some kinds of bacteria, and converts sugars in ethyl alcohol and carbon dioxide in the cytosol.

Tricarboxylic Acid (TCA) cycle or Krebs cycle

Pyruvate molecules are taken up into the mitochondria through specific members of the mitochondrial carrier family. Once in the matrix of mitochondria, the oxidative conversion of pyruvate into acetyl-CoA occurs. Then, acetyl-CoA enters into the Krebs cycle, which conveys the carbon atoms to be oxidized to CO_2 for energy production. In addition, NADH and $FADH_2$, reduced nucleotides with high potential energy, are produced. These nucleosides can be oxidized to NAD^+ and FAD in the inner mitochondrial membrane during the oxidative phosphorylation chain to drive more ATP synthesis. Apart from supplying ATP through catabolic oxidative metabolism, the Krebs cycle also provides metabolic intermediates for biosynthetic processes.

Oxidative phosphorylation chain

In this process, electrons generated from NADH and $FADH_2$ oxidation are transferred through a series of carrier molecules called the electron transport chain. Finally, electrons are ultimately transferred to molecular oxygen, which is reduced to water. This is a multi-step redox process that consists of four respiratory enzyme complexes (Complex I or NADH deshydrogenase, Complex II or succinate deshydrogenase, Complex III or cytochrome bc_1, and Complex IV or cytochrome c oxidase) arranged in a specific orientation in the mitochondrial inner membrane. These reactions are coupled to the creation of a proton gradient across the mitochondrial inner membrane that is used by the F_OF_1 ATP synthase to make ATP from ADP and phosphate.

Although electron transport occurs with great efficiency, a small percentage of electrons are prematurely leaked to oxygen, resulting in the formation of reactive oxygen species (ROS).

High levels of ROS can oxidize cell constituents, such as lipids, proteins and DNA, and thus pose a threat to cell integrity. In mammal cells, such cumulative damage is hypothesized to be a factor in aging, neurological degeneration and cancer (Ames *et al.*, 2005;Beckman and Ames, 1998). However, it is important to highlight that mitochondria, in addition of being the main source of ROS, are as well the site for the detoxification of ROS in yeast. The importance of the antioxidant function of the mitochondria is reflected by the fact that the majority of mitochondrial mutants in yeast are hypersensitive to the hydrogen peroxide ROS (Grant *et al.*, 1997;Martínez-Pastor *et al.*, 2009). The antioxidant defense mechanisms of cells include glutathione, thioredoxin, superoxide dismutase (SOD), catalase and peroxidase enzymes (Gutteridge, 1994;Thorpe *et al.*, 2004). Therefore, mitochondrial metabolism is regulated under physiological conditions and upon environmental challenges which cause oxidative stress (Grant *et al.*, 1997;Martínez-Pastor *et al.*, 2009;Zuin *et al.*, 2008).

4.2.- Retrograde signaling: the RTG pathway

The maintenance of mitochondrial function depends on contributions from the mitochondrial genome and the nuclear genome. The mitochondrial genome encodes a limited but essential number of mitochondrial proteins, most of which are components of the oxidative phosphorylation apparatus, whereas the nuclear genome encodes the vast majority of the mitochondrial proteins. Therefore, it is required a communication between mitochondria and the nuclear genome (Poyton and McEwen, 1996). This nuclear-mitochondrial interaction involves what is known as the *anterograde* and *retrograde* communication. Anterograde communication is the control of the flow of material and signals from the nucleus and cytoplasm to the mitochondria. Contrary, retrograde communication is the response to changes in the functional state of the mitochondria by mediating changes in nuclear gene expression (Liao and Butow, 1993;Liu and Butow, 2006).

The most detailed information on the retrograde signaling, also termed RTG pathway, has been obtained with the budding yeast *S. cerevisiae*. In *S. cerevisiae*, the RTG pathway functions as a homeostatic or stress response mechanism, which senses mitochondrial dysfunction and transmits this information to the nucleus to regulate gene expression, leading to adjustment of various biosynthetic and metabolic activities (Liao *et al.*, 1993;Liu and Butow, 1999;Liu *et al.*, 2006). The retrograde response is induced when mitochondrial function is compromised. For instance, in mutants of one or more genes encoding enzymes of the TCA cycle, or when mitochondrial respiratory function declines or is absent, for instance in *petites*

mutants (also called rhoo and ρ° cells), characterized by large deletions in their mtDNA or a complete lack of the mitochondrial genome (Liao *et al.*, 1991;Small *et al.*, 1995).

4.3.- Positive regulators of the RTG pathway

In budding yeast, several positive and negative regulators of the retrograde response have been identified. Rtg1, Rtg2 and Rtg3 are components of the RTG pathway activation, whereas Mks1, Bmh1 and Bmh2 have been involved in the RTG pathway repression (Liu and Butow, 2006) (Figure 8). The functional characterization of the components and the molecular details that underlie the retrograde pathway regulation are still being clarified.

The Rtg1 and Rtg3 transcription factors

The RTG pathway relies on Rtg1 and Rtg3, which are basic helix-loop-helix leucine zipper (bHLH/Zip) type transcription factors that heterodimerize to activate transcription. Unlike most bHLH/Zip type transcription factors, which bind to the consensus sequence CANNTG, called “E box” (Massari and Murre, 2000), the Rtg1/3 complex binds to an unusual site, GTCAC, termed “R box” (Jia *et al.*, 1997;Liao *et al.*, 1993).

Rtg3 contains both N-terminal and C-terminal transcriptional activation domains (Rothermel *et al.*, 1997). The N-terminal transactivation domain is related to a so-called activation domain 1 (AD1), which is specific to type I HLH transcription factors (Massari *et al.*, 1996;Quong *et al.*, 1993). AD1 has a highly conserved LDFS motif, which activates transcription by recruitment of the SAGA histone acetyltransferase complex (Massari *et al.*, 1999). The C-terminal activation domain of Rtg3 also interacts with Cyc8(Ssn6)-Tup1, a well-known transcriptional repressor complex (Conlan *et al.*, 1999). Deletion of *CYC8* reduces RTG-dependent gene expression, suggesting that besides its well-established repressor activity, Cyc8 can also function as a coactivator in these genes (Conlan *et al.*, 1999). Therefore, Rtg3 has both N-terminal and C-terminal transcriptional activation domains, which function synergistically to activate transcription of RTG-target genes by recruiting coactivators.

Contrary to Rtg3, Rtg1 does not contain a transactivation domain. Moreover, only Rtg3 contains consensus residues that contact DNA (Jia *et al.*, 1997). However, transcriptional activation requires both Rtg1 and Rtg3 as they function as a hetreodimer because neither protein alone is able to bind to an R box site (Jia *et al.*, 1997). Therefore, it has been suggested

that Rtg1 may facilitate the binding of Rtg3 to R box sites, through its interaction with Rtg3 (Sekito *et al.*, 2000; Jia *et al.*, 1997).

The Rtg2 protein

Rtg2 is a cytoplasmatic protein with an N-terminal ATP binding domain, similar to that found in the Hsp70/actin/sugar kinase superfamily of ATP binding proteins (Koonin, 1994;Liao *et al.*, 1993). Integrity of this domain is essential for Rtg2 function (Liu *et al.*, 2003). Although there is much to be learned about this protein, current data suggest that Rtg2 may act as a proximal sensor of mitochondrial dysfunction and promotes the activation and nuclear accumulation of the Rtg1/3 complex in cells with respiratory dysfunction (Sekito *et al.*, 2000).

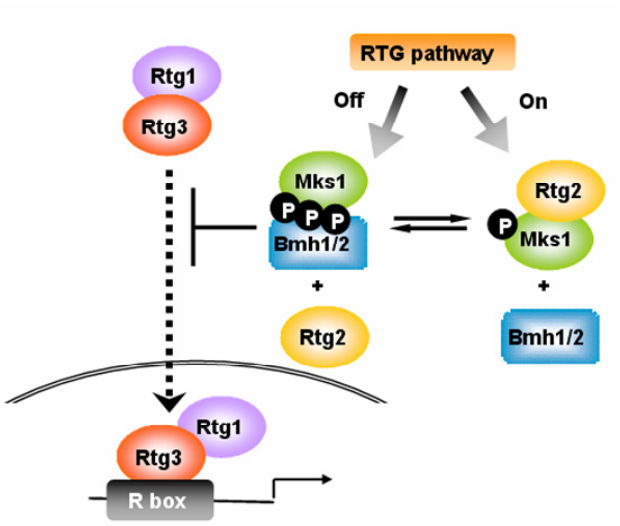

Figure 8. Regulation of the RTG pathway by a dynamic interaction between Rtg2 and the negative regulator Mks1.
Binding of Rtg2 to Mks1 leads to activation of the RTG pathway. Interaction of Mks1 with Bmh1/2 after its dissociation from Rtg2 leads to inhibition of nuclear translocation of Rtg1/3 complex (adapted from Butow and Avadhani 2004).

4.4.- Regulation of the RTG pathway

Activation and nuclear translocation of Rtg1/3

A key regulatory step in the regulation of the RTG pathway is the translocation of Rtg1/3 proteins from the cytoplasm to the nucleus. When the RTG pathway is inactive, Rtg1 and Rtg3 are sequestered together in the cytoplasm. Upon activation of the RTG pathway, Rtg3 undergoes changes in its phosphporylation state and translocates into the nucleus together with Rtg1 (Sekito *et al.*, 2000). Once in the nucleus, Rtg1 and Rtg3 bind as a heterodimer to the

R box sequences in the promoter region of target genes, for transcription activation (Jia *et al.*, 1997;Liu *et al.*, 1999) (see Figure 8).

It has been described that cytoplasmatic Rtg3 is phosphorylated at multiple sites and, in response to respiratory dysfunction (i.e. in ρ^{o} mutants), Rtg3 becomes partially dephosphorylated and localize into the nucleus (Sekito *et al.*, 2000). These events are dependent on Rtg2, since in *rtg2*Δ cells, Rtg3 is hyperphosphorylated and cannot be translocated to the nucelus (Sekito *et al.*, 2002;Sekito *et al.*, 2000). Rtg3 is a phosphoprotein with multiple phospho-sites and a complex pattern of phosphorylations. The RTG pathway is known to be negatively regulated by the TOR pathway (Wullschleger *et al.*, 2006) (see below). Contrary to what happens under respiratory dysfunction, in response to rapamycin treatment, which inhibits TOR signaling, the RTG pathway is activated by the phosphorylation of Rtg3 (Komeili *et al.*, 2000).

The basic region of the Rtg3 bHLH domain also functions as a nuclear localization signal (NLS) (Sekito *et al.*, 2000). Of note, cells lacking *RTG3* show a constitutive cytoplasmic localization of Rtg1. On the other hand, deletion of *RTG1* results in a constitutive nuclear localization of Rtg3 even in *rtg2*Δ cells, suggesting a negative regulatory role of Rtg1 for nuclear translocation (Komeili *et al.*, 2000;Sekito *et al.*, 2000). Taken together, Rtg1 and Rtg3 are interdependent for the dynamic intracellular localization.

Dynamic interaction of Mks1 with Rtg2 or Bmh1/2

Rtg2 is required for the activation and nuclear translocation of Rtg1/3 complex but it does not directly act on the Rtg1/3 proteins. The role of Rtg2 can be explained by its dynamic interaction with Mks1, a cytoplasmic protein that functions as a negative regulator of the RTG pathway. As described in figure 8, when the RTG pathway is active, Mks1 is present in a largely dephosphorylated form, in a complex with Rtg2. However, when the RTG pathway is inactive, Mks1 becomes phosphorylated, no longer interacts with Rtg2, and forms a repressive complex with the redundant 14-3-3 proteins, Bmh1 and Bmh2. This repressive complex sequesters Rtg1/3 complex in the cytoplasm (Dilova *et al.*, 2004;Ferreira Junior *et al.*, 2005;Liu *et al.*, 2003).

As mentioned before, the integrity of the N-terminal ATP binding domain of Rtg2 is essential for its function, probably due to its ability to regulate the interaction of Rtg2 C-terminal region with Mks1 (Liu *et al.*, 2003). However, the signals that regulate the interaction of Rtg2 and Mks1 are not completely understood. Liu and Butow suggested the possibility that

Rtg2 may sense ATP. A defect in mitochondrial respiratory function would reduce cellular ATP levels, thus favoring an interaction between Rtg2 and Mks1 (Liu *et al.*, 2006).

4.5.- Physiological functions of the RTG pathway

One of the main functions of the RTG pathway is to maintain homoeostasis of the non-essential aminoacid glutamate. A clear indication of the RTG pathway involvement in glutamate metabolism is that all *rtg* mutants are glutamate auxotrophs (Jia *et al.*, 1997;Liao *et al.*, 1993). Indeed, the RTG pathway activates the expression of genes involved in glutamate biosynthesis. For instance, genes encoding the three first enzymes of the TCA cycle, namely *CIT1* (encoding citrate sinthase), *ACO1* (encoding aconitase), and *IDH1/*2 (encoding NAD+-dependent isocitrate dehydrogenase) (Liu *et al.*, 1999). In addition, *CIT2*, which encodes a peroxisomal isoform of citrate synthase, is the prototypical target gene regulated by the RTG pathway (Liao *et al.*, 1991). All these gene products control the production of α-ketoglutarate, the direct precursor for glutamate (Figure 9).

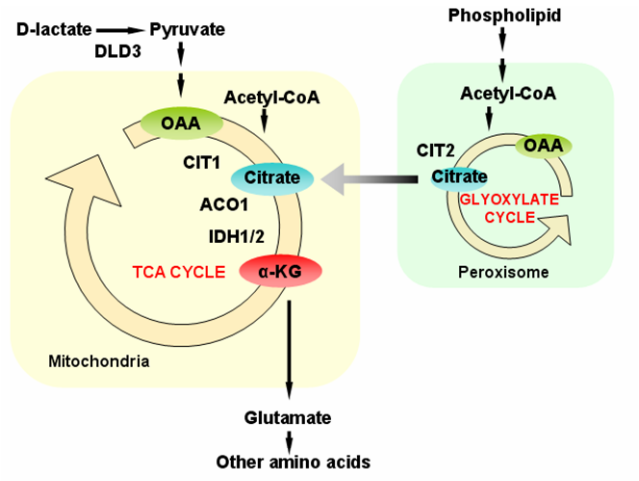

Figure 9. Metabolic reconfiguration in respiratory-deficient cells.
Only genes involved in glutamate biosynthesis and anaplerotic pathways are shown. The retrograde response leads to a metabolic reconfiguration to ensure sufficient levels of α-ketoglutarate for glutamate synthesis, to meet the demand of nitrogen for biosynthetic reactions (adapted from Butow and Avadhani 2004).

Glutamate, together with its downstream metabolite glutamine, provides all the nitrogen used in biosynthetic reactions (Magasanik and Kaiser, 2002). Consistently, increasing glutamate or glutamine production via a mutation in *MKS1*, which leads to activation of the RTG pathway, results in a 43% increase of total cellular aminoacids (Chen and Kaiser, 2002). Therefore, one main function of the RTG pathway is to ensure sufficient levels of α-

ketoglutarate for glutamate synthesis, in order to meet the demand of nitrogen supply for biosynthetic reactions. When cells have compromised mitochondrial function or key metabolic pathways are disrupted, decreased levels of glutamate and glutamine activate the RTG pathway (Butow and Avadhani, 2004). In contrast, when increased extracellular levels of glutamate or glutamine are sensed by cells, the RTG pathway is repressed, which can be viewed as a feedback control of glutamate biosynthesis (Komeili *et al.*, 2000;Liao *et al.*, 1993;Liu *et al.*, 2003). The biochemical mechanisms by which glutamate or glutamine repress the RTG pathway are not known. In this regard, glutamine has been proposed to signal the TOR pathway, which is involved in nutrient and nitrogen sensing (Wullschleger *et al.*, 2006), to inhibit Rtg1 and Rtg3 transcription factors (Crespo *et al.*, 2002;Komeili *et al.*, 2000).

Another function of the RTG pathway is the maintenance of the mtDNA through regulation of the RTG-target gene *ACO1,* thus linking retrograde signaling to mitochondrial biogenesis (Chen *et al.*, 2005). Indeed, Aco1 was identified among proteins cross-linked to mtDNA and mtDNA unstablility was observed in *aco1* deficient cells (Kaufman *et al.*, 2000;McCammon *et al.*, 2003). Therefore, under the transcriptional control of the RTG pathway, aconitase clearely diplays two different activities: an enzymatic activity in the TCA cycle, and a mtDNA mainenance activity. Moreover, another target gene that shows a robust RTG response is *DLD3*, encoding a cytoplasmic isoform of D-lactate dehydrogenase (Chelstowska *et al.*, 1999). A physiological function for the retrograde expression of *DLD3* is unknown, but it is likely involved in regenerating NAD^+ due to the potential buildup of NADH in respiration deficient cells. Finally, besides mitochondrial homeostasis, retrograde signaling is involved in nutrient sensing, growth control, programmed cell death, aging, development and other signaling processes that function in metabolic and organelle homeostasis (Liu and Butow, 2006). In the second part of this thesis, we study the role of Hog1 in regulating the Rtg1/3 transcription factors for RTG-dependent gene expression in response to osmostress.

OBJECTIVES

The basic objective of the research in our laboratory is to understand the mechanisms by which cells respond and adapt to environmental stress, such as heat stress or osmostress. In response to such environmental stresses, a major adaptive response is the regulation of gene expression. Therefore, we aimed to investigate and further characterize mechanisms for gene expression regulation in response to heat stress and osmostress.

The specific objectives of this PhD project were:

I. To characterize the yeast adaptive response to heat stress.

II. To characterize the role of the Rpd3 HDAC complex in the regulation of transcription activation in response to heat stress.

III. To identify novel factors involved in the Hog1-mediated osmostress adaptive response.

IV. To characterize the molecular mechanisms by which Hog1 regulates the Rtg1/3 transcription factors for transcriptional activation upon osmostress.

RESULTS AND DISCUSSION

The Rpd3L HDAC complex is essential for the heat stress response in yeast

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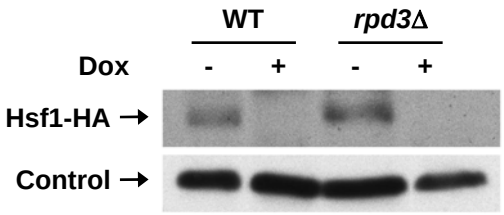
Figure S1

A



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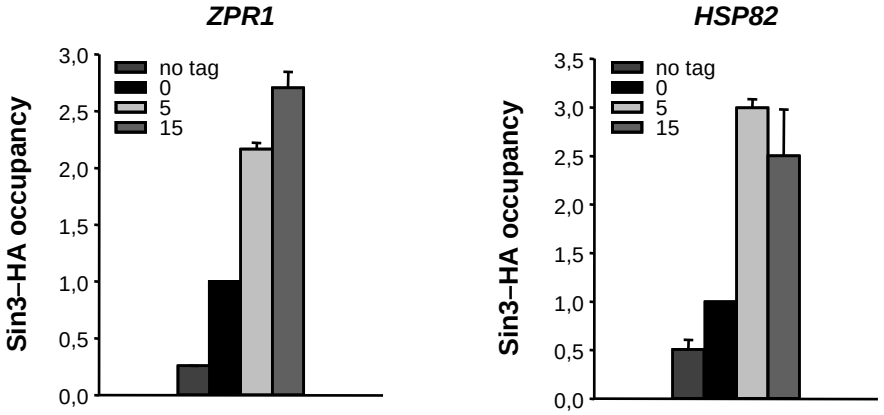

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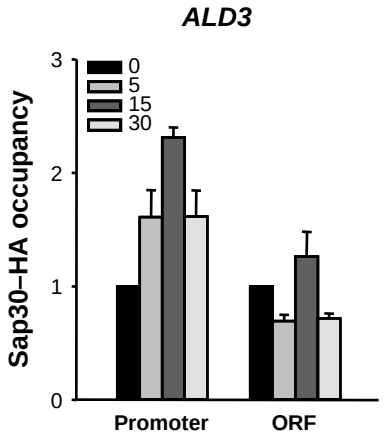

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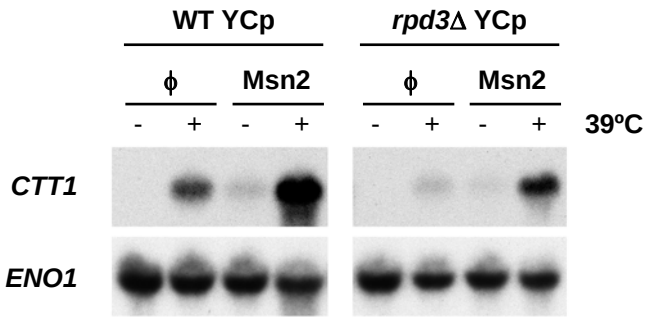
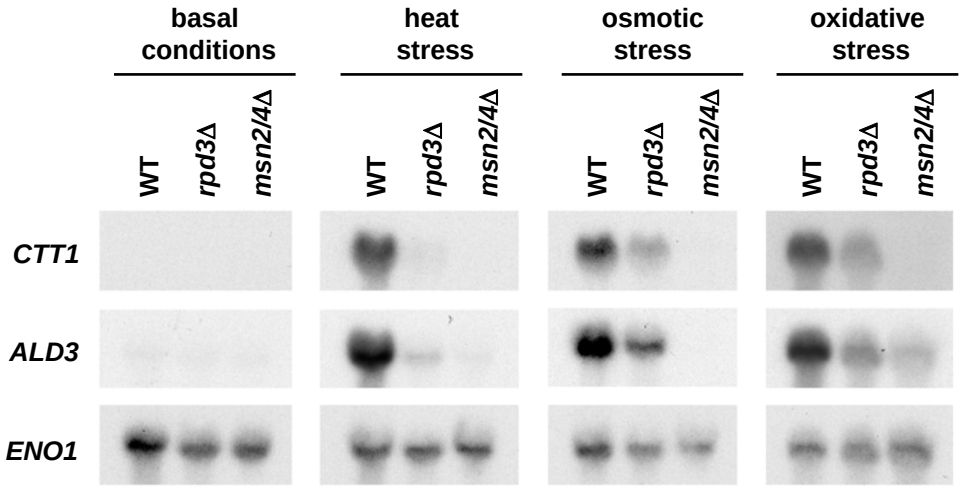

WT YCp
rpd3Δ YCp
ϕ
Msn2
ϕ
Msn2
- + - + - + - +
39ºC
CTT1
ENO1

B


basal conditions
heat stress
osmotic stress
oxidative stress
WT
rpd3Δ
msn2/4Δ
CTT1
ALD3
ENO1

SUPPLEMENTARY FIGURE LEGENDS

Fig. S1 A. Hsf1 levels under the control of the Tet-off promoter. Hsf1-TetOFF-HA tagged in wild type and *rpd3*Δ strains were treated with or without doxycicline (dox) at final concentration of 10μg/ml for 3 hours. TCA extracts and western analysis with a HA monoclonal antibody were performed. **B.** Rpd3 binding to Hsf1-dependent genes. Wild type strain carrying Sin3-HA was grown to mid-log phase and heat shocked at 39ºC for the indicated times (minutes). Proteins were immunoprecipitated with anti-HA monoclonal antibodies and binding to *ZPR1* and *HSP82* promoters was analyzed. Results are shown as the fold induction of treated against the untreated cultures normalized to a telomere internal control. **C.** Binding of the Rpd3L deacetylase complex to the *ALD3* Msn2/4-dependent promoter and ORF. Wild type strain carrying Sap30-HA was treated as in (B). Proteins were immunoprecipitated with anti-HA monoclonal antibodies and binding to *ALD3* promoter and ORFs was analyzed. Results are shown as the fold induction of treated against the untreated cultures normalized to a telomere internal control.

Fig. S2 A. The Rpd3 complex is required to activate Msn2/4-dependent genes per se. Wild-type and *rpd3*Δ mutant strains carrying an empty plasmid or overexpressing Msn2 under the *ADH1* promoter were grown in minimal medium up to mid-log phase and subjected to 39ºC for 15 minutes. Total RNA was assayed by northern blot for transcript levels of *CTT1* and *ENO1* as a loading control. **B.** The Rpd3 complex is required for the transcriptional response upon different cellular stresses. Wild-type, *rpd3*Δ and *msn2/4*Δ mutant strains were grown in YPD medium up to mid-log phase and subjected to heat stress (39ºC, 15 minutes), osmotic stress (0.4M NaCl, 15 minutes) and oxidative stress (1mM H_2O_2, 60 minutes). Total RNA was assayed by northern blot for transcript levels of the indicated genes and *ENO1* as a loading control.

Table S1 List of yeast mutant strains that rendered cellular growth impairment in heat-stress conditions. From 4.644 viable haploid deletion mutants, 277 rendered cells unable to growth at high temperature. Mutants picked from a thermo-sensitive high-throughput genetic screen were individually spotted in YPD plates at 25ºC or 39ºC and classified depending on the degree of heat-sensitive phenotype. Data are classified by phenotype degree, meaning strong (absence of growth) **(A)**, medium **(B)** and weak (formation of smaller colonies than wild type strain) **(C)** thermo-sensitive phenotype.

A. Strong heat-sensitive phenotype

ORF code	Gene name	Gene description
YAL021C	*CCR4*	Transcriptional regulator
YBL007C	*SLA1*	Cytoskeleton assembly control protein
YBL024W	*NCL1*	tRNA (cytosine-5-)-methyltransferase
YBL058W	*SHP1*	Potential regulatory subunit for Glc7p
YBL094C		
YBR065C	*ECM2*	Protein involved in cell wall biogenesis and architecture
YBR106W	*PHO88*	Protein involved in phosphate transport
YBR119W	*MUD1*	U1 snRNP-specific A protein
YBR126C	*TPS1*	Alpha,alpha-trehalose-phosphate synthase, 56 KD subunit
YBR171W	*SEC66*	ER protein-translocation complex subunit
YBR173C	*UMP1*	Proteasome maturation factor
YCL032W	*STE50*	Pheromone response pathway protein
YCL036W	*GFD2*	Great for Full DEAD box protein activity
YCR002C	*CDC10*	Cell division control protein
YCR009C	*RVS161*	Protein involved in cell polarity development
YCR020W-B	*HTL1*	High-Temperature Lethal protein
YCR063W	*BUD31*	Protein involved in bud-site selection
YDL006W	*PTC1*	Protein serine/threonine phosphatase 2c
YDL047W	*SIT4*	Ser/thr protein phosphatase
YDL081C	*RPP1A*	60S large subunit acidic ribosomal protein a1
YDL090C	*RAM1*	Protein farnesyltransferase, beta subunit
YDL116W	*NUP84*	Nuclear pore protein
YDL160C	*DHH1*	DExD/H-box helicase, stimulates mRNA decapping
YDL182W	*LYS20*	Homocitrate synthase
YDL191W	*RPL35A*	60S large subunit ribosomal protein
YDL192W	*ARF1*	Small GTP-binding protein of the ARF family
YDL232W	*OST4*	Oligosaccharyltransferase subunit
YDR017C	*KCS1*	Potential transcription factor of the BZIP type
YDR069C	*DOA4*	Ubiquitin-specific protease
YDR129C	*SAC6*	Actin filament bundling protein, fimbrin
YDR136C	*VPS61*	Vacuolar Protein Sorting
YDR137W	*RGP1*	Reduced growth phenotype protein
YDR173C	*ARG82*	Arginine metabolism transcription factor
YDR195W	*REF2*	RNA 3`-end formation protein
YDR207C	*UME6*	Negative transcriptional regulator
YDR260C	*SWM1*	Developmentally regulated gene, is required for spore wall assembly
YDR264C	*AKR1*	Ankyrin repeat-containing protein
YDR388W	*RVS167*	Reduced viability upon starvation protein
YDR457W	*TOM1*	E3 ubiqitin ligase required for G2/M transition
YDR470C	*UGO1*	Outer membrane protein required for mitochondrial fusion

ORF code	Gene name	Gene description
YEL036C	*ANP1*	Protein required for protein glycosylation in the golgi
YEL044W	*IES6*	Ino Eighty Subunit
YEL059W	*SOM1*	Protein required for mitochondrial Imp1 peptidase function
YER122C	*GLO3*	Zinc finger protein
YER155C	*BEM2*	GTPase-activating protein
YFL001W	*DEG1*	Pseudouridine synthase
YFR036W	*CDC26*	Subunit of anaphase-promoting complex (cyclosome)
YGL020C	*GET1*	Protein involved in determination of mitochondrial structure
YGL038C	*OCH1*	Alpha-1,6-mannosyltransferase
YGL070C	*RPB9*	DNA-directed RNA polymerase II, 14.2 KD subunit
YGL072C		
YGL167C	*PMR1*	Ca++-transporting P-type ATPase located in Golgi
YGL168W	*HUR1*	HydroxyUrea Resistance
YGL173C	*KEM1*	Multifunctional nuclease
YGL218W		
YGL240W	*DOC1*	Component of the anaphase promoting complex
YGL244W	*RTF1*	Regulates DNA binding properties of TBP
YGL246C	*RAI1*	Nuclear protein that binds to and stabilizes the exoribonuclease Rat1p
YGR092W	*DBF2*	Ser/thr protein kinase related to Dbf20p
YGR104C	*SRB5*	DNA-directed RNA polymerase II holoenzyme and SRB subcomplex subunit
YGR135W	*PRE9*	20S proteasome subunit Y13 (alpha3)
YGR163W	*GTR2*	GTp binding protein Resemblance
YHR030C	*SLT2*	Ser/thr protein kinase of MAP kinase family
YHR041C	*SRB2*	DNA-directed RNA polymerase II holoenzyme and SRB subcomplex subunit
YHR081W	*LRP1*	Like rRNA Processing protein
YHR167W	*THP2*	Subunit of the THO complex
YIL076W	*SEC28*	Epsilon-COP coatomer subunit
YJL062W	*LAS21*	Protein required for addition of a side chain to the GPI core structure
YJL063C	*MRPL8*	Mitochondrial ribosomal protein, large subunit
YJL075C	*APQ13*	
YJL080C	*SCP160*	Protein required for maintenance of exact ploidy
YJL115W	*ASF1*	Anti-silencing protein
YJL140W	*RPB4*	DNA-directed RNA polymerase II, 32 kDa subunit
YJL179W	*PFD1*	Prefoldin subunit 1
YJL183W	*MNN11*	Related to Mnn10p, and in a complex containing other MNN gene products
YJL184W	*GON7*	Transfer of mannosylphosphate groups onto N-ed oligosaccharides
YJR090C	*GRR1*	Protein required for glucose repression and for glucose and cation transport
YKL054C	*DEF1*	Coordinates repair and RNA pol II proteolysis in response to DNA damage
YKL113C	*RAD27*	ssDNA endonuclease and 5`-3`exonuclease
YKL126W	*YPK1*	Ser/thr-specific protein kinase
YLR039C	*RIC1*	Protein involved in transcription of ribosomal proteins and ribosomal RNA
YLR182W	*SWI6*	Transcription factor
YLR200W	*YKE2*	Gim complex component
YLR226W	*BUR2*	Divergent CDK-cyclin complex
YLR233C	*EST1*	Telomere elongation protein
YLR242C	*ARV1*	Protein involved in sterol uptake and distribution into the plasma membrane
YLR261C	*VPS63*	Vacuolar Protein Sorting
YLR262C	*YPT6*	GTP-binding protein of the rab family
YLR315W	*NKP2*	Non-essential Kinetochore Protein
YLR322W	*VPS65*	Vacuolar Protein Sorting
YLR337C	*VRP1*	Verprolin
YLR338W	*OPI9*	OverProducer of Inositol
YLR370C	*ARC18*	Subunit of the Arp2/3 complex
YLR396C	*VPS33*	Vacuolar sorting protein
YML035C	*AMD1*	AMP deaminase
YML062C	*MFT1*	Mitochondrial fusion target protein
YMR021C	*MAC1*	Metal binding activator
YMR032W	*HOF1*	Protein involved in cytokinesis
YMR038C	*CCS1*	Copper chaperone for superoxide dismutase Sod1p
YMR060C	*SAM37*	Component of the mitochondrial outer membrane sorting and SAM complex

ORF code	Gene name	Gene description
YMR091C	*NPL6*	Nuclear protein localization factor
YMR198W	*CIK1*	Spindle pole body associated protein
YMR205C	*PFK2*	6-phosphofructokinase, beta subunit
YMR231W	*PEP5*	Vacuolar biogenesis protein
YMR263W	*SAP30*	Subunit of the histone deacetylase B complex
YMR307W	*GAS1*	Glycophospholipid-anchored surface glycoprotein
YMR326C		
YNL098C	*RAS2*	GTP-binding protein
YNL171C		
YNL199C	*GCR2*	Glycolytic genes transcriptional activator
YNL236W	*SIN4*	Global regulator protein
YNL307C	*MCK1*	Ser/thr/tyr protein kinase
YNL330C	*RPD3*	Histone deacetylase B
YNR052C	*POP2*	Protein required for glucose derepression
YOL004W	*SIN3*	Transcription regulatory protein
YOL051W	*GAL11*	DNA-directed RNA polymerase II holoenzyme and SRB subcomplex subunit
YOL076W	*MDM20*	Necessary for mitochondrial inheritance and organisation of the actin cytoskeleton
YOR001W	*RRP6*	Exonuclease component of the nuclear exosome
YOR026W	*BUB3*	Cell cycle arrest protein
YOR027W	*STI1*	Stress-induced protein
YOR035C	*SHE4*	Protein required for mother cell-specific gene expression
YOR036W	*PEP12*	Syntaxin (T-SNARE), vacuolar
YOR141C	*ARP8*	Actin-Related Protein
YOR209C	*NPT1*	Nicotinate phosphoribosyltransferase
YOR320C	*GNT1*	N-acetylglucosaminyltransferase transferase
YOR344C	*TYE7*	Basic helix-loop-helix transcription factor
YOR379C		
YPL031C	*PHO85*	Cyclin-dependent protein kinase
YPL045W	*VPS16*	Vacuolar sorting protein
YPL139C	*UME1*	Negative regulator of meiosis
YPL144W	*POC4*	PrOteasome Chaperone
YPL161C	*BEM4*	Bud emergence protein
YPL193W	*RSA1*	Nucleoplasmic protein involved in assembly of 60S ribosomal subunits
YPL213W	*LEA1*	U2 A` snRNP protein
YPL268W	*PLC1*	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase
YPR036W	*VMA13*	H+-ATPase V1 domain 54 KD subunit, vacuolar
YPR101W	*SNT309*	Splicing factor
YPR133W-A	*TOM5*	Mitochondrial outer membrane protein
YPR163C	*TIF3*	Translation initiation factor eIF4B

B. Medium heat-sensitive phenotype

ORF code	Gene name	Gene description
YAL013W	*DEP1*	Regulator of phospholipid metabolism
YAL056W	*GPB2*	G protein subunit mimic and signaling effector
YAR002W	*NUP60*	Nuclear pore protein
YAR003W	*SWD1*	Subunit of the COMPASS complex
YAR029W		
YBR035C	*PDX3*	Pyridoxamine-phosphate oxidase
YBR078W	*ECM33*	Protein involved in cell wall biogenesis and architecture
YCL060C	*MRC1*	Mediator of the Replication Checkpoint
YCR008W	*SAT4*	Serine/threonine-specific protein kinase
YDL074C	*BRE1*	E3 ubiquitin ligase for Rad6p, required for the ubiquitination of histone H2B
YDR074W	*TPS2*	Alpha,alpha-trehalose-phosphate synthase, 102 KD subunit
YDR159W	*SAC3*	Leucine permease transcriptional regulator
YDR300C	*PRO1*	Glutamate 5-kinase

ORF code	Gene name	Gene description
YDR363W-A	*SEM1*	Regulator of exocytosis and pseudohyphal differentiation
YEL013W	*VAC8*	Protein required for vacuole inheritance and protein targeting
YER044C	*ERG28*	Protein involved in synthesis of ergosterol
YER070W	*RNR1*	Ribonucleoside-diphosphate reductase, large subunit
YGL043W	*DST1*	TFIIS (transcription elongation factor)
YGL095C	*VPS45*	Vacuolar protein sorting-associated protein
YGL163C	*RAD54*	DNA-dependent ATPase of the Snf2p family
YGR155W	*CYS4*	Cystathionine beta-synthase
YGR252W	*GCN5*	Histone acetyltransferase
YIL040W	*APQ12*	Protein involved in nucleocytoplasmic transport of mRNA
YIL084C	*SDS3*	Transcriptional regulator
YJL136C	*RPS21B*	40S small subunit ribosomal protein S21.e
YJL172W	*CPS1*	Gly-X carboxypeptidase YSCS precursor
YJR018W		
YJR118C	*ILM1*	Increased Loss of Mitochondrial DNA
YKL204W	*EAP1*	Translation initiation factor 4E-associated protein
YLR168C		Probably involved in intramitochondrial protein sorting
YLR268W	*SEC22*	Synaptobrevin (V-SNARE)
YNL197C	*PHO23*	Probable component of the Rpd3 histone deacetylase complex
YNL257C	*SIP3*	Snf1p protein kinase interacting protein
YOL050C		
YOR014W	*RTS1*	Potential regulatory subunit of protein phosphatase 2A
YOR043W	*WHI2*	Growth regulation protein
YOR080W	*DIA2*	Protein involved in invasive and pseudohyphal growth
YOR115C	*TRS33*	TRAPP subunit involved in targeting and fusion of ER to golgi transport vesicles
YOR123C	*LEO1*	Extremely hydrophilic protein
YOR258W	*HNT3*	Histidine triad NucleoTide-binding
YOR322C	*LDB19*	Protein of unknown function localised to golgi-vacuole transport vesicles
YPL090C	*RPS6A*	Ribosomal protein S6.e
YPL103C	*FMP30*	Found in Mitochondrial Proteome
YPL174C	*NIP100*	Component of the dynactin complex
YPL192C	*PRM3*	Pheromone-regulated protein required for karyogamy
YPR024W	*YME1*	Protease of the SEC18/CDC48/PAS1 family of ATPases (AAA)
YPR164W	*MMS1*	Drug resistance

C. Weak heat-sensitive phenotype

ORF code	Gene name	Gene description
YAL051W	*OAF1*	Peroxisome proliferating transcription factor
YBR015C	*MNN2*	Type II membrane protein
YBR030W		Protein involved in phospholipid metabolism
YBR036C	*CSG2*	Calcium dependent regulatory protein
YBR111C	*YSA1*	Sugar-nucleotide hydrolase
YBR168W	*PEX32*	PEroXisome related protein
YBR245C	*ISW1*	ATPase component of a four subunit chromatin remodeling complex
YBR272C	*HSM3*	Mismatch repair protein
YCR044C	*PER1*	Protein involved in manganese homeostasis
YDL001W	*RMD1*	Protein required for Meiotic Division
YDL107W	*MSS2*	COX2 pre-mRNA splicing factor
YDL155W	*CLB3*	Cyclin, G2/M-specific
YDL206W		Weak similarity to transporter proteins
YDL243C	*AAD4*	Aryl-Alcohol Dehydrogenase involved in the oxidative stress response
YDR057W	*YOS9*	ER to golgi transport of GPI-anchored proteins
YDR192C	*NUP42*	Nuclear pore protein
YDR233C	*RTN1*	ReTiculoN-like
YDR245W	*MNN10*	Subunit of mannosyltransferase complex

ORF code	Gene name	Gene description
YDR248C		Protein of unknown function localised to cytoplasm
YDR276C	*PMP3*	Sensitive to natrium
YDR335W	*MSN5*	Multicopy supressor of snf1 mutation
YDR385W	*EFT2*	Translation elongation factor eEF2
YDR392W	*SPT3*	General transcriptional adaptor or co-activator
YDR440W	*DOT1*	Protein-lysine N-methyltransferase
YDR477W	*SNF1*	Carbon catabolite derepressing ser/thr protein kinase
YER058W	*PET117*	Cytochrome c oxidase assembly factor
YER151C	*UBP3*	Ubiquitin-specific proteinase
YFL018C	*LPD1*	Dihydrolipoamide dehydrogenase precursor
YFL023W	*BUD27*	Protein involved in bipolar bud site selection
YFL036W	*RPO41*	DNA-directed RNA polymerase, mitochondrial
YFR010W	*UBP6*	Ubiquitin-specific protease
YGL081W		
YGL101W		
YGL110C	*CUE3*	Coupling of Ubiquitin conjugation to ER degradation
YGL147C	*RPL9A*	Ribosomal protein L9.e
YGL148W	*ARO2*	Chorismate synthase
YGL196W	*DSD1*	D-serine dehydratase
YGL200C	*EMP24*	Component of the COPII-coated vesicles, 24 kDa
YGL256W	*ADH4*	Alcohol dehydrogenase IV
YGR061C	*ADE6*	5`-phosphoribosylformyl glycinamidine synthetase
YGR071C		
YGR217W	*CCH1*	Calcium channel (alpha subunit) of the plasma membrane
YHL040C	*ARN1*	Transporter of ferrirubin, ferrirhodin and other ferrichromes
YHR031C	*RRM3*	DNA helicase involved in rDNA replication and Ty1 transposition
YHR048W		Putative mediator of drug efflux
YHR150W	*PEX28*	Peroxisomal integral membrane protein, regulation of peroxisome size and number
YHR163W	*SOL3*	Possible 6-phosphogluconolactonase
YHR182W		
YIL017C	*VID28*	Vacuole import and degradation
YIL039W	*TED1*	Trafficking of Emp24p/Erv25p-dependent cargo Disrupted
YIL092W		
YIR003W	*AIM21*	Altered Inheritance rate of Mitochondria
YJL093C	*TOK1*	Voltage-gated, outward-rectifying K+ channel protein of the plasma membrane
YJL138C	*TIF2*	Translation initiation factor eIF4A
YJL185C		
YJL196C	*ELO1*	Fatty acid elongation protein
YJL209W	*CBP1*	Apo-cytochrome b pre-mRNA processing protein
YJL214W	*HXT8*	Hxt family protein with intrinsic hexose transport activity
YJR032W	*CPR7*	Member of the cyclophilin family
YKL006W	*RPL14A*	Ribosomal protein
YKL016C	*ATP7*	F1F0-ATPase complex, FO D subunit
YKL048C	*ELM1*	Ser/thr-specific protein kinase
YKL062W	*MSN4*	Transcriptional activator
YKL139W	*CTK1*	Carboxy-terminal domain (CTD) kinase, alpha subunit
YKL208W	*CBT1*	Apo-cytochrome b pre-mRNA processing protein
YKL211C	*TRP3*	Anthranilate synthase component II
YKR001C	*VPS1*	Member of the dynamin family of GTPases
YKR007W	*MEH1*	Multicopy suppressor of Ers1 Hygromycin B sensitivity
YLL018C-A	*COX19*	Protein required for expression of mitochondrial cytochrome oxidase
YLR055C	*SPT8*	Transcriptional adaptor or co-activator
YLR241W		
YLR258W	*GSY2*	UDP-glucose--starch glucosyltransferase, isoform 2
YLR269C		
YLR360W	*VPS38*	Protein involved in vacuolar sorting
YLR373C	*VID22*	Vacuole import and degradation
YML129C	*COX14*	Cytochrome-c oxidase assembly protein
YMR003W	*AIM34*	Altered Inheritance rate of Mitochondria
YMR016C	*SOK2*	Regulatory protein in the PKA signal transduction pathway

ORF code	Gene name	Gene description
YMR039C	*SUB1*	Transcriptional coactivator
YMR143W	*RPS16A*	Ribosomal protein S16.e
YMR223W	*UBP8*	Ubiquitin-specific protease that is a component of the SAGA
YNL025C	*SSN8*	DNA-directed RNA polymerase II holoenzyme and SRB subcomplex subunit
YNL064C	*YDJ1*	Mitochondrial and ER import protein
YOR125C	*CAT5*	Protein involved in coenzyme Q (ubiquinone) biosynthesis
YOR144C	*ELG1*	Protein required for S phase progression and telomere homeostasis
YOR180C	*DCI1*	Enoyl-CoA Hydratase, peroxisomal
YOR251C		Putative thiosulfate sulfurtransferases
YPL037C	*EGD1*	GAL4 DNA-binding enhancer protein
YPR116W		
YPR119W	*CLB2*	Cyclin, G2/M-specific

Table S2 List of genes whose expression in response to heat stress was affected by the *RPD3* mutant. Out of the 582 genes induced **(A)** or the 594 genes repressed **(B)** at least 2-fold in response to heat stress (39ºC, for 20 min.), 65% and 60% respectively showed a significant reduction on gene expression when *RPD3* was deleted. Fold changes of both wild type and *RPD3* mutant strains are indicated and genes were grouped according to the degree of gene expression observed in the absence of *RPD3*: Strongly dependent (less than 25% of the activation or repression observed in wild type cells), moderately dependent (from 25% to 50%) and weakly dependent (from 51% to 75%). Dependence on Msn2/4 or Hsf1 transcription factors is indicated based on previously published data where the heat shock response program was compared between wild type cells and cells lacking either functional *HSF1* (Yamamoto *et al.*, 2005) or *MSN2/4* genes (Gasch *et al.*, 2000; Berry and Gasch, 2008).

A. Genes induced in response to heat stress that were affected by the *RPD3* mutant.

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep	Msn2/4 dep	Hsf1 dep
YCL041C		2,18	-1,94	Stronlgy dep		
YHL048W	COS8	2,00	-1,72	Strongly dep		
YGL125W	MET13	2,01	-1,66	Strongly dep		
YNL270C	ALP1	2,05	-1,47	Strongly dep		
YIL113W	SDP1	2,37	-1,62	Strongly dep		
YER039C-A		2,02	-1,32	Strongly dep		
YPL264C		2,28	-1,46	Strongly dep		
YBR284W		2,03	-1,28	Strongly dep		
YJL088W	ARG3	2,01	-1,24	Strongly dep		
YLR356W		2,84	-1,75	Strongly dep		
YDR100W		2,36	-1,44	Strongly dep		
YJL017W		2,10	-1,18	Strongly dep	X	
YNR064C		2,44	-1,36	Strongly dep		
YMR052C-A		2,35	-1,30	Strongly dep		
YCR030C	SYP1	2,07	-1,13	Strongly dep		
YGL010W		2,21	-1,17	Strongly dep		
YNL241C	ZWF1	2,35	-1,19	Strongly dep		
YFR017C		2,99	-1,51	Strongly dep		
YGR143W	SKN1	2,02	-1,01	Strongly dep		
YPL203W	TPK2	2,21	-1,08	Strongly dep		
YJL163C		2,56	-1,21	Strongly dep	X	
YLR304C	ACO1	2,27	-1,06	Strongly dep		
YIL087C		2,70	-1,25	Strongly dep	X	
YKL086W		2,36	-1,06	Strongly dep		
YEL059W		2,82	-1,26	Strongly dep		

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep	Msn2/4 dep	Hsf1 dep
YNL202W	SPS19	3,16	-1,38	Strongly dep		
YER039C	HVG1	2,50	-1,07	Strongly dep		
YMR315W		2,61	-1,11	Strongly dep		
YJR020W		2,81	-1,17	Strongly dep		
YBR214W	SDS24	3,34	-1,13	Strongly dep		
YDR379C-A		3,04	-1,02	Strongly dep		
YOR185C	GSP2	3,14	-1,03	Strongly dep		
YPL017C		6,72	-1,13	Strongly dep		
YDR034W-B		12,85	1,72	Strongly dep		
YOL084W	PHM7	33,92	5,67	Strongly dep	X	
YPL281C	ERR2	7,22	1,22	Strongly dep		
YPL223C	GRE1	6,88	1,18	Strongly dep	X	
YOR393W	ERR1	7,38	1,28	Strongly dep		
YNL195C		14,97	2,73	Strongly dep	X	
YBR116C		50,47	11,60	Strongly dep	X	
YKL217W	JEN1	8,65	2,03	Strongly dep		
YBR117C	TKL2	42,42	9,98	Strongly dep	X	
YDL085W	NDE2	8,92	2,14	Strongly dep		
YHL024W	RIM4	8,23	2,04	Strongly dep		
YOR348C	PUT4	6,14	1,56	Strongly dep		
YAL054C	ACS1	9,67	2,46	Strongly dep		
YER053C-A		5,67	1,45	Strongly dep		
YMR323W		4,08	1,07	Moderately dep.		
YER067W		8,90	2,35	Moderately dep.	X	
YBR230C		5,89	1,66	Moderately dep.	X	
YCR061W		3,80	1,10	Moderately dep.		
YGL121C	GPG1	4,03	1,18	Moderately dep.	X	
YKL093W	MBR1	4,61	1,40	Moderately dep.		
YGR088W	CTT1	22,16	6,73	Moderately dep.	X	
YER096W	SHC1	5,32	1,62	Moderately dep.	X	
YPL280W		5,12	1,58	Moderately dep.		
YKL221W	MCH2	3,94	1,21	Moderately dep.		
YMR322C		4,67	1,44	Moderately dep.		
YBL049W		4,02	1,25	Moderately dep.		
YGR087C	PDC6	5,14	1,65	Moderately dep.		
YAR035W	YAT1	4,57	1,47	Moderately dep.		
YOL153C		4,02	1,31	Moderately dep.		
YEL060C	PRB1	5,05	1,65	Moderately dep.		
YDL238C		3,50	1,17	Moderately dep.		
YGR256W	GND2	15,47	5,24	Moderately dep.	X	
YJL161W		4,27	1,47	Moderately dep.	X	
YPL230W		4,65	1,61	Moderately dep.	X	
YMR189W	GCV2	3,65	1,29	Moderately dep.		
YDR453C	TSA2	8,64	3,05	Moderately dep.	X	
YNL194C		18,99	6,75	Moderately dep.	X	
YDL199C		3,21	1,14	Moderately dep.	X	
YDR380W	ARO10	33,74	12,03	Moderately dep.		
YBR241C		3,74	1,34	Moderately dep.		
YDR178W	SDH4	3,01	1,09	Moderately dep.		

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep	Msn2/4 dep	Hsf1 dep
YGR213C	RTA1	2,91	1,05	Moderately dep.		
YPR184W	GDB1	9,02	3,27	Moderately dep.	X	
YNR014W		2,78	1,02	Moderately dep.		
YDL181W	INH1	3,85	1,42	Moderately dep.		
YOR100C	CRC1	29,56	10,92	Moderately dep.		
YGR110W		2,85	1,07	Moderately dep.		
YBR132C	AGP2	4,79	1,81	Moderately dep.		
YKL148C	SDH1	3,13	1,20	Moderately dep.		
YKR046C		3,92	1,53	Moderately dep.	X	
YCR010C	ADY2	8,01	3,12	Moderately dep.		
YPL119C	DBP1	2,85	1,11	Moderately dep.	X	
YGL205W	POX1	5,19	2,03	Moderately dep.		
YHR016C	YSC84	2,97	1,17	Moderately dep.		
YHR140W		4,73	1,86	Moderately dep.		
YAL004W		3,44	1,36	Moderately dep.		
YMR271C	URA10	2,61	1,03	Moderately dep.		
YBR183W	YPC1	4,00	1,58	Moderately dep.	X	
YMR105C	PGM2	21,07	8,42	Moderately dep.	X	
YCR068W	CVT17	2,80	1,12	Moderately dep.		
YDL079C	MRK1	2,64	1,07	Moderately dep.		
YNL092W		2,77	1,12	Moderately dep.		
YBL048W		4,09	1,66	Moderately dep.		
YBL043W	ECM13	3,06	1,24	Moderately dep.		
YIL160C	POT1	7,66	3,13	Moderately dep.		
YPL247C		4,63	1,90	Moderately dep.		
YLR177W		2,83	1,16	Moderately dep.		
YOR391C		3,08	1,27	Moderately dep.		
YGR287C		3,44	1,42	Moderately dep.		
YBR280C		4,24	1,79	Moderately dep.		
YMR206W		2,48	1,05	Moderately dep.		
YGR019W	UGA1	2,60	1,10	Moderately dep.	X	
YOR328W	PDR10	3,70	1,57	Moderately dep.		
YHL021C		11,34	4,81	Moderately dep.	X	
YNL015W	PBI2	2,44	1,05	Moderately dep.	X	
YGR144W	THI4	2,54	1,09	Moderately dep.		
YOR161C		10,78	4,65	Moderately dep.	X	
YHR138C		2,46	1,06	Moderately dep.		
YOR010C	TIR2	2,44	1,06	Moderately dep.		
YDR018C		2,40	1,04	Moderately dep.		
YOR347C	PYK2	4,66	2,02	Moderately dep.		
YOR003W	YSP3	3,67	1,60	Moderately dep.		
YER119C		2,91	1,27	Moderately dep.		
YJL016W		2,46	1,07	Moderately dep.	X	
YML054C	CYB2	7,55	3,30	Moderately dep.		X
YBL101C	ECM21	2,81	1,24	Moderately dep.		
YJL045W		4,78	2,11	Moderately dep.		
YLR137W		2,54	1,12	Moderately dep.		
YGR292W	MAL12	3,57	1,60	Moderately dep.		
YGR288W	MAL13	2,27	1,02	Moderately dep.		

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep	Msn2/4 dep	Hsf1 dep
YPL003W	ULA1	2,27	1,03	Moderately dep.		
YCL027C-A		4,77	2,16	Moderately dep.		
YLR446W		2,26	1,03	Moderately dep.		
YJL185C		2,23	1,02	Moderately dep.		
YKL163W	PIR3	10,93	5,01	Moderately dep.	X	
YDR216W	ADR1	6,99	3,21	Moderately dep.		
YBL015W	ACH1	4,23	1,95	Moderately dep.	X	
YHR033W		2,53	1,17	Moderately dep.		
YBR299W	MAL32	2,85	1,32	Moderately dep.		
YLL041C	SDH2	2,32	1,08	Moderately dep.		
YMR250W	GAD1	12,07	5,62	Moderately dep.	X	
YPR028W	YOP1	2,20	1,03	Moderately dep.		X
YLR149C		6,20	2,90	Moderately dep.		
YDL130W-A	STF1	2,21	1,04	Moderately dep.		
YNR002C	FUN34	15,76	7,39	Moderately dep.	X	
YLR164W		2,20	1,03	Moderately dep.		
YMR110C		3,22	1,52	Moderately dep.	X	
YDL023C		2,27	1,07	Moderately dep.	X	
YIL169C		2,88	1,36	Moderately dep.		
YHR139C	SPS100	5,12	2,45	Moderately dep.	X	
YPR026W	ATH1	3,82	1,83	Moderately dep.	X	
YHR202W		2,21	1,06	Moderately dep.		
YGL258W-A		2,72	1,30	Moderately dep.		
YDR513W	TTR1	2,59	1,25	Moderately dep.	X	
YDR074W	TPS2	5,11	2,46	Moderately dep.	X	
YJR078W	BNA2	3,22	1,56	Moderately dep.		
YHR001W-A	QCR10	2,32	1,12	Moderately dep.		
YOR173W		11,13	5,39	Moderately dep.	X	
YHR137W	ARO9	21,11	10,31	Moderately dep.		
YGR149W		3,38	1,65	Moderately dep.		
YLR174W	IDP2	3,40	1,67	Moderately dep.		
YBR285W		4,84	2,37	Moderately dep.	X	
YCL044C		2,10	1,04	Moderately dep.		
YLR307W	CDA1	2,50	1,23	Moderately dep.		
YJL089W	SIP4	3,87	1,91	Moderately dep.		X
YJL141C	YAK1	2,62	1,29	Moderately dep.		
YKL037W		2,84	1,40	Moderately dep.		
YER179W	DMC1	2,35	1,16	Moderately dep.		
YNL033W		2,12	1,05	Moderately dep.		
YFR054C		2,09	1,05	Moderately dep.		
YMR181C		2,26	1,13	Moderately dep.	X	
YDR533C		4,42	2,23	Moderately dep.	X	
YNL019C		2,04	1,03	Moderately dep.		
YEL039C	CYC7	4,63	2,35	Moderately dep.	X	
YIL033C	BCY1	2,03	1,03	Moderately dep.	X	
YMR136W	GAT2	2,19	1,12	Weakly dep.		
YHR022C		3,69	1,89	Weakly dep.		
YIR038C	GTT1	2,94	1,51	Weakly dep.	X	
YGL104C	VPS73	3,64	1,87	Weakly dep.		

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep	Msn2/4 dep	Hsf1 dep
YOL119C	MCH4	2,55	1,31	Weakly dep.		
YIL099W	SGA1	5,94	3,06	Weakly dep.		
YER037W	PHM8	4,25	2,19	Weakly dep.		
YOR228C		2,01	1,04	Weakly dep.		
YCR064C		3,45	1,79	Weakly dep.		
YML042W	CAT2	9,39	4,91	Weakly dep.	X	
YPL136W		2,85	1,49	Weakly dep.		
YFL055W	AGP3	2,50	1,31	Weakly dep.		
YIL101C	XBP1	2,06	1,08	Weakly dep.		
YBR054W	YRO2	9,65	5,05	Weakly dep.		
YJL083W		2,04	1,07	Weakly dep.		
YKR049C		3,07	1,62	Weakly dep.		
YLR120C	YPS1	2,08	1,10	Weakly dep.		
YGR023W	MTL1	3,44	1,81	Weakly dep.		
YJL066C	MPM1	2,74	1,46	Weakly dep.		
YGL259W	YPS5	2,27	1,22	Weakly dep.	X	
YKR076W	ECM4	2,12	1,14	Weakly dep.	X	
YDL138W	RGT2	2,55	1,38	Weakly dep.		
YOR288C	MPD1	2,01	1,08	Weakly dep.		
YHR096C	HXT5	47,80	25,88	Weakly dep.	X	
YGL072C		2,23	1,21	Weakly dep.		
YKR091W	SRL3	5,58	3,02	Weakly dep.		
YGR205W		2,59	1,41	Weakly dep.		
YDL218W		2,25	1,23	Weakly dep.		
YMR262W		2,16	1,18	Weakly dep.		
YLR112W		2,18	1,19	Weakly dep.		
YBR105C	VID24	2,62	1,44	Weakly dep.	X	
YJL217W		3,99	2,20	Weakly dep.		
YPR192W	AQY1	2,14	1,18	Weakly dep.		
YMR013W-A		3,13	1,74	Weakly dep.		
YLR312C		2,50	1,39	Weakly dep.		
YJL159W	HSP150	3,50	1,96	Weakly dep.		
YLR251W	SYM1	5,26	2,95	Weakly dep.		
YNL117W	MLS1	2,56	1,44	Weakly dep.		
YBR240C	THI2	2,02	1,14	Weakly dep.		
YHR199C		2,00	1,13	Weakly dep.		
YLR438W	CAR2	4,08	2,31	Weakly dep.		
YDR204W	COQ4	2,81	1,60	Weakly dep.		
YMR053C	STB2	2,93	1,67	Weakly dep.		
YJR073C	OPI3	2,22	1,27	Weakly dep.		
YGL146C		3,07	1,75	Weakly dep.		
YMR261C	TPS3	2,89	1,65	Weakly dep.	X	
YGR146C		2,62	1,51	Weakly dep.		
YNR019W	ARE2	4,23	2,43	Weakly dep.		
YJL068C		2,08	1,20	Weakly dep.		
YOR065W	CYT1	2,26	1,30	Weakly dep.		
YLR267W	BOP2	7,90	4,55	Weakly dep.		
YEL041W		2,56	1,47	Weakly dep.		
YOR178C	GAC1	7,95	4,62	Weakly dep.		

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep	Msn2/4 dep	Hsf1 dep
YER095W	RAD51	2,05	1,19	Weakly dep.		
YER163C		2,00	1,16	Weakly dep.		
YHR171W	APG7	2,06	1,20	Weakly dep.		
YOL155C		3,18	1,86	Weakly dep.		
YLR245C	CDD1	2,93	1,72	Weakly dep.		
YNL274C		8,47	4,97	Weakly dep.	X	
YML100W	TSL1	15,99	9,38	Weakly dep.	X	X
YLR133W	CKI1	2,40	1,41	Weakly dep.		
YLR252W		5,50	3,24	Weakly dep.		
YJL172W	CPS1	2,52	1,49	Weakly dep.		
YEL024W	RIP1	2,18	1,29	Weakly dep.		
YMR280C	CAT8	6,24	3,69	Weakly dep.		
YEL011W	GLC3	5,44	3,23	Weakly dep.		
YOL083W		2,39	1,42	Weakly dep.	X	
YIL155C	GUT2	3,88	2,31	Weakly dep.		
YDR085C	AFR1	3,31	1,97	Weakly dep.		
YLR205C	HMX1	5,16	3,07	Weakly dep.		
YOR134W	BAG7	6,19	3,69	Weakly dep.	X	
YOL025W	LAG2	2,01	1,20	Weakly dep.		
YNL200C		4,19	2,50	Weakly dep.	X	
YDL124W		2,40	1,43	Weakly dep.	X	
YNR034W-A		13,83	8,26	Weakly dep.		
YJL116C	NCA3	5,86	3,51	Weakly dep.		
YIR028W	DAL4	2,63	1,58	Weakly dep.		
YJR096W		4,82	2,90	Weakly dep.	X	
YJR036C	HUL4	2,22	1,33	Weakly dep.		
YIR039C	YPS6	5,23	3,17	Weakly dep.	X	
YDR255C	RMD5	2,05	1,24	Weakly dep.		
YNL125C	ESBP6	3,62	2,20	Weakly dep.		
YPL250C	ICY2	2,13	1,29	Weakly dep.		
YLR142W	PUT1	4,60	2,80	Weakly dep.	X	
YKL091C		4,39	2,67	Weakly dep.	X	
YKL141W	SDH3	2,23	1,36	Weakly dep.		X
YDL149W	APG9	2,25	1,37	Weakly dep.		
YBR056W		2,82	1,72	Weakly dep.	X	
YMR170C	ALD2	3,91	2,39	Weakly dep.	X	
YML131W		2,39	1,46	Weakly dep.	X	
YBR287W		2,42	1,48	Weakly dep.	X	
YOL052C-A	DDR2	22,46	13,84	Weakly dep.	X	
YLR337C	VRP1	2,03	1,25	Weakly dep.		
YBR149W	ARA1	2,36	1,46	Weakly dep.	X	
YFL054C		2,80	1,73	Weakly dep.		
YJL103C		2,46	1,52	Weakly dep.		
YJL102W	MEF2	2,23	1,38	Weakly dep.		
YDL024C	DIA3	10,81	6,70	Weakly dep.	X	
YBR186W	PCH2	2,12	1,32	Weakly dep.		
YDL174C	DLD1	2,05	1,28	Weakly dep.		
YOR285W		2,01	1,26	Weakly dep.		
YMR041C		2,41	1,51	Weakly dep.		

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep	Msn2/4 dep	Hsf1 dep
YGR201C		3,69	2,31	Weakly dep.	X	
YNR034W	SOL1	2,00	1,26	Weakly dep.		
YGR268C		2,91	1,84	Weakly dep.		
YDR391C		2,15	1,37	Weakly dep.	X	
YOL151W	GRE2	2,07	1,32	Weakly dep.	X	
YBR018C	GAL7	3,44	2,19	Weakly dep.		
YLR178C	TFS1	14,07	9,00	Weakly dep.	X	
YOR386W	PHR1	3,80	2,43	Weakly dep.		
YOR292C		2,06	1,32	Weakly dep.		
YDR504C		2,13	1,37	Weakly dep.	X	
YPR001W	CIT3	8,47	5,47	Weakly dep.		
YPL222W		3,47	2,24	Weakly dep.		
YML004C	GLO1	3,85	2,49	Weakly dep.	X	
YDL223C	HBT1	12,51	8,10	Weakly dep.	X	
YER033C	ZRG8	2,40	1,55	Weakly dep.		
YIL124W	AYR1	2,57	1,66	Weakly dep.		
YLR080W	EMP46	2,54	1,65	Weakly dep.		
YNL305C		3,39	2,20	Weakly dep.		
YJR103W	URA8	2,25	1,47	Weakly dep.		
YJL048C		2,65	1,74	Weakly dep.		
YGR194C	XKS1	2,76	1,81	Weakly dep.		
YDL072C		2,30	1,52	Weakly dep.	X	
YDL032W		2,23	1,47	Weakly dep.		
YKL051W		2,51	1,65	Weakly dep.	X	
YGR127W		2,38	1,57	Weakly dep.	X	
YKL171W		2,18	1,44	Weakly dep.		
YBR053C		3,45	2,28	Weakly dep.	X	
YBR005W		2,23	1,48	Weakly dep.		
YLL023C		2,62	1,73	Weakly dep.	X	X
YJR160C		2,45	1,63	Weakly dep.		
YMR152W	YIM1	2,17	1,44	Weakly dep.	X	
YDL247W		2,34	1,56	Weakly dep.		
YLR089C		2,07	1,38	Weakly dep.		
YFR015C	GSY1	3,06	2,05	Weakly dep.		
YBR046C	ZTA1	2,65	1,78	Weakly dep.		
YER062C	HOR2	2,59	1,73	Weakly dep.		
YDL222C		28,04	18,83	Weakly dep.	X	
YPR030W	CSR2	4,41	2,96	Weakly dep.		
YKL218C	SRY1	2,15	1,45	Weakly dep.		
YDR096W	GIS1	2,30	1,55	Weakly dep.		
YMR104C	YPK2	4,28	2,90	Weakly dep.		
YMR118C		5,70	3,86	Weakly dep.		
YMR196W		12,63	8,56	Weakly dep.	X	
YBR147W		3,27	2,23	Weakly dep.		
YER024W	YAT2	3,77	2,57	Weakly dep.		
YMR139W	RIM11	2,01	1,37	Weakly dep.		
YDR436W	PPZ2	2,12	1,45	Weakly dep.		
YNL242W	APG2	2,32	1,58	Weakly dep.		
YOR052C		2,70	1,84	Weakly dep.		

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep	Msn2/4 dep	Hsf1 dep
YNL237W	YTP1	3,17	2,17	Weakly dep.		
YNL014W	HEF3	2,69	1,84	Weakly dep.		
YBR137W		2,35	1,61	Weakly dep.		
YEL020C		2,36	1,63	Weakly dep.		
YBR126C	TPS1	6,24	4,31	Weakly dep.	X	
YGR044C	RME1	2,17	1,50	Weakly dep.		
YDR032C	PST2	2,38	1,65	Weakly dep.		
YJL070C		2,04	1,41	Weakly dep.		
YGR239C	PEX21	2,12	1,47	Weakly dep.		
YPL123C	RNY1	3,57	2,48	Weakly dep.	X	
YLL019C	KNS1	3,01	2,10	Weakly dep.		
YJR149W		2,86	1,99	Weakly dep.		
YML070W	DAK1	3,41	2,38	Weakly dep.	X	
YJL057C	IKS1	3,42	2,39	Weakly dep.		
YBR203W		2,48	1,73	Weakly dep.		
YOR230W	WTM1	2,02	1,41	Weakly dep.		
YDR040C	ENA1	5,68	4,00	Weakly dep.		
YGL156W	AMS1	3,26	2,30	Weakly dep.	X	
YAL005C	SSA1	5,08	3,58	Weakly dep.		X
YDL026W		2,57	1,81	Weakly dep.		
YDL057W		2,01	1,42	Weakly dep.		
YHR087W		16,34	11,58	Weakly dep.		
YCL047C		2,20	1,56	Weakly dep.		
YBR026C	MRF1	2,62	1,86	Weakly dep.	X	
YNL293W	MSB3	2,33	1,66	Weakly dep.		
YER079W		2,31	1,65	Weakly dep.	X	
YOR374W	ALD4	15,78	11,25	Weakly dep.		
YKL103C	LAP4	3,58	2,55	Weakly dep.	X	
YDR070C		9,60	6,86	Weakly dep.	X	
YLR070C	XYL2	3,75	2,68	Weakly dep.		
YGR243W		4,06	2,91	Weakly dep.		
YFL044C		2,49	1,78	Weakly dep.		
YBR006W	UGA2	3,20	2,30	Weakly dep.		
YIL055C		3,02	2,17	Weakly dep.		
YGR183C	QCR9	2,11	1,52	Weakly dep.		
YDL204W		19,15	13,82	Weakly dep.	X	
YDL169C	UGX2	2,29	1,66	Weakly dep.		
YDR122W	KIN1	2,27	1,64	Weakly dep.		
YMR090W		4,02	2,90	Weakly dep.	X	
YJL108C	PRM10	4,01	2,90	Weakly dep.		
YLR258W	GSY2	10,97	7,99	Weakly dep.	X	
YFL015C		2,92	2,13	Weakly dep.		
YEL035C	UTR5	2,53	1,85	Weakly dep.		
YLR047C		2,45	1,79	Weakly dep.		
YHR008C	SOD2	2,25	1,65	Weakly dep.		
YIL111W	COX5B	3,20	2,36	Weakly dep.		
YMR103C		4,80	3,54	Weakly dep.		
YPR160W	GPH1	24,71	18,23	Weakly dep.	X	
YGL062W	PYC1	6,20	4,58	Weakly dep.		

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep	Msn2/4 dep	Hsf1 dep
YLR241W		2,09	1,54	Weakly dep.		
YKR011C		2,14	1,59	Weakly dep.		
YOR136W	IDH2	2,02	1,50	Weakly dep.		
YDL214C	PRR2	2,10	1,56	Weakly dep.		
YGL227W	VID30	2,30	1,71	Weakly dep.		
YPR002W	PDH1	8,89	6,60	Weakly dep.		
YLL020C		3,06	2,27	Weakly dep.		
YML007C-A		2,73	2,04	Weakly dep.		
YJL213W		4,00	2,99	Weakly dep.		
YLR152C		2,10	1,58	Weakly dep.	X	
YMR174C	PAI3	3,62	2,73	Weakly dep.	X	
YJR091C	JSN1	2,51	1,89	Weakly dep.		
YJR008W		4,22	3,19	Weakly dep.	X	

B. Genes repressed in response to heat stress that were affected by the *RPD3* mutant.

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep
YDL058W	USO1	-2,09	2,74	Strongly dep
YAL019W	FUN30	-2,25	1,94	Strongly dep
YKL082C		-2,45	2,03	Strongly dep
YBR155W	CNS1	-2,04	1,62	Strongly dep
YOL144W	NOP8	-2,35	1,68	Strongly dep
YHR169W	DBP8	-2,09	1,48	Strongly dep
YDR365C		-2,29	1,63	Strongly dep
YBR104W	YMC2	-2,2	1,55	Strongly dep
YKR024C	DBP7	-2,01	1,37	Strongly dep
YMR239C	RNT1	-2,11	1,42	Strongly dep
YOL124C		-2,05	1,37	Strongly dep
YLL021W	SPA2	-2,96	1,95	Strongly dep
YDR097C	MSH6	-2,49	1,63	Strongly dep
YBR142W	MAK5	-2,09	1,33	Strongly dep
YDR211W	GCD6	-2,1	1,34	Strongly dep
YNL308C	KRI1	-2,7	1,66	Strongly dep
YER082C	UTP7	-2,13	1,29	Strongly dep
YDR312W	SSF2	-2,21	1,25	Strongly dep
YHR065C	RRP3	-2,02	1,13	Strongly dep
YPL217C	BMS1	-2,04	1,13	Strongly dep
YNL313C		-2,17	1,2	Strongly dep
YIL091C		-2,09	1,16	Strongly dep
YPL094C	SEC62	-2,08	1,15	Strongly dep
YBR267W		-2,01	1,1	Strongly dep
YDR267C		-2	1,09	Strongly dep
YDR398W	UTP5	-2,67	1,43	Strongly dep
YMR301C	ATM1	-2,26	1,21	Strongly dep
YML074C	FPR3	-2,55	1,34	Strongly dep
YJR007W	SUI2	-2	1,04	Strongly dep
YKL143W	LTV1	-2,88	1,49	Strongly dep
YGL111W	NSA1	-2,26	1,17	Strongly dep

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep
YHR196W	UTP9	-2,65	1,36	Strongly dep
YER007C-A		-2,31	1,18	Strongly dep
YKL144C	RPC25	-2,06	1,05	Strongly dep
YNR051C	BRE5	-2,45	1,24	Strongly dep
YIL019W		-2,58	1,31	Strongly dep
YLR221C	RSA3	-2,28	1,15	Strongly dep
YJL145W	SFH5	-2,21	1,09	Strongly dep
YAL036C	FUN11	-2,54	1,25	Strongly dep
YPR143W		-2,11	1,02	Strongly dep
YKR092C	SRP40	-3,08	1,48	Strongly dep
YOR207C	RET1	-2,12	1,01	Strongly dep
YJR072C		-2,9	1,37	Strongly dep
YCR016W		-2,14	1	Strongly dep
YHR066W	SSF1	-2,97	1,39	Strongly dep
YDL031W	DBP10	-2,2	1,02	Strongly dep
YLR420W	URA4	-2,31	1,06	Strongly dep
YAL025C	MAK16	-2,61	1,18	Strongly dep
YLR401C		-3,02	1,34	Strongly dep
YHR154W	RTT107	-2,43	1,07	Strongly dep
YER049W		-2,44	1,07	Strongly dep
YOR056C	NOB1	-2,83	1,23	Strongly dep
YDR037W	KRS1	-2,63	1,14	Strongly dep
YDL153C	SAS10	-3,48	1,51	Strongly dep
YHR084W	STE12	-2,59	1,11	Strongly dep
YNR054C		-2,52	1,08	Strongly dep
YHR088W	RPF1	-2,4	1,02	Strongly dep
YOR048C	RAT1	-2,38	1,01	Strongly dep
YNL182C		-2,6	1,09	Strongly dep
YBR247C	ENP1	-2,62	1,09	Strongly dep
YGR280C	PXR1	-3,1	1,28	Strongly dep
YOL041C	NOP12	-2,81	1,14	Strongly dep
YNL292W	PUS4	-2,6	1,05	Strongly dep
YGR145W	ENP2	-2,71	1,09	Strongly dep
YGL120C	PRP43	-2,84	1,13	Strongly dep
YCL054W	SPB1	-3,27	1,28	Strongly dep
YIL103W		-2,61	1,02	Strongly dep
YPR112C	MRD1	-3,27	1,27	Strongly dep
YKL021C	MAK11	-2,66	1,03	Strongly dep
YLL034C		-2,6	1,01	Strongly dep
YPL263C	KEL3	-2,96	1,13	Strongly dep
YMR128W	ECM16	-3,95	1,51	Strongly dep
YNL248C	RPA49	-3,11	1,16	Strongly dep
YLR183C	TOS4	-2,75	1,02	Strongly dep
YLR129W	DIP2	-3,35	1,24	Strongly dep
YPL043W	NOP4	-3,14	1,11	Strongly dep
YLL008W	DRS1	-3,57	1,22	Strongly dep
YDL112W	TRM3	-3,01	1,01	Strongly dep
YPL126W	NAN1	-3,26	1,04	Strongly dep
YLR409C	UTP21	-3,57	1,1	Strongly dep

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep
YJR041C		-3,43	1,03	Strongly dep
YDR087C	RRP1	-4,47	1,28	Strongly dep
YCR087C-A		-3,99	1,11	Strongly dep
YLR106C	MDN1	-4,46	1,2	Strongly dep
YOR272W	YTM1	-4,35	1,02	Strongly dep
YNL175C	NOP13	-7,92	-1,03	Strongly dep
YJL050W	MTR4	-5,98	-1,07	Strongly dep
YPL012W	RRP12	-5,77	-1,11	Strongly dep
YGL078C	DBP3	-4,2	-1,01	Strongly dep
YMR049C	ERB1	-4,86	-1,24	Strongly dep
YBL039C	URA7	-5,16	-1,44	Moderately dep
YOR206W	NOC2	-3,71	-1,05	Moderately dep
YMR290C	HAS1	-4,34	-1,25	Moderately dep
YER006W	NUG1	-4,47	-1,3	Moderately dep
YOL077C	BRX1	-4,29	-1,26	Moderately dep
YGL099W	LSG1	-3,46	-1,03	Moderately dep
YHR197W		-3,57	-1,09	Moderately dep
YDR465C	RMT2	-3,85	-1,19	Moderately dep
YNL002C	RLP7	-3,63	-1,13	Moderately dep
YGR103W	NOP7	-6,48	-2,02	Moderately dep
YDR447C	RPS17B	-7,24	-2,3	Moderately dep
YER131W	RPS26B	-10,95	-3,49	Moderately dep
YGR173W		-3,33	-1,07	Moderately dep
YHR170W	NMD3	-4,31	-1,41	Moderately dep
YMR229C	RRP5	-5,41	-1,77	Moderately dep
YLR197W	SIK1	-6,73	-2,2	Moderately dep
YBR084W	MIS1	-5,02	-1,65	Moderately dep
YPR163C	TIF3	-4,91	-1,64	Moderately dep
YLR180W	SAM1	-7,62	-2,61	Moderately dep
YDR429C	TIF35	-3,52	-1,23	Moderately dep
YIL078W	THS1	-3,2	-1,12	Moderately dep
YJL033W	HCA4	-3,13	-1,11	Moderately dep
YOR271C		-3,03	-1,08	Moderately dep
YDL157C		-3,18	-1,14	Moderately dep
YJR070C		-3,86	-1,4	Moderately dep
YGL171W	ROK1	-3,08	-1,12	Moderately dep
YLR002C	NOC3	-3,25	-1,19	Moderately dep
YOR021C		-2,78	-1,02	Moderately dep
YNL075W	IMP4	-3,1	-1,14	Moderately dep
YJL200C		-4	-1,49	Moderately dep
YLR449W	FPR4	-3,77	-1,4	Moderately dep
YOL022C		-3,02	-1,12	Moderately dep
YBL083C		-3,21	-1,2	Moderately dep
YMR131C	RRB1	-3,25	-1,21	Moderately dep
YOR108W		-5,64	-2,11	Moderately dep
YPR010C	RPA135	-5,56	-2,09	Moderately dep
YDR060W	MAK21	-3,42	-1,3	Moderately dep
YPL226W	NEW1	-3,15	-1,2	Moderately dep
YBL076C	ILS1	-3,27	-1,26	Moderately dep

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep
YOR310C	NOP58	-4,27	-1,64	Moderately dep
YJL109C	UTP10	-3,64	-1,4	Moderately dep
YOR051C		-2,6	-1	Moderately dep
YIR026C	YVH1	-3,01	-1,17	Moderately dep
YDL060W	TSR1	-3,22	-1,27	Moderately dep
YBL082C	RHK1	-3,12	-1,24	Moderately dep
YKL078W	DHR2	-3	-1,19	Moderately dep
YLR068W	FYV7	-2,54	-1,02	Moderately dep
YDR101C	ARX1	-3,65	-1,48	Moderately dep
YPL266W	DIM1	-3,68	-1,49	Moderately dep
YER126C	NSA2	-3,63	-1,47	Moderately dep
YKL216W	URA1	-6,4	-2,6	Moderately dep
YLL022C	HIF1	-3,01	-1,23	Moderately dep
YDR165W	TRM82	-3,04	-1,25	Moderately dep
YOL080C	REX4	-2,52	-1,04	Moderately dep
YHR020W		-5,7	-2,36	Moderately dep
YDR023W	SES1	-3,3	-1,38	Moderately dep
YDR324C	UTP4	-2,37	-1	Moderately dep
YNR003C	RPC34	-3,12	-1,33	Moderately dep
YCL059C	KRR1	-2,72	-1,16	Moderately dep
YNL247W		-3,88	-1,67	Moderately dep
YCR057C	PWP2	-2,57	-1,11	Moderately dep
YMR246W	FAA4	-4,93	-2,13	Moderately dep
YNL110C	NOP15	-2,91	-1,26	Moderately dep
YMR146C	TIF34	-2,82	-1,23	Moderately dep
YMR217W	GUA1	-3,85	-1,69	Moderately dep
YOR341W	RPA190	-6,88	-3,01	Moderately dep
YKR081C	RPF2	-3,05	-1,35	Moderately dep
YDR399W	HPT1	-2,89	-1,28	Moderately dep
YPR033C	HTS1	-2,64	-1,18	Moderately dep
YPL211W	NIP7	-4,58	-2,05	Moderately dep
YPL207W		-2,67	-1,2	Moderately dep
YKL014C		-4,05	-1,82	Moderately dep
YDR083W	RRP8	-2,91	-1,32	Moderately dep
YGR200C	ELP2	-2,62	-1,19	Moderately dep
YKL191W	DPH2	-2,42	-1,1	Moderately dep
YIL133C	RPL16A	-4,46	-2,03	Moderately dep
YFR001W	LOC1	-2,47	-1,13	Moderately dep
YGR123C	PPT1	-4,29	-1,96	Moderately dep
YBL004W	UTP20	-3,73	-1,71	Moderately dep
YGR083C	GCD2	-2,19	-1,01	Moderately dep
YBR092C	PHO3	-3,48	-1,6	Moderately dep
YDR496C	PUF6	-2,45	-1,13	Moderately dep
YDL201W	TRM8	-3,21	-1,48	Moderately dep
YLR214W	FRE1	-3,49	-1,61	Moderately dep
YGR128C	UTP8	-2,22	-1,03	Moderately dep
YAL035W	FUN12	-2,36	-1,09	Moderately dep
YLR172C	DPH5	-2,91	-1,35	Moderately dep
YDL208W	NHP2	-5	-2,33	Moderately dep

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep
YLR222C	UTP13	-2,6	-1,22	Moderately dep
YDL213C	FYV14	-2,56	-1,2	Moderately dep
YDL167C	NRP1	-2,48	-1,17	Moderately dep
YLR435W		-2,83	-1,34	Moderately dep
YDR361C	BCP1	-3,23	-1,53	Moderately dep
YNR044W	AGA1	-2,89	-1,37	Moderately dep
YMR321C		-3,6	-1,71	Moderately dep
YJR129C		-2,24	-1,07	Moderately dep
YBR121C	GRS1	-3,27	-1,56	Moderately dep
YOR309C		-4,41	-2,1	Moderately dep
YIL094C	LYS12	-2,26	-1,08	Moderately dep
YNL087W		-3,4	-1,62	Moderately dep
YEL040W	UTR2	-2,12	-1,01	Moderately dep
YPL273W	SAM4	-3,79	-1,81	Moderately dep
YAR008W	SEN34	-2,15	-1,03	Moderately dep
YAL059W	ECM1	-3,91	-1,88	Moderately dep
YNL113W	RPC19	-2,17	-1,04	Moderately dep
YPL093W	NOG1	-2,82	-1,37	Moderately dep
YDR234W	LYS4	-3,85	-1,87	Moderately dep
YNL062C	GCD10	-2,49	-1,22	Moderately dep
YDR412W		-2,55	-1,26	Moderately dep
YPL086C	ELP3	-2,41	-1,19	Moderately dep
YEL055C	POL5	-2,79	-1,38	Moderately dep
YER070W	RNR1	-4,31	-2,13	Moderately dep
YLR325C	RPL38	-5,99	-3,02	Moderately dep
YNL061W	NOP2	-3,15	-1,59	Moderately dep
YOR361C	PRT1	-2,67	-1,35	Moderately dep
YOR236W	DFR1	-3,29	-1,67	Moderately dep
YCR047C	BUD23	-2,29	-1,16	Moderately dep
YJL069C	UTP18	-2	-1,02	Weakly dep
YHR149C		-2,51	-1,28	Weakly dep
YOR095C	RKI1	-4,39	-2,25	Weakly dep
YDR346C	SGI1	-2,22	-1,14	Weakly dep
YOR091W		-2,07	-1,07	Weakly dep
YOR168W	GLN4	-2,06	-1,06	Weakly dep
YNR043W	MVD1	-2,03	-1,05	Weakly dep
YMR260C	TIF11	-3,01	-1,55	Weakly dep
YKL099C	UTP11	-2,08	-1,08	Weakly dep
YDR507C	GIN4	-2,35	-1,22	Weakly dep
YDR161W	TCI1	-3,02	-1,58	Weakly dep
YPR110C	RPC40	-2,79	-1,46	Weakly dep
YER073W	ALD5	-3,34	-1,75	Weakly dep
YNR012W	URK1	-2,3	-1,2	Weakly dep
YNL102W	POL1	-2,39	-1,26	Weakly dep
YLR264W	RPS28B	-2,92	-1,54	Weakly dep
YDL003W	MCD1	-2	-1,05	Weakly dep
YDL063C		-2,34	-1,24	Weakly dep
YBR088C	POL30	-2,66	-1,41	Weakly dep
YKR079C		-2	-1,06	Weakly dep

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep
YGR229C	SMI1	-2,04	-1,08	Weakly dep
YER118C	SHO1	-2,17	-1,16	Weakly dep
YLR430W	SEN1	-2,05	-1,11	Weakly dep
YLR432W	IMD3	-4,45	-2,42	Weakly dep
YLL045C	RPL8B	-5,92	-3,22	Weakly dep
YLR196W	PWP1	-2,99	-1,63	Weakly dep
YLR003C		-2,21	-1,22	Weakly dep
YNL132W	KRE33	-2,85	-1,57	Weakly dep
YGR162W	TIF4631	-3,49	-1,92	Weakly dep
YLR042C		-2,07	-1,14	Weakly dep
YJL111W	CCT7	-2,11	-1,16	Weakly dep
YBR242W		-2,14	-1,19	Weakly dep
YNL151C	RPC31	-2,84	-1,58	Weakly dep
YDR382W	RPP2B	-4,21	-2,34	Weakly dep
YLR276C	DBP9	-2,03	-1,14	Weakly dep
YHR064C	SSZ1	-2,73	-1,53	Weakly dep
YPL212C	PUS1	-2,36	-1,32	Weakly dep
YLR186W	EMG1	-2,89	-1,62	Weakly dep
YDR263C	DIN7	-2,13	-1,2	Weakly dep
YJL157C	FAR1	-2,33	-1,32	Weakly dep
YLR372W	SUR4	-3,11	-1,76	Weakly dep
YIL121W		-2,44	-1,4	Weakly dep
YIL131C	FKH1	-2,16	-1,24	Weakly dep
YDL082W	RPL13A	-5,51	-3,18	Weakly dep
YJL198W	PHO90	-2,6	-1,5	Weakly dep
YLR029C	RPL15A	-3,93	-2,27	Weakly dep
YDR300C	PRO1	-2,82	-1,63	Weakly dep
YPL160W	CDC60	-3,88	-2,24	Weakly dep
YLR384C	IKI3	-2,52	-1,46	Weakly dep
YLR017W	MEU1	-2,21	-1,28	Weakly dep
YDR341C		-2,25	-1,31	Weakly dep
YDR321W	ASP1	-4,31	-2,54	Weakly dep
YCR059C	YIH1	-2,44	-1,44	Weakly dep
YOR001W	RRP6	-2,31	-1,37	Weakly dep
YMR263W	SAP30	-2,02	-1,2	Weakly dep
YOR340C	RPA43	-2,4	-1,43	Weakly dep
YGR234W	YHB1	-12,47	-7,49	Weakly dep
YBR084C-A	RPL19A	-4,11	-2,48	Weakly dep
YLR388W	RPS29A	-5,01	-3,04	Weakly dep
YOR326W	MYO2	-2	-1,22	Weakly dep
YOR369C	RPS12	-4,39	-2,69	Weakly dep
YNL069C	RPL16B	-3,24	-2	Weakly dep
YDL121C		-3,25	-2,01	Weakly dep
YJL122W		-2,64	-1,63	Weakly dep
YDL075W	RPL31A	-6,08	-3,76	Weakly dep
YKR060W		-2,03	-1,26	Weakly dep
YLR333C	RPS25B	-2,96	-1,85	Weakly dep
YKL009W	MRT4	-2,23	-1,39	Weakly dep
YMR259C		-2,19	-1,38	Weakly dep

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep
YKL029C	MAE1	-2,17	-1,37	Weakly dep
YJL177W	RPL17B	-5,11	-3,24	Weakly dep
YBR040W	FIG1	-2,01	-1,28	Weakly dep
YDR492W		-2,2	-1,39	Weakly dep
YJL080C	SCP160	-3,23	-2,05	Weakly dep
YMR142C	RPL13B	-6,47	-4,12	Weakly dep
YKL113C	RAD27	-2,09	-1,34	Weakly dep
YHR062C	RPP1	-2,26	-1,45	Weakly dep
YKL056C		-2,36	-1,53	Weakly dep
YHR019C	DED81	-3,43	-2,22	Weakly dep
YLR175W	CBF5	-2,59	-1,68	Weakly dep
YMR235C	RNA1	-2,01	-1,3	Weakly dep
YLR448W	RPL6B	-4,68	-3,05	Weakly dep
YFL004W	VTC2	-2,37	-1,55	Weakly dep
YLR441C	RPS1A	-4,68	-3,05	Weakly dep
YNR021W		-2,01	-1,31	Weakly dep
YNL141W	AAH1	-2,19	-1,44	Weakly dep
YPL245W		-2,85	-1,88	Weakly dep
YEL053C	MAK10	-2,02	-1,35	Weakly dep
YGL148W	ARO2	-4,44	-2,96	Weakly dep
YBR249C	ARO4	-5,43	-3,62	Weakly dep
YHR193C	EGD2	-2,6	-1,74	Weakly dep
YLR212C	TUB4	-2,12	-1,42	Weakly dep
YMR079W	SEC14	-2,78	-1,86	Weakly dep
YGR085C	RPL11B	-3,55	-2,38	Weakly dep
YPL183C		-2,1	-1,41	Weakly dep
YHR208W	BAT1	-4,54	-3,06	Weakly dep
YNL255C	GIS2	-2,83	-1,91	Weakly dep
YOR243C		-3,54	-2,4	Weakly dep
YMR185W		-2,22	-1,52	Weakly dep
YJL207C		-2,23	-1,52	Weakly dep
YHR089C	GAR1	-3,8	-2,6	Weakly dep
YBR143C	SUP45	-2,38	-1,64	Weakly dep
YJL138C	TIF2	-2,27	-1,56	Weakly dep
YCR051W		-2,29	-1,58	Weakly dep
YNL148C	ALF1	-2,32	-1,6	Weakly dep
YCL037C	SRO9	-2,96	-2,04	Weakly dep
YBR162W-A	YSY6	-2,29	-1,58	Weakly dep
YOR101W	RAS1	-2,2	-1,52	Weakly dep
YGR264C	MES1	-2,74	-1,9	Weakly dep
YGL234W	ADE5,7	-2,09	-1,45	Weakly dep
YPR187W	RPO26	-2,52	-1,75	Weakly dep
YNR009W		-2,57	-1,79	Weakly dep
YIL069C	RPS24B	-4,66	-3,26	Weakly dep
YHR068W	DYS1	-2,73	-1,91	Weakly dep
YGR035C		-2,2	-1,54	Weakly dep
YJR031C	GEA1	-2,11	-1,48	Weakly dep
YGR090W	UTP22	-2,15	-1,51	Weakly dep
YEL051W	VMA8	-2,24	-1,57	Weakly dep

Name	Gene name	FC wt	FC rpd3Δ	Rpd3 dep
YJL136C	RPS21B	-4,96	-3,51	Weakly dep
YGL031C	RPL24A	-7,69	-5,48	Weakly dep
YLR061W	RPL22A	-7,3	-5,21	Weakly dep
YLR363W-A		-2,04	-1,46	Weakly dep
YBR073W	RDH54	-2,2	-1,58	Weakly dep
YPL131W	RPL5	-3,53	-2,53	Weakly dep
YOR239W	ABP140	-2,09	-1,5	Weakly dep
YGL123W	RPS2	-3,75	-2,7	Weakly dep
YKR059W	TIF1	-2,11	-1,53	Weakly dep
YER025W	GCD11	-2,82	-2,05	Weakly dep
YPL249C-A	RPL36B	-3,93	-2,86	Weakly dep
YNL178W	RPS3	-3,95	-2,88	Weakly dep
YDR044W	HEM13	-2,57	-1,89	Weakly dep
YLR150W	STM1	-3,82	-2,8	Weakly dep
YLR083C	EMP70	-3,14	-2,31	Weakly dep
YGR285C	ZUO1	-3,04	-2,23	Weakly dep
YKL181W	PRS1	-2,35	-1,73	Weakly dep
YML026C	RPS18B	-6,34	-4,66	Weakly dep
YOL039W	RPP2A	-3,98	-2,93	Weakly dep
YOL121C	RPS19A	-4,78	-3,52	Weakly dep
YOR294W	RRS1	-2,25	-1,66	Weakly dep
YFR031C-A	RPL2A	-5,17	-3,82	Weakly dep
YDL055C	PSA1	-3,14	-2,32	Weakly dep
YGL189C	RPS26A	-2,71	-2,01	Weakly dep
YHR025W	THR1	-2,42	-1,8	Weakly dep
YBR031W	RPL4A	-3,05	-2,27	Weakly dep
YDR345C	HXT3	-3,77	-2,81	Weakly dep
YLR075W	RPL10	-3,16	-2,36	Weakly dep
YML056C	IMD4	-3,66	-2,74	Weakly dep
YJL130C	URA2	-2,66	-1,99	Weakly dep
YIL064W		-3,28	-2,46	Weakly dep

The Rpd3L HDAC complex is essential for the heat stress response in yeast

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(1) A high-throughput genetic screen reveals a network of genes required for heat stress adaptation

In this study, we identified the network of essential activities required for cell adaptation to heat stress. We performed a genome-wide genetic screen that searched for gene deletion mutants of *S. cerevisiae* unable to grow at high temperature. Similar analyses have been done in other stresses, such as osmotic stress, alteration of carbon source or low temperature (Abe and Minegishi, 2008;Giaever *et al.*, 2002;Zapater *et al.*, 2007). However, for the study of heat stress adaptation, numerous works focused in investigating the heat stress response at the level of gene expression or using proteomic approaches in different organisms (Causton *et al.*, 2001;Eisen *et al.*, 1998;Gasch *et al.*, 2000;GuhaThakurta *et al.*, 2002;Larkindale and Vierling, 2008;Matsuura *et al.*, 2010). Therefore, the identification of thermo-sensitive mutants reported in this work is an important contribution to better understand the heat-specific stress adaptation elements in yeast. Remarkably, the 48% of the mutants identified in our genetic screen had not been previously involved in the heat stress response, revealing new activities required for survival in response to temperature increase. On the other hand, the additional 52% of the mutants were already described as heat-sensitive, thus validating the approach.

The genome-wide genetic screen identified 305 yeast viable mutants as thermo-sensitive. To confirm the thermo-sensitivity of these mutants, we individually spotted them on YPD plates, at both control temperature (30º) and high temperature (39ºC), and validated 277 mutants unable to grow at high temperature. Therefore, the initial genetic screen yielded 28 false positive results (which represent the 10% of the mutants identified), that were discarded in the second manual screen. The genome-wide genetic screen consisted of a robot which replicated a collection of the complete yeast non-essential gene deletion mutants, by pinning each mutant on YPD plates at control and high temperature (Tong et al., 2001). The 10% of false positive mutants could be justified by the technical features of the genetic screen (mutants that did not grow due to wrong pinning by the robot).

The final 277 thermo-sensitive mutants represent the 6% of the total of genes analysed. This result indicates that an important fraction of genes in the genome are required for the

adaptive response to high temperature. Nevertheless, we should take into account that in the genetic screen only the non-essential genes of *S. cerevisiae* were analysed, which represent approximately the 80% of the genome. Thus, there may be still several essential genes playing an important role for the heat stress adaptation that we have not identifyed.

One advantage of the second manual screen is that it is easier to evaluate the degree of cell growth defect of the mutants. Therefore, the thermo-sensitive mutants identified in the screen were grouped according to their degree of heat-sensitivity. This classification permits to discriminate genes that are less important from those more important for adaptation to high temperature. The mutant strains with medium or weak thermo-sensitive phenotype identify genes that encode for activities required to adapt in heat stress. On the other hand, the mutant strains that render a strong growth defect, or absence of growth in high temperature, identify genes that encode for essential activities in heat stress adaptation and survival.

When cells are exposed to heat stress, several cellular functions are damaged. To describe the physiological response of cells in front of a heat shock, we performed a functional classification of the large-scale data of thermo-sensitive mutants. The identified heat-essential genes covered almost all general biological functions, from metabolism to protein fate. This is reasonable because protein unfolding and a general imbalance of protein homeostasis are produced when cells are exposed to a heat shock, thus altering almost all cellular physiological aspects (Richter *et al.*, 2010). The most overrepresented functional categories were the following:

- Regulation of carbohydrate metabolism. It is known that, in response to stress, a change in cellular metabolism is produced. Changes in metabolic pathways may be needed to reorganize and stabilize the energy supply of the cell (Malmendal *et al.*, 2006;Voit and Radivoyevitch, 2000).

- Transcriptional factors for the regulation of the mRNA synthesis. For instance, components of the mediator and SAGA complexes, and transcriptional elongating factors, which are essential to promote rapid and efficient gene expression changes required for the heat adaptive response (Zanton and Pugh, 2004;Zanton and Pugh, 2006b).

- Proteins controlling cell morphogenesis and differentiation, such as budding, cell polarity or filament formation. Upon heat stress, membrane proteins may be important to maintain or restore membrane stability and function (Richter *et al.*, 2010).

- Control of cell cycle, which similar to in other environmental stresses, is delayed to allow cells for adaptation (Jenkins and Hannun, 2001;Riezman, 2004).

- Protein fate. It is known that, upon heat stress, genes encoding for chaperones and proteins of the proteolytic system are strongly induced (Richter *et al.*, 2010;Riezman, 2004). They are needed to prevent the defects of wrong protein folding and protein aggregation and to remove misfolded or irreversible aggregated proteins from the cell, thus playing essential roles for heat stress adaptation.

- Biogenesis of cellular compartments, which are expected to be important due to the fact that high temperature severely alters cytoskeleton and organelles organization (Richter *et al.*, 2010).

Notably, only 18% of the heat-essential genes identified in the genetic screen (51 of the 277) were also crucial for cell survival in osmostress, according to data from a genome-wide genetic screen described in Zapater *et al.* (2007). Thus, the network of genes required for heat stress survival seems to be specific, albeit there is some overlapping of genes required for stress survival in different kind of stresses (Auesukaree *et al.*, 2009).

The large-scale data of thermo-sensitive mutants obtained in the screen was further analyzed to identify over-represented protein complexes. Interestingly, the Rpd3 histone deacetylase (HDAC) complex was the most enriched in the screen, with several subunits of the complex displaying a strong heat-sensitive phenotype. Actually, a role of Rpd3 for adaptation to heat stress had already been suggested before (de Nadal *et al.* 2004). Moreover, in addition to the Rpd3 HDAC complex, other protein complexes with previously defined roles in heat shock response were also identified. For instance, the Isw1 remodeling complex, which is involved in chromatin remodeling of heat-responsive genes and is required for proper activation of gene expression upon heat shock (Erkina *et al.*, 2010). Also, mutants in several subunits of the RNA Polymerase II Mediator complex displayed a strong heat-sensitive phenotype. It is known that Mediator plays an important role in both constitutive and inducible expression of Hsf1-dependent genes (Lee *et al.*, 1999;Singh *et al.*, 2006). Moreover, Mediator is involved in the hyperphosphorylation and degradation of Msn2 during stress (Lallet *et al.*, 2006). Other protein complexes enriched in the screen were related to vacuole and endosome function (e.g. Class C Vps complex). A protective role of vacuolar function, based on a possible autophagic system for protein clearance, has been suggested for the heat stress response (Richter *et al.*, 2010).

Overall, the results from the heat-sensitive high-throughput genetic screen, with the functional analysis and the analysis of complex enrichment, will help to further identify and characterize the activities required for an accurate response to heat stress.

(2) The Rpd3 HDAC complex is essential for acute transcriptional response upon heat stress

As the Rpd3 HDAC complex is required for cell viability upon heat stress, we decided to further investigate its functional role in response to high temperature. It is known that Rpd3 regulates both activation and repression of gene expression. Thus, we analyzed transcription by northern blot assays and found that expression of both heat inducible genes (*CTT1* and *ALD3)* and heat-repressed genes (*GAR1*, *RPL16A*, *KRI1*, and *MAK5*) was affected in the *rpd3* mutant strain. Therefore, Rpd3 is required for the regulation of gene expression in response to heat stress, having a role both in the activation as well as in the repression of transcription upon stress.

To define the overall role of Rpd3 in gene expression regulation upon heat stress, we performed a global gene expression analysis comparing gene expression of wild-type and *rpd3*Δ strains upon heat shock (20 minutes at 39ºC). Of the total 6301 genes analyzed, 582 genes were induced at least two fold in response to heat shock, whereas 594 genes were repressed at least two fold. Most of the genes repressed encoded for protein synthesis and ribosomal protein genes. In response to different stresses, these genes are repressed to stop cellular growth, to allow cells to adapt to the new environmental conditions. On the other hand, the strongest induced genes encoded for Heat Shock Proteins (Hsp). Therefore, our data are consistent with other similar analysis, in terms of number and type of genes that change their expression upon heat stress (Alejandro-Osorio *et al.*, 2009;Causton *et al.*, 2001;Gasch *et al.*, 2000).

65% of the genes induced upon heat shock showed a significant reduction in gene expression in the *rpd3* mutant strain. Similarly, 60% of the genes repressed upon heat shock were dependent on Rpd3. Therefore, a high proportion of the heat-responsive genes, either heat-induced or heat-repressed genes are affected by Rpd3 deletion. These data suggest an important role for Rpd3 in modulating global gene expression in response to heat shock, controlling both activation and repression of transcription. These results are consistent with a recent report by Alejandro-Osorio *et al.*, where DNA microarrays were performed to compare gene expression of wild-type and *rpd3*Δ strains, stressing cells over time at 37ºC. This study

also demonstrated that Rpd3 was required for normal expression of repressed and induced genes upon heat stress (Alejandro-Osorio *et al.*, 2009).

The genes altered in the *rpd3* mutant displayed different degrees of dependence on Rpd3. Some genes were strongly or moderately Rpd3-dependent (the 29% of the induced genes and the 35% of the repressed genes), whereas a significant number of genes were weekly dependent on Rpd3 (the 36% of induced genes and the 25% of repressed genes). These results suggest that Rpd3 has differential contribution for the regulation of gene expression. In the case of weekly dependent genes, Rpd3 does not have a crucial role for the expression of these genes, but rather contributes likely in coordination with other regulators to the activation or repression of transcription.

It is interesting to highlight the role of Rpd3 in the activation of gene expression. Although many genetic studies have demonstrated a repressive role of Rpd3 for gene expression, several studies have reported that the deacetylase may also have a positive role in gene expression. For instance, it is required for induction of the osmoresponsive genes (de Nadal *et al*., 2004), the DNA damage-inducible genes (Sharma *et al*., 2007), the anaerobic *DAN*/*TIR* genes (Sertil *et al*., 2007), the yeast transcriptional regulator *HAP1* gene (Xin *et al*., 2007), and the multidrug resistance efflux pump *PDR5* gene (Borecka-Melkusova *et al*., 2008). Moreover, the Rpd3 HDAC complex is recruited to specific stress-activated promoters upon heat shock and H_2O_2 treatment and is required for their activation (Alejandro-Osorio *et al*., 2009). Overall, our data and other reports strongly suggest that Rpd3 plays a role in regulating stress-responsive gene activation and that Rpd3 might be directly involved in transcriptional activation of genes whose expression requires a rapid response to environmental changes.

(3) The role of Rpd3 HDAC complex is different depending on the transcription factor governing gene expression in heat stress

The results of the genome-wide expression analysis suggested that Rpd3 is not required for the whole heat stress transcriptional program. The 34% of the heat-activated genes and the 40% of the heat-repressed genes were independent on the deacetylase. Therefore, we attempted to determine if any special distinction exists between Rpd3-dependent and independent genes, focusing on the heat stress-activated genes. To achieve this objective, we wondered if there was a dependency on the different transcription factors controlling gene expression upon heat stress.

Hsf1 and Msn2/4 are the main transcription factors that mediate gene expression in response to heat shock. In addition, the Rlm1 transcription factor is also required to activate transcription of certain genes under these conditions. Induction of Msn2/4-dependent genes (such as *CTT1*, *ALD3*, *TPS1* or *PNS1*) was highly affected in the *rpd3* mutant strain. These results were previously observed by others (Alejandro-Osorio *et al.*, 2009;de Nadal *et al.*, 2004). In contrast, induction of Hsf1-dependent genes (such as *ZPR1*, *HSP82* and *SSA3*) was not affected by *rpd3* deletion, as neither was the induction of the Rlm1-dependent genes (such as *SED1* and *HSP150*). The dependence on Msn2/4 or Hsf1 transcription factors of the genes affected in the *rpd3* mutant was analyzed in the microarrays data, and we confirmed that Hsf1-dependent genes are not affected in the *rpd3* mutant, whereas the Msn2/4-dependent genes are highly dependent on Rpd3. These data indicate that, upon heat stress, Rpd3 has a differential effect depending on the transcription factor that specifically governs gene expression. Indeed, Rpd3 is required for the activation of genes specifically under the control of the Msn2/4 transcription factors.

Interestingly, it has been described that Rpd3 acts as a repressor at the Hsf1-dependent promoters, since expression of Hsf1-dependent genes is increased in an *rpd3* mutant compared to a wild-type (Kremer *et al.*, 2009) (Figure 10). This repressive role of Rpd3 on Hsf1-dependent genes is not detected in our northern blot assays, although we do observe hyper-activation of some Hsf1-dependent genes in the *rpd3*Δ strain in the microarrays data. Overall, these results suggest that while Rpd3 has a positive role in expression of Msn2/4-regulated genes in response to heat stress as well as to other cellular stresses, it functions as a repressor for Hsf1-dependent genes. From this observation, it could be anticipated that Hsf1-dependent promoters display different requirements for gene expression than Msn2/4-dependent promoters. Indeed, several studies have shown that, for the induction of heat-responsive genes, chromatin regulators contribute differently on genes that depend on Hsf1 or that are under the redundant control of Hsf1 and Msn2/4 transcription factors (Erkina *et al.*, 2006;Erkina *et al.*, 2008;Erkina *et al.*, 2010). Therefore, it is reasonable to assume that the requirements of the STRE-dependent promoters are different than the requirements of the HSE-dependent promoters and that in heat stress, Rpd3 plays a different role in a promoter context manner.

It would be interesting to analyze which are the requirements for those genes under a dual regulation of Hsf1 and Msn2/4 transcription factors, such as *HSP12* or *HSP26*. Actually, it seems that entry of Msn2/4 occurs before the binding of Hsf1 and subsequent entry of

polymerase (Erkina *et al.*, 2008). In fact, activation of *HSP12* is reduced in an *rpd3*Δ strain upon heat stress (de Nadal *et al.*, 2004).

It is worth noting that in response to osmostress, Rpd3 is also required for activation of gene expression, although the scenario is different than in heat stress (Figure 10). In response to osmostress, the Hog1 MAPK is activated and enters into the nucleus to mediate control of gene expression. Hog1 is recruited to osmoresponsive promoters through different specific transcription factors. Then, Hog1 binding at promoters facilitates direct recruitment of the Rpd3 HDAC complex, leading to entry of the polymerase and induction of gene expression. In osmostress, Rpd3 is required to activate transcription of almost all Hog1-dependent genes as a general mechanism, since Rpd3 is recruited by Hog1, regardless of which specific transcription factor governs gene expression (de Nadal *et al*., 2004). However, in response to heat stress, the Rpd3 effect in gene induction is limited to only the genes controlled by Msn2/4.

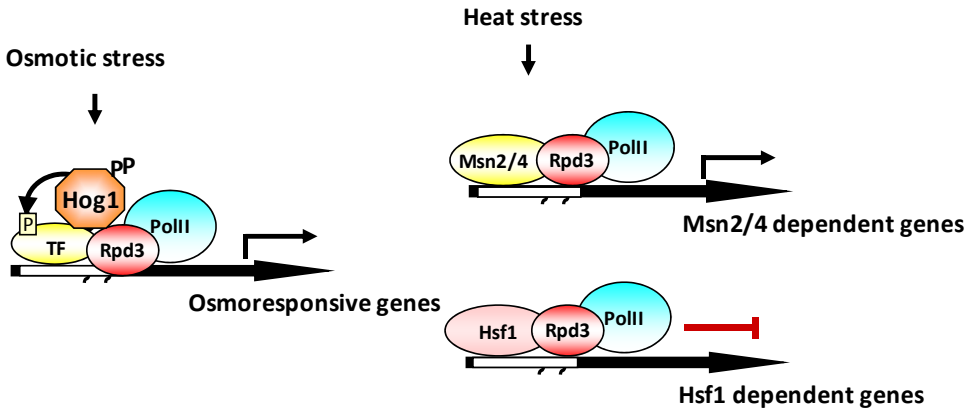

Figure 10. Rpd3 is required for induction of osmostress and heat stress responsive genes.
Upon osmostress, Rpd3 plays a general role for activation of Hog1-dependent osmoresponsive genes. The Hog1 MAPK recruits the deacetylase at the osmoresponsive promoters, depending on different transcription factors. However, upon heat stress, Rpd3 has a differential effect depending on the transcription factor that specifically governs gene expression. It is required for activation of Msn2/4-dependent genes and repression of Hsf1-dependent genes.

The relationship of Rpd3 and Msn2/4 transcription factors is not only restricted to transcription activation upon heat stress. Alejandro-Osorio and coworkers demonstrated that Rpd3 is required for the activation of a wide range of genes in response to different stresses, suggesting that Rpd3 may function as a cofactor for the regulation of the General Stress Response genes, regulated by the Msn2/4 transcription factors (Alejandro-Osorio *et al.*, 2009).

Overexpression of Msn2 or Msn4 activates transcription of stress-dependent genes in absence of any stress (Gasch *et al*., 2000). We demonstrated that Rpd3 was required to

activate gene expression when Msn2 was overexpressed, suggesting that the Rpd3 deacetylase complex is required to transcribe Msn2/4-dependent genes independently of the upstream incoming signal. Rpd3 is required for activation of the *CTT1* and *HSP12* genes upon osmostress in a Hog1-dependent manner, whereas it is also required for *CTT1* and *HSP12* induction upon heat stress, but in a Hog1-independent manner. Therefore, it seems that the role of Rpd3 as a cofactor with Msn2/4 persists despite the different upstream regulators. It would be interesting to decipher the mechanisms by which Rpd3 associates with Msn2/4-dependent promoters despite distinct condition-specific upstream pathways.

(4) The large Rpd3L complex is responsible for transcriptional activation upon heat stress

Rpd3 can be part of the small Rpd3S or the large Rpd3L complex. Cells deficient for the common subunits (*RPD3*, *SIN3* and *UME1*) and for the specific subunits of the large Rpd3L complex (*SAP30*, *SDS3*, *DEP1* and *PHO23*) displayed impaired transcription activation of the Msn2/4-dependent genes upon heat shock. However, cells deficient for the subunits of the small complex (*RCO1* and *EAF3*) displayed normal gene induction. These results indicated that the large Rpd3L complex is the one involved in the regulation of transcription activation upon heat stress.

Correspondingly, mutants of the central components of the complex and mutants of the large Rpd3L complex displayed a thermo-sensitive phenotype, whereas *rco1* mutant of the small complex did not. Thus, the large Rpd3L complex is the one required for cell adaptation to heat stress. Remarkably, cells deficient for *EAF3,* which encodes for a subunit of the small complex, did render thermo-sensitive cells. Recently it has been described that Esa1, the catalytic subunit of the NuA4 histone acetyl transferase (HAT) complex is required for the activation of the *HSP82* gene, suggesting that NuA4 may act directly in the regulation of Hsf1-target genes (Kramer and Gross 2009). Eaf3, in addition to being a subunit of the small Rpd3S complex, also acts as a subunit of the NuA4 HAT complex. Therefore, the fact that the *eaf3* mutant displayed a thermo-sensitive phenotype could be explained because Eaf3 may regulate proper expression of the Hsf1-dependent genes in response to heat stress by a different mechanism.

The role of Rpd3 in transcriptional activation when belongs to the large Rpd3L complex has been shown in other studies, and it seems well established that the large complex is recruited at promoters but not at coding regions to mediate gene activation (Sertil *et al.,* 2007; Sharma *et al*., 2007). Chromatin immunoprecipitation (ChIP) experiments showed that the

large complex (identified by Sap30) was recruited at promoters but not at ORF regions of the genes. It would be interesting to study whether the small Rpd3S complex is as well recruited at the ORF regions of the Msn2/4-dependent genes.

(5) The binding of Rpd3 HDAC complex to heat stress promoters depends on Msn2/4

When we analyzed the recruitment of Rpd3 to heat stress-responsive promoters, we found that the Rpd3 complex was recruited to Msn2/4-dependent promoters (*CTT1*, *ALD3* and *HSP12*) in response to heat stress. In contrast, Rpd3 complex associated with Hsf1-dependent promoters (*ZPR1* and *HSP82)* under basal conditions and this recruitment increased in response to heat stress. As described before, Rpd3 acts as a repressor of Hsf1-dependent gene expression (Kremer *et al.*, 2009). Thus, the fact that Rpd3 is present in Hsf1-dependent genes in the absence of stress suggests that Rpd3 is critical to repress basal transcription that occurs commonly among most heat shock genes. Upon induction, the negative contribution of Rpd3 may serve to balance the induction of gene expression by alternative factors, acting to fine-tune the heat shock transcriptional response. In the case of Msn2/4-dependent genes basal transcription is repressed without the help of Rpd3 and the role of Rpd3 is important for activation of gene expression upon stress.

Promoter binding of the Rpd3 complex upon stress was altered in the *msn2/4*Δ strain, suggesting that the Msn2/4 transcription factors may target Rpd3. However, whereas the Rpd3 complex recruitment was totally impaired at the *ALD3* promoter, it was only partially reduced at the *CTT1* and *HSP12* promoters. Therefore, the presence of Msn2/4 at promoters is not totally required for targeting Rpd3. Further experiments could be done to analyze whether Msn2/4 are the only factors that recruit the deacetylase in response to heat stress. In any case, the signaling molecule that recruits the deacetylase at the Msn2/4-dependent promoters upon heat stress remains to be identified.

(6) Role of the Rpd3 HDAC complex in transcriptional activation upon heat stress

By ChIP experiments of RNA Pol II, we observed that association of polymerase at the Msn2/4 promoters in response to heat stress was not abolished, but more than 50% reduced in the *rpd3* mutant compared to wild-type. These results are consistent with the results of the northern blot assays, in which we observe that expression of these genes is not abolished but strongly affected in the *rpd3* mutant. Therefore, even without Rpd3 and only with the presence of Msn2/4 transcription factors at the promoter, there may be a little entry of polymerase and small levels of induction. In any case, it is clear that Rpd3 has an important

role for entry of polymerase at promoters and is positively regulating transcription initiation upon heat stress.

It is known that a transient eviction of nucleosomes at heat-dependent genes is produced in response to heat stress (Erkina *et al.*, 2008;Erkine *et al.*, 2003;Gross *et al.*, 1993;Petesch *et al.*, 2008;Zhao *et al.*, 2005). By ChIP experiments monitoring loss of histone 4 at the *CTT1* promoter upon a heat shock time course, we found that whereas in the *msn2/4* mutant, histone eviction was strongly impaired, in the *rpd3* mutant, eviction of histones was modestly affected (35% less of eviction than the wild-type at 10 minutes of stress). These results are in agreement with the impaired transcription of the *msn2/4*Δ strain and the partially affected transcription of the *rpd3*Δ strain, although we expected a higher effect in the *rpd3* mutant. Of note, whereas the role of Rpd3 for entry of polymerase is clear, it seems that Rpd3 is not essential for chromatin remodeling. These results suggest that, similar to other examples, such as the DNA damage-inducible genes (Sharma *et al*., 2007), Rpd3 may regulate later stages in the assembly of the preinitiation complex or may facilitate multiple rounds of polymerase recruitment, whereas it is not involved in the remodeling of chromatin structure.

(7) The function of Rpd3 in heat stress gene induction requires its histone deacetylase activity

To determine if deacetylase activity was required for Rpd3 function in heat stress, we used a catalytically inactive Rpd3 mutant, which is unable to deacetylate its substrates (Kadosh *et al.*, 1998). This mutant did not complement the heat sensitivity of an *rpd3*Δ strain, indicating that the Rpd3 deacetylase activity is required for cell adaptation and viability upon heat stress. Moreover, activation of the Msn2/4-dependent genes *CTT1* and *ALD3* upon heat stress was affected in the catalytically inactive mutant to the same extent than the *rdp3*Δ strain. Correspondingly, the catalytically inactive *rpd3* mutant showed reduced binding of RNA Pol II to promoters compared to wild-type. Overall, these results indicate that the deacetylase activity of Rpd3 is required for proper recruitment of RNA Pol II to stress-dependent genes and for activation of transcription in response to heat stress, allowing cells to adapt and survive in heat stress.

Although histone deacetylation has been traditionally associated with repression of transcription, it has been demonstrated that deacetylation of histones may also be associated with transcriptional activation (Bernstein *et al.*, 2000;Wang et al., 2002;Deckert and Struhl, 2001). Moreover, as mentioned before, Rpd3 has been shown to activate transcription in response to specific situations (de Nadal et al., 2004;Sertil et al., 2007;Sharma et al.,

2007;Alejandro-Osorio *et al.*, 2009;Xin *et al*., 2007;Borecka-Melkusova *et al.,* 2008). In some cases it has been demonstrated that a decrease of histone acteylation levels by the direct action of Rpd3 is associated with the positive effect on transcription (de Nadal et al., 2004; Sertil et al., 2007; Sharma et al., 2007). However, in the case of heat stress, although we know that the deacetylase activity of Rpd3 is required for the positive effect on transcription, we still do not know whether histones are the substrates of the deacetylation.

In order to test whether Rpd3 is deacetylating histones on the Msn2/4-dependent genes for their proper activation in response to heat shock, we performed ChIP assays to analyze Histone 4 Lysine acteylation marks on *CTT1* and *ALD3* promoters upon heat stress. We found no differences in the *rpd3* mutant strain compared to the wild-type strain (results not shown). One explanation for this result is that other core histones may be the specific substrate of deacetylation by Rpd3. To test this hypothesis, ChIP experiments of other acetylated histones should be performed. Another possibility is that other factors than histones are substrates for the Rpd3 deacetylase activity. It has been described that some histone deacetylases can deacetylate other proteins different than histones, such as transcription factors (Sakurai and Enoki, 2010;Swingler *et al.*, 2010). Indeed, Msn2/4 undergoes posttranslational modifications previous to their activation (Garreau *et al.*, 2000;Gorner *et al.*, 1998;Lallet *et al.*, 2006). Therefore, we cannot discard the possibility that, in response to heat stress, Rpd3 deacetylates Msn2/4 or another co-transcription factor. The fact that Rpd3 seems to have not a direct role for chromatin remodeling upon heat stress would support the idea that histones are not the substrates of the deacetylase. In any case, deacetylation by Rpd3, whether at histones or other proteins, is a modification with a key role in gene induction upon stress.

(8) The Rpd3 HDAC complex may act in coordination with other chromatin regulators

As described in the introduction, in addition to histone acetylation and deacetylation, other post-translational chromatin modifications are required for regulating gene expression in response to heat stress. Thus, in addition to Rpd3, other histone modifying factors may also coordinate gene expression under heat stress. For instance, H3 lysine trimethylation is rapidly lost in *HSP104* in response to stress (Zhang et al., 2005; Dai et al., 2008).

To identify other chromatin regulators involved in the adaptive response to heat stress, we can use the data from the genetic screen described here. Actually, we found that mutations in other chromatin-modifying activities, such as *UBP8* (encoding a histone deubiquitinase), *DOT1* (encoding a histone methylase) or *SNF1* (encoding a kinase involved in chromatin

remodeling) caused cell growth defects at high temperature. Thus, our analyses provide a powerful tool to further investigate essential activities for the heat stress adaptive response.

Personal contribution to this work: Except for the development of the genome-wide genetic screen, I have been fully involved in the design, execution and discussion of the experiments and results described in this article.

Targeting the basic helix-loop-helix leucine Zipper Rtg1 and Rtg3 transcription factors by the Stress-Activated Protein Kinase Hog1

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Running Head: The Hog1 SAPK controls Rtg1/3 upon osmostress.

ABSTRACT

Cells modulate expression of nuclear genes in response to alterations in mitochondrial function, a response termed retrograde (RTG) regulation. In budding yeast, the RTG pathway relies on Rtg1 and Rtg3 basic helix-loop-helix leucine Zipper transcription factors. Exposure of yeast to external hyperosmolarity activates the Hog1 stress-activated protein kinase (SAPK), which is essential for the induction of diverse osmoadaptive responses, such as regulation of gene expression. Five transcription factors, including Sko1, Hot1, the redundant Msn2 and Msn4, and Smp1, have been shown to be controlled by the Hog1 SAPK. The mechanisms by which Hog1 regulates their function differ from one to another. Here, we show that Rtg1 and Rtg3 transcription factors are new targets of the Hog1 SAPK. In response to osmostress, RTG-dependent genes are induced in a Hog1-dependent manner and Hog1 is required for Rtg1/3 complex nuclear translocation. In addition, Hog1 activity regulates Rtg1/3 binding to chromatin and transcriptional activity. Therefore, Hog1 modulates Rtg1/3 complex by several mechanisms. Overall, our data suggest that Hog1, through activation of the RTG pathway, contributes to ensure mitochondrial function as part of the Hog1-mediated osmoadaptive response.

INTRODUCTION

Cells can monitor and respond to changes in the state of mitochondria by mediating changes in the expression of nuclear genes, a response termed retrograde (RTG) regulation (Liao and Butow, 1993;Parikh *et al.*, 1987). In yeast, the RTG signaling pathway functions as a homeostatic or stress response mechanism to adjust various biosynthetic and metabolic activities when mitochondrial dysfunction is produced (Liao *et al.*, 1991;Liu and Butow, 1999;Small *et al.*, 1995). The key transcriptional activators of the RTG pathway are the Rtg1 and Rtg3 basic helix-loop-helix leucine Zipper (bHLH-Zip) transcription factors, which activate transcription by binding as a heterodimer to the consensus sequence GTCAC, termed R box (Jia *et al.*, 1997;Liao and Butow, 1993). Rtg1 and Rtg3 function as a heterodimer because neither protein alone is able to bind to R box sites (Jia *et al.*, 1997). Although transcription activation requires both Rtg1 and Rtg3, only Rtg3 has been shown to contain transcriptional activation domains (Rothermel *et al.*, 1997). In respiratory competent cells, Rtg1 and Rtg3 exist as a complex largely in the cytoplasm, whereas in cells with mitochondrial dysfunction, such as cells without mtDNA (ρ^o petites), they exist as a complex predominantly localized in the nucleus (Sekito *et al.*, 2000). Another member of the RTG pathway is Rtg2, a cytoplasmic protein that may act as a proximal sensor of mitochondrial dysfunction, being required for Rtg1/3 complex nuclear translocation and subsequent activation of gene expression (Liu and Butow, 2006;Rothermel *et al.*, 1997). Remarkably, the RTG pathway is known to be negatively regulated by the TOR pathway (Wullschleger *et al.*, 2006). Nuclear accumulation of Rtg1/3, as well as expression of their target genes, is induced by addition of rapamycin (Komeili et al 2000).

Exposure of yeast cells to increases in external osmolarity activates the Hog1 stress-activated protein kinase (SAPK), which is essential for the induction of diverse osmoadaptive responses. One of the main functions of activated Hog1 SAPK is the regulation of gene expression (de Nadal and Posas, 2010;Martinez-Montanes *et al.*, 2010;Weake and Workman, 2010). Indeed, genome-wide transcriptional analyses showed that a large number of genes are

regulated by osmostress in a Hog1-dependent manner, including genes that encode proteins implicated in carbohydrate metabolism, general stress protection, protein production, and signal transduction (Hohmann, 2002;Posas *et al.*, 2000;Rep *et al.*, 2000). One mechanism by which Hog1 modulates gene expression is by regulation of transcription factors. Sko1, Hot1, the redundant Msn2 and Msn4, and Smp1 have been proposed to be controlled by the Hog1 SAPK (de Nadal *et al.*, 2003;Proft *et al.*, 2001;Rep *et al.*, 2000;Rep *et al.*, 1999). These factors are unrelated, and the mechanisms by which Hog1 regulates their function differ from one to another. Sko1 is an ATF/CREB factor that represses gene expression under non-stress conditions by the recruitment of the general corepressor complex Cyc8-Tup1. In response to osmostress, Hog1 phosphorylates Sko1, switching its activity from a repressing to an activating state, which involves the recruitment of the SWI/SNF and SAGA complexes (Guha *et al.*, 2007;Kobayashi *et al.*, 2008;Proft *et al.*, 2001;Proft and Struhl, 2002). Hot1 physically interacts with Hog1, and its binding to DNA is regulated by Hog1 kinase activity. Morover, Hog1 mediates RNA Pol II recruitment at Hot1-target promoters (Alepuz *et al.*, 2003;Alepuz *et al.*, 2001;Rep *et al.*, 1999). Msn2 and Msn4 are generic stress factors controlled by PKA and Hog1 (Alepuz *et al.*, 2001;Rep *et al.*, 2000). Finally, Smp1 is phosphorylated by Hog1 in response to osmostress and its transactivation activity is dependent on phosphorylation by Hog1 (de Nadal *et al.*, 2003). However, the activators reported to be under the control of Hog1 cannot account for the regulation of all the Hog1-dependent genes, suggesting that additional transcription factors under the control of the SAPK are required for gene expression in osmostress (Capaldi *et al.*, 2008;Miller *et al.*, 2011).

Here, we show that, in response to osmostress, Hog1 controls Rtg1/3 transcription factor complex to induce RTG-dependent genes. Hog1 interacts with the Rtg1/3 transcription complex *in vivo* and it is required for nuclear translocation of the complex upon osmostress and for recruitment of the complex at Rtg1/3-responsive promoters. We have identified the relevant Hog1 phosphorylation sites in Rtg3 and analyzed their effect on stress-regulated gene expression. Overall, our data suggest that Hog1, through activation of the RTG pathway, ensures that mitochondrial function is maintained for proper adaptation to osmostress.

RESULTS

The Rtg1 and Rtg3 transcription factors are required for cell survival upon osmostress

In an exhaustive genome-wide genetic screen searching for deletions that render cells osmosensitive, we identified several protein complexes involved in transcription such as SAGA, Mediator and RSC (Mas *et al.*, 2009;Zapater *et al.*, 2007). In addition, we found that mutations in *RTG1* and *RTG3* genes rendered cells unable to grow at high osmolarity. *RTG1* and *RTG3* genes encode for basic helix-loop-helix leucine Zipper (bHLH-Zip) transcription factors, which form a heterodimer to bind to the R box sites of promoters and activate gene expression under mitochondrial dysfunction.

To characterize in more detail the phenotype of osmosensitivity of the *rtg1* and *rtg3* mutants, we individually spotted these mutant strains onto YPD plates with different concentrations of salt and sorbitol. Deletion of *RTG1* and *RTG3* genes affected cell growth at high osmolarity both in salt and sorbitol media, indicating that these transcription factors are important for adaptation to osmostress (Figure 1A).

RTG-dependent genes are induced in response to osmostress in a Hog1-dependent manner

The requirement of *RTG1* and *RTG3* for cell survival at high osmolarity prompted us to analyze the expression of genes known to be under the control of Rtg1 and Rtg3 transcription factors, in response to osmostress. The RTG pathway is active in basal conditions, but in the presence of glutamine, Rtg1/3 transcription factors are retained in the cytoplasm and RTG-dependent genes are repressed (Komeili *et al.*, 2000;Liao and Butow, 1993;Liu and Butow, 1999). Thus, to maintain the RTG pathway inactive and Rtg1/3-target genes repressed, growth media for all the experiments was supplemented with glutamine (see Materials and Methods). Typical Rtg1/3-target genes are *CIT2*, encoding a peroxisomal isoform of citrate syntase, *PYC1*, encoding pyrubate carboxylase, and *DLD3*, encoding a cytoplasmic isoform of D-lactate dehydrogenase. These genes show a strong increase in levels of expression, in response to respiratory dysfunction (Chelstowska *et al.*, 1999;Epstein *et al.*, 2001;Liao *et al.*, 1991). These

genes were also strongly induced in response to osmostress and their transcription was dependent on *RTG1* and *RTG3* (Figure 1B).

When cells suffer mitochondrial dysfunction, the cytoplasmic Rtg2 protein is required for the activation of the retrograde response (Liao and Butow, 1993). Remarkably, under osmostress conditions, cells lacking *RTG2* also displayed impaired transcription of the RTG-dependent genes (Figure S1A). These data indicated that the integrity of the RTG pathway is required for gene activation upon osmostress.

The Hog1 MAP kinase plays a crucial role to regulate gene expression upon osmostress. Then, we tested whether induction of the Rtg1/3-target genes in response to high osmolarity was dependent on Hog1. Expression of *CIT2, PYC1 and DLD3* was strongly reduced upon stress in a *hog1*Δ strain when compared to a wild-type (Fig 1B). Therefore, Hog1 is required for activation of RTG-dependent genes upon osmostress. To test whether Hog1 was required for induction of RTG-dependent genes specifically upon osmostress, we analyzed expression of the RTG-dependent genes in response to rapamycin. Activation of RTG-responsive genes was not altered in a *hog1*Δ strain upon addition of rapamycin (Figure S1B). Thus, Hog1-mediated expression is restricted to osmostress

Of note, induction of osmoresponive genes that are regulated by defined transcription factors (e.g. *CTT1*, *STL1*, *GRE2*) was not affected in cells lacking *RTG1* or *RTG3* (FigureS1C). Therefore, Rtg1 and Rtg3 transcription factors are required specifically for activation of a specific subset of osmoresponsive genes.

The Hog1 SAPK interacts with the Rtg1/3 complex in vivo

It was reported that Hog1 is able to interact with some of the transcription factors regulated by the SAPK (de Nadal and Posas, 2010). To investigate the relationship between Hog1 and the Rtg1/3 transcription complex, we tested whether Hog1 was able to interact with Rtg1 and Rtg3 upon osmostress. We performed GST-pull down experiments in extracts from osmotically stressed cells expressing GST-Hog1 and HA-tagged versions of *RTG1* and *RTG3*. Hog1 was able to coprecipitate with both Rtg1 and Rtg3 proteins in osmostress (Figure 2).

Thus, the Hog1 SAPK physically interacts with the Rtg1/3-complex, which provides biochemical evidence for the relationship between the SAPK and the transcription complex.

The Rtg1/3 complex and Hog1 bind to RTG-dependent promoters interdependently

Osmoresponsive transcription factors bind at specific target-promoters to regulate gene expression in response to osmostress and, in some cases, Hog1 is required to modulate their association to DNA (Alepuz *et al.*, 2003;Alepuz *et al.*, 2001). Thus, we asked whether Rtg1/3 complex was recruited at the corresponding target-promoters upon osmostress and whether this recruitment was dependent on Hog1. Because Rtg1 and Rtg3 form a heterodimer to bind to DNA, since neither protein alone is able to bind to a target R box site (Jia *et al.*, 1997), we used chromatin immunoprecipitation (ChIP) of Rtg1-6HA to follow the binding of the complex to *CIT2* and *DLD3* promoters upon osmostress. Rtg1-HA was recruited at *CIT2* and *DLD3* promoters in response to osmostress. However, binding of Rtg1-HA was abolished in a *hog1*Δ strain (Figure 3A). Therefore, binding of the transcription complex to chromatin upon osmostress is dependent on the Hog1 SAPK.

It is known that the Hog1 SAPK is targeted to specific osmostress-responsive genes in response to stress, and this recruitment is dependent on specific transcription factors (Alepuz *et al.*, 2001;Pascual-Ahuir *et al.*, 2006;Pokholok *et al.*, 2006;Proft *et al.*, 2006). Thus, we asked whether Hog1 was recruited at the RTG-dependent promoters in response to stress and whether this recruitment was dependent on the Rtg1 transcription factor complex. By performing ChIP experiments, we followed the binding of Hog1-HA to *CIT2* and *DLD3* promoters before and after osmostress in a wild-type and *rtg1*Δ strains. As shown in Figure 3B, Hog1 associated at these promoters in response to osmostress and its recruitment was dependent on the presence of the Rtg1 transcription factor (Figure 3B). Taken together, these data provide evidence that Hog1 is directly involved in the regulation of transcription of Rtg1/3-target genes in response to osmostress.

Moreover, we assessed the presence of RNA Pol II (Rpb1) at the promoters of the RTG-dependent genes in wild type and *rtg1*Δ cells in response to osmostress. RNA Pol II was recruited at the promoters of *CIT2* and *DLD3* in response to osmostress and, as expected, recruitment of RNA Pol II upon osmostress was impaired in *rtg1*Δ cells (Figure 3C). These results are consistent with the fact that in an *rtg1*Δ strain, *CIT2* and *DLD*3 genes are not expressed in response to stress (Figure 1B).

Hog1 is required for nuclear translocation of the Rtg1/3 complex upon osmostress

The RTG pathway is regulated by the dynamic localization of the Rtg1/3 heterodimer from the cytoplasm to the nucleus (Sekito *et al.*, 2000). Thus, we assessed the subcellular localization of the Rtg1/3 transcription complex upon osmostress. We constructed plasmids containing in-frame GFP fusion protein at the C terminus of full-length Rtg1 and Rtg3 proteins expressed under their native promoters. Rtg1-GFP and Rtg3-GFP were mainly localized in the cytoplasm in the absence of stress, whereas they showed a predominant nuclear localization when cells were subjected to a brief osmotic shock (Figure 4A). We next addressed whether Hog1 regulates the subcellular localization of the Rtg1/3-complex, expressing Rtg1-GFP and Rtg3-GFP fusion proteins in a *hog1*Δ background. As depicted in Figure 4A, the nuclear accumulation of Rtg1-GFP and Rtg3-GFP was abolished upon osmostress in cells lacking *HOG1*, indicating that Hog1 is required for the nuclear translocation of the Rtg1/3 transcription complex in response to osmostress.

Of note, when *hog1*Δ cells expressing Rtg1-GFP were treated with rapamycin, Rtg1-GFP concentrated into the nucleus similar to wild-type (Figure 4A, right panels), showing that Hog1 is not required for nuclear translocation of the Rtg1 in response to rapamycin. Taken together, the data indicate that Hog1 regulates the subcellular localization of the Rtg1/3 complex specifically upon osmostress.

Nuclear translocation of the Rtg1/3 complex upon osmostress is independent on Hog1 catalytic activity

The above results prompted us to investigate whether Hog1 catalytic activity was required for entry of the Rtg1/3 complex into the nucleus upon osmostress. For this purpose, *hog1*Δ cells expressing Rtg1-GFP or Rtg3-GFP were transformed with an empty vector or a monocopy vector containing full length *HOG1* or a catalytically inactive Hog1 mutant (*HOG1*^{KNN}) (Wurgler-Murphy *et al.*, 1997). Cells lacking *HOG1* failed to accumulate Rtg1-GFP and Rtg3-GFP into the nucleus upon osmostress. Expression of wild-type Hog1 restored the ability of cells to translocate Rtg1-GFP and Rtg3-GFP into the nucleus upon stress. Interestingly, cells expressing the catalytically impaired Hog1 concentrated Rtg1-GFP and Rtg3-GFP into the nucleus upon osmostress as the wild-type cells (Figure 4B). Therefore, our results indicated that, upon osmostress, it is not the catalytic activity of Hog1 but the presence of Hog1 that is required for Rtg1/3 complex accumulation into the nucleus.

To confirm that only the presence of Hog1 is required for the localization of the Rtg1/3 complex, we followed the localization of Rtg1-GFP and Rtg3-GFP in cells expressing modified versions of Hog1 with altered subcellular localization. A Hog1 mutant containing point mutations at the phosphorylation sites by the Pbs2 MAPKK, which is unable to translocate into the nucleus upon stress (Hog1^{TAYA}) (Ferrigno *et al.*, 1998) and a Hog1 fused to a nuclear localization site (NLS), which is retained into the nucleus even in the absence of stress were used. As shown in Figure 4C (left panel), cells expressing Hog1^{TAYA} showed Rtg1-GFP and Rtg3-GFP localized throughout the cytoplasm and nucleus under unstressed conditions and their localization did not change upon stress. Correspondingly, cells expressing the nuclear targeted Hog1 (Hog1^{NLS}), showed Rtg1 and Rtg3 concentrated into the nucleus both in unstressed cells and after an osmotic shock (Fig 4C, right panel). Therefore, the localization of the Rtg1/3 complex depends on the localization of Hog1. Overall, these results suggest that it is not the catalytic activity of Hog1 but only the interaction of Hog1 with the Rtg1/3 transcription complex that is required for nuclear translocation of the complex in response to osmostress.

Hog1 catalytic activity is required for binding of Rtg1/3 complex at promoters and proper RTG dependent gene expression

We then asked whether the catalytic activity of Hog1 could play a role in the regulation of the Rtg1/3 complex. We initially analyzed the role of Hog1 catalytic activity in the binding of Rtg1 to chromatin. *hog1*Δ cells with genomically tagged Rtg1 (Rtg1-HA) were transformed with a vector expressing wild-type Hog1, Hog1^{KNN} and an empty vector. As shown previously, deletion of *HOG1* resulted in impaired recruitment of Rtg1/3 complex to chromatin (i.e. *CIT2* and *DLD3* promoters) upon osmostress. In contrast, in cells expressing wild-type Hog1, Rtg1 associated to chromatin upon stress. However, in cells expressing Hog1^{KNN}, the recruitment of Rtg1 to chromatin upon osmostress was clearly impaired (Figure 5A). Therefore, the catalytic activity of the Hog1 SAPK is required for recruitment of the Rtg1/3 complex at the promoters in response to stress. Correspondingly, cells carrying the Hog1^{KNN} mutant were not able to induce transcription of the RTG-dependent genes upon osmostress (Figure 5B). Taken together, our data indicated that, in response to osmostress, catalytic activity of Hog1 is required for chromatin association of the Rtg1/3 complex.

Rtg1 and Rtg3 are phosphorylated directly by the Hog1 SAPK

Then we performed *in vitro* and *in vivo* phosphorylation assays to assess whether Rtg1 and Rtg3 were direct substrates of Hog1. Initially, we tested whether Hog1 phosphorylates Rtg1 directly by using purified proteins in an *in vitro* kinase assay, in which Hog1 was activated in the presence of a constitutive MAPKK allele (Pbs2^{EE}) (Proft *et al.*, 2001). The *in vitro* kinase assay revealed that Rtg1 was directly phosphorylated by Hog1 (Figure 6A), indicating that Rtg1 can be directly phosphorylated by the Hog1 SAPK. Rtg1 contains a unique putative phosphorylation site for SAPKs (Ser-Pro or Thr-Pro), namely Thr60, that is located within the loop region that separates the two amphipathic helices in the bHLH/Zip region of the protein. Then, we created a point mutant version of Rtg1 which contained a Thr replacement with Ala (Rtg1^{T60A}). The mutant protein was purified and used in an *in vitro* kinase assay as before.

Notably, phosphorylation of Rtg1^{T60A} by Hog1 was completely abolished (Figure 6A), indicating that Hog1 phosphorylates *in vitro* Rtg1 specifically at the Thr60 residue.

We then tested whether Hog1 was able to phosphorylate Rtg3 directly by using purified proteins in an *in vitro* kinase assay as before. As shown in Figure 6B (lane 1), full length Rtg3 was phosphorylated by Hog1, indicating that both Rtg1 and Rtg3 can be *in vitro* phosphorylated by the SAPK. Rtg3 contains 11 putative phosphorylation sites for SAPKs. To map the phosphorylation sites for Hog1 in Rtg3, we created several truncated *RTG3* alleles and expressed them as GST-tagged proteins in *E. coli*. After purification, the same amounts of the pure proteins were subjected to in *vitro* phosphorylation. Whereas a truncated form of Rtg3 containing amino acids 1 to 210 (Rtg3-M1) was phosphorylated by Hog1, a region containing amino acids 1 to 183 (Rtg3-M2) was unable to be phosphorylated by the SAPK (Figure 6B, lanes 2 and 3). Rtg3M-1 contains a putative phosphorylation site (Thr197) for SAPKs that is not present in the Rtg3-M2 fragment, suggesting that this residue could be a Hog1 phosphorylation site in Rtg3. Moreover, as shown in Fig 6B, a C-terminus region of Rtg3 containing amino acids 211 to 486 (Rtg3-M3) was also phosphorylated by Hog1, although to a lesser extent than Rtg3-M1 (Figure 6B, lane 4). Four sequences corresponding to the consensus phosphorylation site for SAPKs are present in this region (Ser222, Ser227, Thr249 and Ser376). To further determine the phosphorylation sites in Rtg3, we created an Rtg3 allele with point mutations that replace Ser and Thr residues with Ala of the five candidate residues to be phosphorylated (Rtg3^{5M}) and we tested it for phosphorylation by Hog1. As shown in Figure 6C, Rtg3 phosphorylation was abolished in the Rtg3^{5M} mutant, indicating that Hog1 phosphorylates *in vitro* Rtg3 specifically at these five residues.

Rtg1 and Rtg3 in vivo phosphorylations upon osmostress

We next tested whether Hog1 phosphorylated Rtg1 at Thr60 in response to osmostress *in vivo*. Wild-type and *hog1*Δ strains were transformed with plasmids expressing Rtg1-HA and Rtg1^{T60A}-HA tagged proteins. Cells were subjected to a brief osmostic shock and proteins were probed using specific monoclonal antibodies against the HA epitope. The mobility pattern of

Rtg1 in SDS-PAGE was altered in wild-type cells subjected to an osmotic shock (Figure 7A). When extracts from osmotic-stressed cells were treated with alkaline phosphatase, the mobility pattern of Rtg1 could be reversed, confirming that the mobility change was induced by phosphorylation. Moreover, phosphorylation of Rtg1 in response to osmostress depended on Hog1, since Rtg1 did not undergo the mobility shift in *hog1*Δ cells (Figure 7A). Therefore, Rtg1 is rapidly phosphorylated upon hyperosmotic shock *in vivo* and this modification depends on the Hog1 SAPK. However, mutation of Thr60 did not abolish the mobility shift in response to osmostress (Figure 7B, left panel). These results indicate that additional residues to Thr60 in Rtg1 might be the responsible for the mobility shift. In fact, Rtg1 contains two alternative sites, Ser163 and Ser164, which match with the concensus sequence for PKA. Then, we assessed the ability of these residues to be phosphorylated in response to osmostress. *In vivo* phosphorylation assays were performed as before with plasmids expressing Rtg1, Rtg1$^{S163A/S164A}$ and the triple mutant HA- tagged proteins. While wild-type Rtg1 underwent a mobility shift due to phosphorylation upon osmostress, Rtg1$^{S163A/S164A}$ and Rtg1 triple mutant did not (Figure 7B, middle panel and right panel). Taken together, these results indicate that Hog1 directly phosphorylates Rtg1 at Thr60, and indirectly at Ser163 and Ser164 residues likely through an intermediate kinase, in response to stress.

Next, we attempted to determine whether Rtg3 was phosphorylated *in vivo* in response to omostress. Rtg3 appeared to be phoshorylated in the absence of osmotic treatment, based on its mobility after phosphatase treatment. Moreover, the protein appeared as multiple bands (Figure 7C, left panel). These observations suggested that, as described before, multiple hyperphosphorylated froms of the protein exist in basal conditions (Komeili *et al.*, 2000). However, whereas we could detect a change in mobility in response to rapamycin treatment, like it was previously described (Komeili *et al.*, 2000), in response to osmostress Rtg3 did not undergo a clear mobility shift (Figure 7C).

Phosphorylation at multiple sites of Rtg3 by Hog1 affects Rtg3 function

To assess the role of Rtg1 phosphorylation by Hog1, we studied the effect of the mutation of the phosphorylation sites in gene expression. A vector containing full-length *RTG1* or the *RTG*^{T60A}, *RTG*1^{S163AS164A} and *RTG*1^{3M} alleles were transformed in *rtg1*Δ cells and the effect on transcription was measured *in vivo*. Induction of *CIT2* and *DLD3* genes in response to osmostress was not affected in cells containing any of the mutant alleles compared to wild-type (Figure 8A). These results indicated that Rtg1 phosphorylation by Hog1 is not a key determinant for Rtg1/3-mediated gene expression in response to osmostress.

Next, we aimed to determine whether Rtg3 phosphorylation by Hog1 were important for transcriptional activation upon osmostress. Therefore, we assessed transcriptional activation in *rtg3*Δ cells carrying wild-type *RTG3* or a mutant version of *RTG3* replacing the five Ser and Thr phosphorylation sites for Hog1 (*RTG3*^{5M}). Cells expressing the non-phosphorylatable mutant Rtg3^{5M} showed a partial impairment of induction of transcription compared to wild-type, indicating that phosphorylation of Rtg3 is important for Rtg3 function (Figure 8B).

Because Hog1 catalytic activity is required for the recruitment of the Rtg1/3 transcription complex at the responsive promoters, we asked whether Rtg3 phosphorylation by Hog1 was also required for the association of Rtg1/3 to chromatin. Recruitment of the Rtg1/3 complex in cells expressing the non-phosphorylatable Rtg3 (Rtg3^{5M}) was almost identical compared to cells expressing wild-type Rtg3 (Figure S2). Overall, the results suggested that Rtg3 phosphorylation by Hog1 is important for regulation of the activity of the transcription factor.

DISCUSSION

Yeast cells respond to increases in the external osmolarity by activating the stress-activated Hog1 SAPK. A major outcome of the activation of Hog1 is the regulation of gene expression and several transcription factors have been proposed to be controlled by Hog1 (de Nadal and Posas, 2010;Martinez-Montanes *et al.*, 2010;Weake and Workman, 2010). However, data suggest that additional transcription factors may be required for the osmostress-induced gene expression controlled by the SAPK (Miller *et al.*, 2011). Here, we show that the Rtg1 and Rtg3 bHLH-Zip transcription factors are new targets for Hog1 and are essential for adaptation to osmostress. Deletion of *RTG1* and *RTG3* results in a deficient expression of key genes in the TCA cycle, such as *CIT2*, *DLD3* and *PYC1,* in response to osmostress. These genes are known to be activated upon respiratory dysfunction by the RTG pathway (Chelstowska *et al.*, 1999;Epstein *et al.*, 2001;Liao *et al.*, 1991). Our data indicate that the Rtg1/3 transcription factors are required for induction of a specific set of genes in osmostress.

Previous studies demonstrated that osmostress affects mitochondrial function by reducing the mitochondrial electron transport in plants (Hamilton and Heckathorn, 2001). Moreover, recently it was described that constitutive activation of Hog1 provokes a decrease in respiration, which leads to cell death caused by an increase on reactive oxygen species (ROS) (Vendrell *et al.*, 2011). We assessed whether respiration rate was altered upon osmostress and obtained that, consistent with previous results, oxygen consumption decreased dramatically when cells were exposed to osmostress (Figure S3). Thus, an osmotic shock leads to alterations in mitochondrial function and respiration capability. Moreover, *rtg1* and *rtg3* mutants displayed affected respiration before osmostress treatment and almost impaired respiration after a brief osmotic shock (Figure S3). It is known that mitochondrial biogenesis and function is needed for efficient adaptation to osmostress, since mutants with defects in many different mitochondrial components showed hypersensitivity to increased NaCl concentrations (Martínez-Pastor *et al.*, 2009). Taken together, the data suggest that cells exposed to osmostress conditions have compromised mitochondrial respiration and Rtg1 and Rtg3 transcription factors might be important determinants for maintaining mitochondrial function.

In vivo gene expression analysis showed that, specifically upon osmostress, Hog1 is required for proper induction of the Rtg1/3-target genes. Furthermore, there are several lines of evidence suggesting that the Rtg1/3 complex is directly regulated by the Hog1 SAPK upon osmostress. Hog1 controls the Rtg1/3 heterodimeric transcription complex by a triple mechanism: 1) regulating the nuclear translocation of the heterodimeric complex, 2) stimulating the association of the complex to the responsive promoters and 3) controlling its transcriptional activity.

Hog1 interacts *in vivo* with the Rtg1/3 complex and is required for the nuclear translocation of the complex in response to osmostress conditions. Notably, localization of Rtg1-GFP and Rtg3-GFP, in cells expressing Hog1 mutants with altered catalytic activity and subcellular localization, suggested that this level of control by Hog1 is likely dependent on the interaction of the SAPK with the complex while not on its catalytic activity. Under basal conditions Hog1 appears to be distributed throughout the cytoplasm and the nucleus, whereas Hog1 activation by osmostress causes a rapid and marked concentration of the SAPK in the nucleus (Ferrigno *et al.*, 1998;Reiser *et al.*, 1999). Thus, upon Hog1 phosphorylation and nuclear accumulation, the Rtg1/3 complex is targeted to the nucleus, most likely thanks to its interaction with the SAPK.

In addition to the role of Hog1 in the regulation of the Rtg1/3 subcellular localization, Hog1 further required for Rtg1/3 chromatin binding and transcriptional activity. It is known that, in response to osmostress, Hog1 becomes intimately linked with stress-responsive promoters, and this binding is dependent on the presence of stress-mediating transcriptional activators (Alepuz *et al.*, 2003;Alepuz *et al.*, 2001;Pascual-Ahuir *et al.*, 2006;Pokholok *et al.*, 2006;Proft *et al.*, 2006). For instance, Hog1 is recruited to *STL1*, and this recruitment is dependent on the presence of the Hot1 transcription factor (Alepuz *et al.*, 2001). Similarly, Hog1 was recruited to the RTG-dependent promoters in response to osmostress dependently on Rtg1. Because Rtg1 is found at promoters as a heterodimer with Rtg3, we can assume that binding of Hog1 at the RTG-dependent promoters in response to osmostress is dependent on the presence of the Rtg1/3 complex. Thus, the Rtg1/3 transcription complex targets the SAPK to the

specific promoters. On the other hand, our results also showed that recruitment of Rtg1/3 complex to RTG-dependent promoters during osmostress was strongly reduced in a *hog1*Δ strain. These results are in agreement with the subcellular localization experiments indicating that Hog1 regulates the localization of the complex upon stress. Remarkably, not only the presence of Hog1 but also its catalytic activity was essential for recruitment of the transcription complex to chromatin. Overall, the interdependence of binding of Hog1 and Rtg1 to promoters of target genes reveals, as in the case of other stress-mediating transcription factors, a functional connection between the two factors for making a stable complex at stress-responsive genes.

Moreover, Hog1 directly phosphorylates Rtg1 and Rtg3, and Rtg3 phosphorylation is important for proper gene activation. Hog1 directly phosphorylates Rtg1 at Thr60 residue, and indirectly at Ser163 and Ser164 residues likely through an unknown intermediate kinase. In any case, the Rtg1 non-phosphorylatable mutant is able to induce transcription to the same extent as the wild-type, indicating that the Rtg1 phosphorylations carried directly or indirectly by the SAPK are not essential for gene expression, although might be relevant for some specific aspects of transcription in osmostress. On the other hand, Hog1 directly phosphorylates Rtg3 at five specific residues. Interestingly, gene induction is clearly affected in the Rtg3 non-phosphorylatable mutant, whereas Rtg1/3 recruitment is not altered. Thus, phosphorylation of Rtg3 by Hog1 is important for induction of transcription.

Taken together, our results show that the Rtg1/3 transcription complex is directly regulated by the Hog1 SAPK in osmostress and different levels of regulation control this transcription complex, and links transcriptional regulation of mitochondrial genes to Hog1-mediated adaptive responses.

MATERIALS AND METHODS

Yeast strains

S. cerevisiae strain K699 (MATa leu2-3,112 ura3-1 his3-11 trp1-1 can1-100) and its derivatives YNN15 (*RTG1*::kanMX4), YNN20 (*RTG3*::kanMX4), YNN41 (*RTG2*::kanMX4) and YNN17 (*HOG1*::kanMX4) were used in this work. Genomic disruptions were made by long flanking homology PCR-based gene disruption. C-terminus genomic tagging of Rtg1 in wild-type and *hog1*Δ strains yielded YNN21 (*RTG1-6HA::HIS3*) and YNN23 (*HOG1*::kanMX4 *RTG1-6HA::HIS3*). C-terminus genomic tagging of Rtg3 yielded YNN44 (*RTG3-6HA::HIS3*). For Hog1 ChIP assays, YCR109 (*HOG1-6HA::HIS3*) and YCR110 (*RTG1*::kanMX4 *HOG1-6HA::HIS3*) were used. Tagging of genomic ORFs with HA epitopes was done with a PCR-based strategy.

Plasmids

GFP-fused constructs were generated by amplifying *RTG1* and *RTG3* coding regions plus promoter regions (~-800) from genomic DNA and cloned into the XhoI site of the pRS416-GFP construct (Raitt et al., 2000). To analyse the role of Hog1 catallytic activity, YCpLac111-Hog1-3HA and YCpLac111-Hog1KNN-3HA vectors were used (Wurgler-Murphy *et al.*, 1997). pGBT9 (Gal4DBD-*HOG1*) and pRS426TEG2-*HOG1*TAYA were also used in this study (Ferrigno *et al.*, 1998;Silver *et al.*, 1984). Full length *RTG1* and *RTG3* coding regions were PCR amplified from genomic DNA and cloned into the BamHI site of the bacterial expression vector pGEX-4T (Pharmacia), which allows the expression of GST-tagged proteins in *E. coli*. pGEX-4T plasmids containing mutated alleles described in Results section were generated by PCR amplification with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) from the pGEX-4T plasmids containing the wild-type *RTG1* and *RTG3* ORFs. Each mutation was verified by DNA sequencing. pGEX4T-Hog1 and pGEX4T-Pbs2^{EE} (*PBS2* with Ser514-Glu and Thr518-Glu mutations) are described in (Bilsland-Marchesan *et al.*, 2000). To obtain

hemaglutinin (HA)-fusion proteins, the promoter (~800) and coding regions of wild-type *RTG1* and *RTG3* were PCR amplified and cloned into BamHI site of the pRS415 vector. Mutated alleles described in Results section were generated and verified as before. 6xHA tags were inserted before the stop codon by recombination using a specific cassette containing the HA epitopes. These cassettes were obtained by PCR amplification using the GA2256 plasmid (from Dr. Gustav Ammerer Lab) as template.

Buffers and media

Buffer A consists of 50 mM Tris-HCl (pH 7.5), 15 mM EDTA, 15 mM EGTA, 2 mM dithiothreitol (DTT), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 5 μg of pepstatine per ml, and 5 μg of leupeptin per ml. Alkaline phosphatase (AP) buffer consists of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM MgCl2. STET buffer consists of 10 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1mM EDTA, 5% Triton X-100. Kinase buffer consists of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, and 2 mM DTT. Phosphatase inhibitor cocktail contains 10 mM NaF, 1 mM sodium pyrophosphate, and 10 mM β-glycerophosphate. For all experiments, cell cultures were grown in minimal dextrose (MD) media which contained 0.7% yeast nitrogen base and 2% dextrose, pH 5.5. Glutamine was added to MD media to a final concentration of 0.2%. To supplement the auxotrophic requirements of strains, required amino acids were added (0.006% histidine, 0.008% leucine, 0.006% adenine, 0.005% tryptophan, 20 μg/ml uracile and 20 μg/ml methionine), lacking for the specific ones to select for plasmid maintenance. Cells were grown in YPD (1% yeast extract, 2% peptone and 2% dextrose) when indicated in the text.

Cell viability assays

Yeast cultures were grown to early log phase (0.5-0.8 OD_{660}) and diluted to a 0.05 OD_{660}. Serial dilutions of cultures were spotted directly onto YPD plates containing glutamine and the indicated concentrations of salt and sorbitol, and incubated at 30ºC. Growth was scored after 2-5 days.

Northern blot analysis

Yeast cultures were grown to early log phase (0.5-0.8 OD_{660}) and either subjected to stress (0.4 M NaCl, indicated times in the figure legends) or untreated. Total RNA was extracted from 15 ml of culture by acid phenol treatment as described previously (Lyer and Sthul 1996). 20μg of total RNA per sample were run in 1% agarose gels by electrophoresis. RNAs were transferred to a nylon membrane (Roche) by a Vacuum blotter model 785 (Bio-Rad, Hercules, CA). Total RNA and expression of specific genes was probed by using radiolabeled PCR fragments containing the ORF region of *CIT2*, *DLD3*, *PYC1*, *CTT1*, *STL1*, *GRE2* or the noncoding exon of *RDN18* as a loading control. Signals were quantified by using a storage phosphor screen, a Typhoon 8600 phosphorimager.

In vivo coprecipitation assays

In vivo interaction of Rtg1-HA and Rtg3-HA fusion proteins with GST-Hog1 was determinated by GST pull-down experiments. Exponential growing cells (0.5-0.8 OD_{660}) were subjected to a brief osmotic shock (0.4M NaCl, 10 min). Two milligrams of yeast extract in a mixture of buffer A plus 150 mM NaCl and phosphatase inhibitors were prepared and incubated with glutathione-Sepharose beads overnight at 4ºC. Beads were washed extensively with buffer A, resuspended in loading buffer, and separated on SDS-15% polyacrylamide gel electrophoresis (Rtg1-HA) or SDS-10% polyacrylamide gel electrophoresis (Rtg3-HA). Immunoblotting was done by using anti-HA monoclonal antibody 12CA5 (Roche) and anti-GST monoclonal antibody (Pharmacia) together with ECL reagment (GE Healhtcare).

Chromatin immunoprecipitation

ChIP experiments were performed as described before (Zapater *et al.*, 2007). Yeast cultures were grown to early log phase (0.6–0.8 OD_{660}) before samples of the culture were exposed to osmotic stress (0.4 M NaCl, indicated times in the figure legends). For cross-linking, yeast cells were treated with 1% formaldehyde for 20 min at room temperature. Antibodies used were

mouse polyclonal anti-Rpb1 (8WG16; Covance), and monoclonal anti-HA 12CA5. For quantitative PCR analysis of osmoresponsive genes and constitutively expressed genes, the following primers, with locations indicated by the distance from the respect ATG initiation codon, were used: *CIT2,* –460/–141; *DLD3*, –253/+46; and *TEL2* (telomeric region on the right arm of chromosome VI).

GFP florescence microscopy

Exponential growing cells (0.6–0.8 OD_{660}) were observed without fixation using a Nikon Eclipse 901 microscope with an ORCA II CCD camera (Hamamatsu). Images were taken at 100X magnification and converted to Photoshop 7.0.1 (Adobe Systems).

Purification of GST proteins in E. coli and in vitro kinase assays

GST fusion proteins encoding $Pbs2^{EE}$, Hog1, and wild-type or mutant Rtg1 and Rgt3 were constructed by using pGEX-4T (Pharmacia), expressed in *E. coli* and purified by using glutathione-Sepharose beads (Pharmacia) in STET buffer as descibed previosly (Posas *et al.,* 1996). Phosphorylations of Rtg1, Rtg3 and mutant versions by Hog1 were monitored as follows. One microgram of recombinant GST-Hog1 was activated by phosphorylation by using 0.5 μg of GST-$PBS2^{EE}$ in the presence of kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl2 and 2 mM DTT) and 50 μM ATP. After 15 min at 30°C, 5 μg of GST-Rtg1, GST-Rtg2 or GST-Rtg3 was added to the previous mixture together with [γ-32P]-ATP (0.2 μCi/μl). The mixture was then incubated for 10 min at 30°C, and the reactions were stopped by the addition of 2X SDS loading buffer. The labelled proteins were resolved by SDS-PAGE, stained with Comassie blue and detected by autoradiography.

In vivo phosphorylation assays

Cells containing the *RTG1* and *RTG3* ORF fused to haemagglutinin (6xHA) epitope were grown to mid-log phase (0.6-0.8 OD_{660}), subjected to osmotic stress treatment (0.4 M NaCl, 5 min) and harvested by centrifugation. Yeast extracts were obtained in a mixture of buffer A plus 150 mM

NaCl and phosphatase inhibitors. Protein concentration was determined by Bradford analysis (Bio-Rad Protein Assay). When necessary, 1mg of total yeast extracts was treated for 2h at 37ºC with 1 µl phosphatase alkaline (20U/µl; Roche). In experiments that did not require the use of alkaline phosphatase, protein extraction was performed using the trichloroacetic acid (TCA) protocol, described (Macia *et al.*, 2009). Total crude extracts were loaded onto SDS-15% polyacrylamide gel, and the Rtg1-HA and Rtg3-HA fusion proteins were detected by immunoblotting using an anti-HA monoclonal antibody 12CA5 (Roche).

Oxygen consumption measures

Oxygen consumption was measured using a HI 9146 Oxygen measurer from HANNA Instruments (East Drive Woonsocket, RI) and readings were recorded every 15 seconds for 10 minutes. Results are reported as per cent O_2 consumed per minute per 1 X 10^8 cells. Data represent the mean and standard deviation of three independent experiments.

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FIGURE LEGENDS

Figure 1. The Rtg1 and Rtg3 bHLH/Zip transcription factors are essential for adaptation to osmostress. **(A)** Mutations in *RTG1* and *RTG3* genes render cells osmosensitive. Wild-type (WT) and the indicated mutant strains were grown to logarithmic phase and diluted to 0.05 OD_{660}. 10-fold serial dilution series were platted on MD-Gln plates without or with NaCl or sorbitol at the indicated concentrations. Growth was scored after 2-5 days. **(B)** Rtg1, Rtg3 and the Hog1 SAPK are required for the induction of *CIT2, PYC1* and *DLD3* genes in response to osmostress. Indicated yeast strains were grown on MD-Gln to 0.5-0.8 OD_{660} and treated with 0.4M NaCl at the indicated times. Total RNA was analyzed by northern blot analysis for transcript levels of *CIT2, PYC1*, *DLD3* and *RDN18* as a loading control.

Figure 2. *In vivo* binding of Hog1 to Rtg1 and Rtg3. Strains expressing Rtg1-HA or Rtg3-HA from the wild type locus were transformed with a plasmid expressing GST or GST-Hog1 under the P_{TEF1} promoter. Cells were grown in MD-Gln to logarithmic phase and samples were taken 10 minutes after 0.4M NaCl treatment. GST- Hog1 was pulled-down by glutathione-Sepharose 4B and the presence of Rtg1-HA and Rtg3-HA was probed by immunobloting using anti-HA specific monoclonal antibody (upper panel). Total extracts are presented in the middle panel. The amount of precipitated GST proteins was detected using anti-GST specific monoclonal antibody (lower panel).

Figure 3. Occupancy of Rtg1, Hog1 and RNA Pol II at RTG-dependent promoters. **(A)** Association of Rtg1/3 complex to *CIT2* and *DLD3* promoters in response to osmostress is dependent on Hog1. Wild-type (WT) and *hog1*Δ strains carrying HA-tagged Rtg1 were grown to mid-log phase and subjected to osmostress (0.4 M NaCl) for the indicated times. Proteins were immunoprecipitated with anti-HA monoclonal antibodies and binding to the promoter regions of *CIT2* and *DLD3* loci was analysed by PCR. Results are shown as the fold induction of treated relative to non-treated (time zero) samples normalized to *TEL2*. Data are the mean

and standard deviation of three independent experiments. **(B)** Recruitment of the Hog1 SAPK to *CIT2* and *DLD3* promoters in response to osmostress is dependent on Rtg1. Wild-type (WT) and *rtg1*Δ strains were analysed by ChIP as described for (A) **(C)** Rtg1 is required for recruitment of RNA Pol II at *CIT2* and *DLD3* promoters in response to osmostress. Wild-type (WT) and *rtg1*Δ strains were analysed by ChIP as described for (A) using anti-Rpb1 antibody (8WG16, Covance).

Figure 4. Role of Hog1 in regulating the subcellular localization of Rtg1/3 complex upon osmostress. **(A)** Rtg1/3 complex translocates into the nucleus upon osmostress in a Hog1-dependent manner. Wild-type (WT) and *hog1*Δ strains were transformed with plasmids carrying GFP-tagged Rtg1 or Rtg3 expressed under their own promoters. Cells were grown in MD-Gn to 0.5-0.7 OD_{660} and not stressed or stressed with 0.4M NaCl for 5 min before used for fluorescence microscopy analysis. Cells expressing Rtg1-GFP were treated with 1μg/ml of rapamycin (Rap) for 15 minutes. The nuclear disposition the GFP-based fluorescence was confirmed by its localization with DAPI stained nuclear DNA (data not shown). **(B)** Rtg1/3 nuclear translocation is not dependent on Hog1 catalytic activity. *hog1*Δ strains expressing Rtg1-GFP and Rtg3-GFP were transformed with an empty monocopy vector YCpLac111 or a YCpLac111 vector containing full length *HOG1* or a *HOG1* mutant allele in ATP binding site (*HOG1*^{KNN}), which is unable to phosphorylate its substrates. Cells were treated as in (A) **(C)** Subcellular localization of the Rtg1/3 complex depends on the subcellular localization of Hog1. *hog1*Δ strains expressing Rtg1-GFP and Rtg3-GFP were transformed with a monocopy vector carrying wild-type *HOG1*, *HOG1*^{TAYA}, which codifies for an enzyme unable to translocate to the nucleus, or a vector carrying NLS-containing *HOG1* (*HOG1*^{NLS}). Cells were treated as in (A).

Figure 5. Hog1 catalytic activity is required for Rtg1/3 chromatin binding and RTG-dependent gene expression. **(A)** Association of Rtg1/3 complex to *CIT2* and *DLD3* promoters in response to osmostress is dependent on Hog1 catalytic activity. *hog1*Δ cells containing genomically

tagged Rtg1 were transformed with the empty vector YCpLac111 or an YCpLac111 vector containing full length *HOG1* or *HOG1*^{KNN} expressed under their own promoters. Strains were grown on MD-Gln to 0.5-0.8 OD_{660} and treated with 0.4M NaCl at the indicated times. Proteins were immunoprecipitated with anti-HA monoclonal antibodies and binding to the promoter regions of *CIT2* and *DLD3* loci was analysed by PCR. Results are shown as the fold induction of treated relative to non-treated (time zero) samples normalized to *TEL2*. Data are the mean and standard deviation of three independent experiments. **(B)** Hog1 SAPK activity is required for the induction of *CIT2* and *DLD3* genes in response to osmostress. The same strains and growth conditions as in (A) were used. Total RNA was analyzed by northern blot analysis for transcript levels of *CIT2*, *DLD3* and *RDN18* as a loading control.

Figure 6. Hog1 directly phosphorylates Rtg1 and Rtg3 *in vitro.* **(A)** Rtg1 is phosphorylated at Thr60 by Hog1 *in vitro*. Full length Rtg1 and Rtg1^{T60A} were tested for their ability to be phosphorylated by an *in vitro*-activated Hog1. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography (upper panel). GST-tagged Rtg1 proteins were detected by comassie staining (lower panel). **(B)** Hog1 directly phosphorylates Rtg3 *in vitro.* Full length Rtg3 and Rtg3 fragments Rtg3-M1 (amino acids 1 to 210), Rtg3-M2 (1 to 183) and Rtg3-M3 (211 to 486) were tested for their ability to be phosphorylated by an *in vitro*-activated Hog1. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography (left panel). GST-tagged Rtg3 proteins were detected by comassie staining (right panel). Arrows indicate the positions of Rtg3 fragments. **(C)** Mutations of Rtg3 Thr197, Ser222, Ser227, Thr249 and Ser376 to Ala (Rtg3^{5M}) abolish phosphorylation by Hog1. Recombinant tagged proteins were purified from *E. coli* and subjected to phosphorylation by activated Hog1 as before. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography (upper panel). The GST-tagged Rtg3 and Rtg3^{5M} protein were detected by comassie staining (lower panel).

Figure 7. Hog1 phosphorylates Rtg1 *in vivo.* **(A)** Rtg1 is phosphorylated upon osmostress in a Hog1-dependent manner. Wild-type (WT) and *hog1*Δ cells expressing Rtg1-HA were grown to 0.6-1OD_{660}. Samples were taken before (-) or 5 min after (+) the addition of NaCl to a final concentration of 0.4M, and the extracts were treated (+) or not (-) with 10U of alkaline phosphatase (AP). Rtg1-HA was detected by immunoblotting using anti-HA monoclonal antibody. **(B)** Rtg1 is phosphorylated at Ser163/Ser164 by Hog1 *in vivo*. *rtg1*Δ and *hog1*Δ strains were transformed with HA-tagged Rtg1 and the indicated mutants. Cells were grown and samples were taken before (-) or 5 min after (+) the addition of 0.4M NaCl. Rtg1-HA mobility was assessed as in (A). (C) Rtg3 phosphorylation by Hog1upon osmostress is not detected *in vivo*. Wild-type cells expressing Rtg3-HA were grown to 0.6-1 OD_{660}. Samples were taken before or after a brief osmotic shock (0.4M NaCl for 5 minutes), and the extracts were treated or not with 10U of alkaline hosphatase (AP). For rapamycin control, 1μg/ml of rapamycin was added to cell cultures for 15 minutes. Rtg3-HA was detected by immunoblotting using anti-HA monoclonal antibody.

Figure 8. Effect of Rtg1 and Rtg3 phosphorylations by Hog1 in gene expression**. (A)** Rtg1 phosphorylation by Hog1 is not essential for activation of gene expression upon osmostress. The *rtg1*Δ yeast strain was transformed with plasmids expressing wild-type Rtg1 or the indicated mutants, grown to mid-log phase and subjected to osmotic stress (0.4 M NaCl) for the indicated times. Total RNA was assayed by northern blot for transcript levels of *CIT2*, *DLD3* and *RDN18* (as a loading control). **(B)** Hog1 phosphorylation sites in Rtg3 are required for transcriptional activity. *rtg3*Δ yeast strain was transformed with plasmids expressing wild-type (WT) Rtg3 or the Rtg3 non-phosphorylatable mutant ($Rtg3^{5M}$), grown to mid-log phase and subjected to osmotic stress (0.4 M NaCl) for the indicated length of time. Total RNA was assayed by northern blot for transcript levels of *CIT2*, *DLD3* and *RDN18* (as a loading control).

SUPPLEMENTAL MATERIAL

Figure S1. RTG-dependent gene expression in osmostress is controlled by Hog1. **(A)** The integrity of the RTG pathway is required for gene expression upon osmostress. Wild-type cells and cells deleted for *RTG2* were grown in MD-Gln to logarithmic phase and subjected to osmotic shock (0,4M NaCl) for the indicated times. Total RNA was assayed by northern blot analysis for *CIT2* and *RDN18* as a loading control. **(B)** Hog1 is not required for induction of RTG-dependent genes under rapamycin treatment. The indicated strains were grown in MD-Gln to logarithmic phase and not treated or treated with 1μg/ml of rapamycin (Rap) for 15 minutes**.** Total RNA was assayed by northern blot analysis for the indicated transcripts and *RDN18* as a loading control. **(C)** Rtg1 transcription factor is required specifically for induction of RTG-dependent genes in osmostress. The indicated strains were grown and treated as in (A). Total RNA was assayed as for (A).

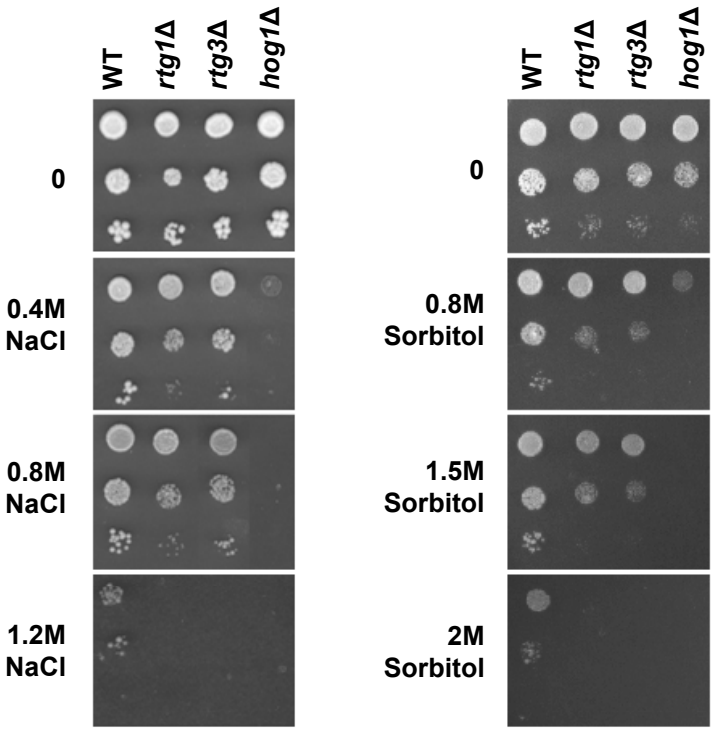
Figure S2. Hog1 phosphorylation sites in Rtg3 are not essential for Rtg1/3 chromatin binding. *rtg3*Δ yeast strain with genomically tagged Rtg1 was transformed with empty plasmid or plasmids expressing wild-type Rtg3 or the Rtg3 non-phosphorylatable mutant ($Rtg3^{5M}$), grown to mid-log phase and subjected to osmotic stress (0.4 M NaCl) for the indicated length of time. Tagged Rtg1was immunoprecipitated with anti-HA monoclonal antibodies and binding to the promoter regions of *CIT2* and *DLD3* loci was analysed by PCR. Results are shown as the fold induction of treated relative to non-treated (time zero) samples normalized to *TEL2*. Data are the mean and standard deviation of three independent experiments.

Figure S3. Cells suffer a reduction in oxygen consumption when exposed to osmostress conditions. Strains were grown in YPD media to mid-log phase and subjected or not to an osmotic shock of 10 min 0.4M NaCl. Then, the % of soluble oxygen in YPD was measured for a length of 5 minutes (oxygen consumption). Actynomicin A, which functions as an inhibitor of

mitochondrial electron transport by blocking the roxidation of reduced cytochrome b, was added to wild-type cells as a control of respiration inhibition. Data represent the mean and standard deviation of three independent experiments.

Figure 1

A


WT
rtg1Δ
rtg3Δ
hog1Δ
0
0.4M NaCl
0.8M NaCl
1.2M NaCl
0.8M Sorbitol
1.5M Sorbitol
2M Sorbitol

B

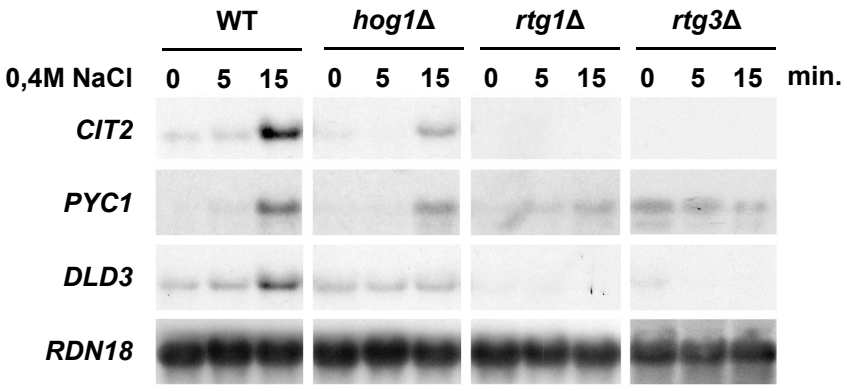

WT
hog1Δ
rtg1Δ
rtg3Δ
0,4M NaCl
0 5 15 0 5 15 0 5 15 0 5 15 min.
CIT2
PYC1
DLD3
RDN18

Figure 2

A

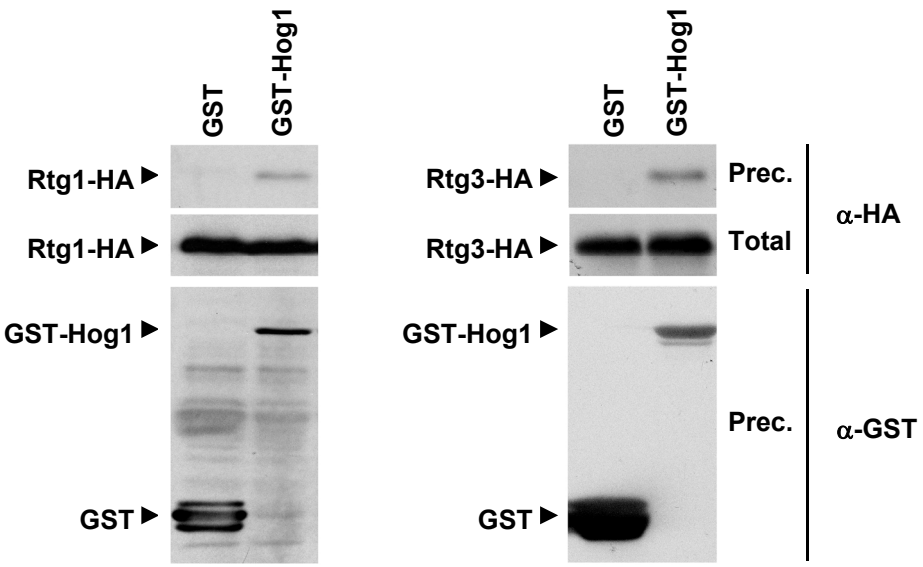

GST
GST-Hog1
Rtg1-HA
Rtg1-HA
GST-Hog1
GST
GST
GST-Hog1
Rtg3-HA
Rtg3-HA
GST-Hog1
GST
Prec.
Total
Prec.
α-HA
α-GST

Figure 3

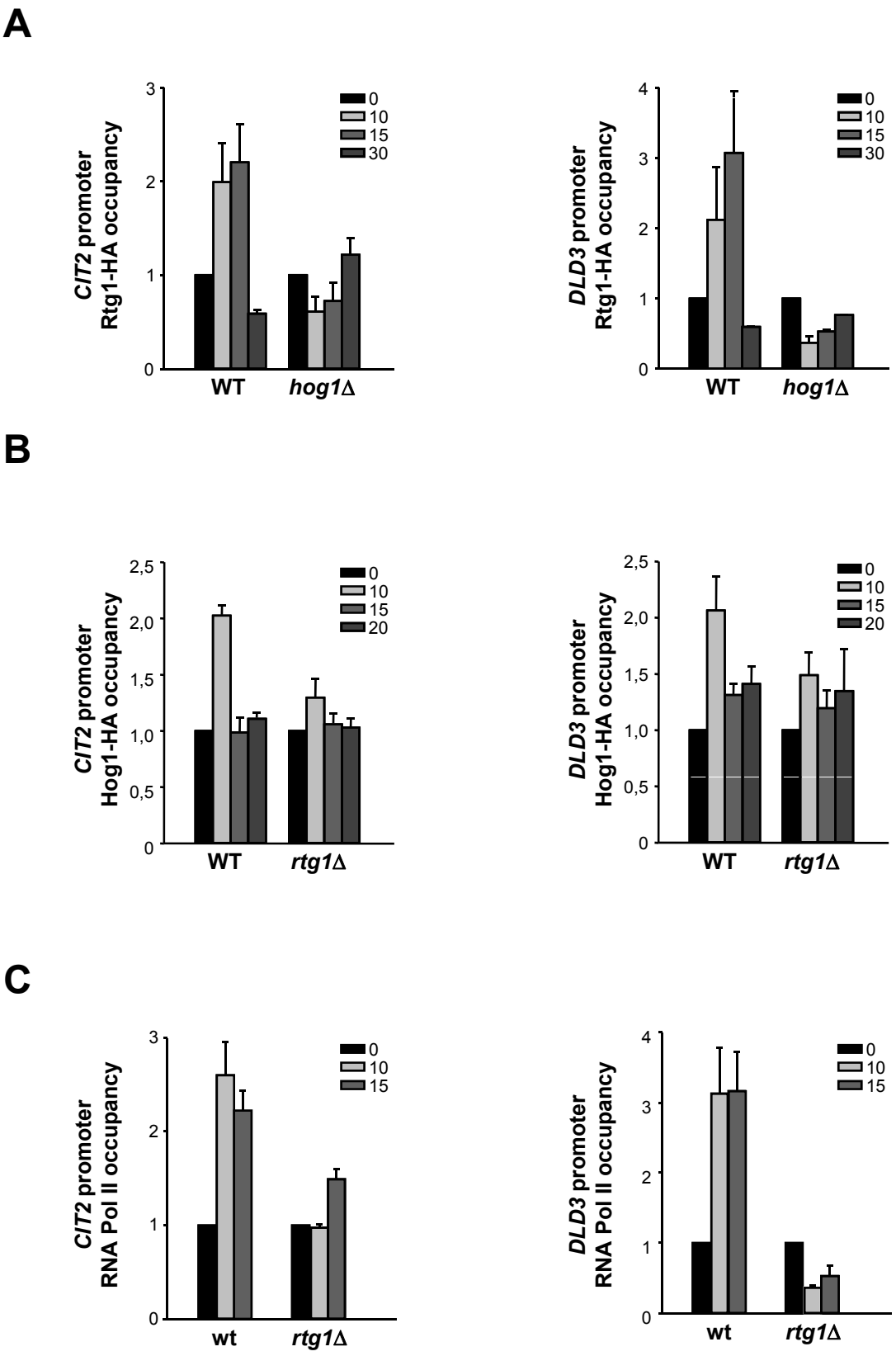

A
CIT2 promoter
Rtg1-HA occupancy
0
10
15
30
WT
hog1Δ
DLD3 promoter
Rtg1-HA occupancy
0
10
15
30
WT
hog1Δ
B
CIT2 promoter
Hog1-HA occupancy
0
10
15
20
WT
rtg1Δ
DLD3 promoter
Hog1-HA occupancy
0
10
15
20
WT
rtg1Δ
C
CIT2 promoter
RNA Pol II occupancy
0
10
15
wt
rtg1Δ
DLD3 promoter
RNA Pol II occupancy
0
10
15
wt
rtg1Δ

Figure 4

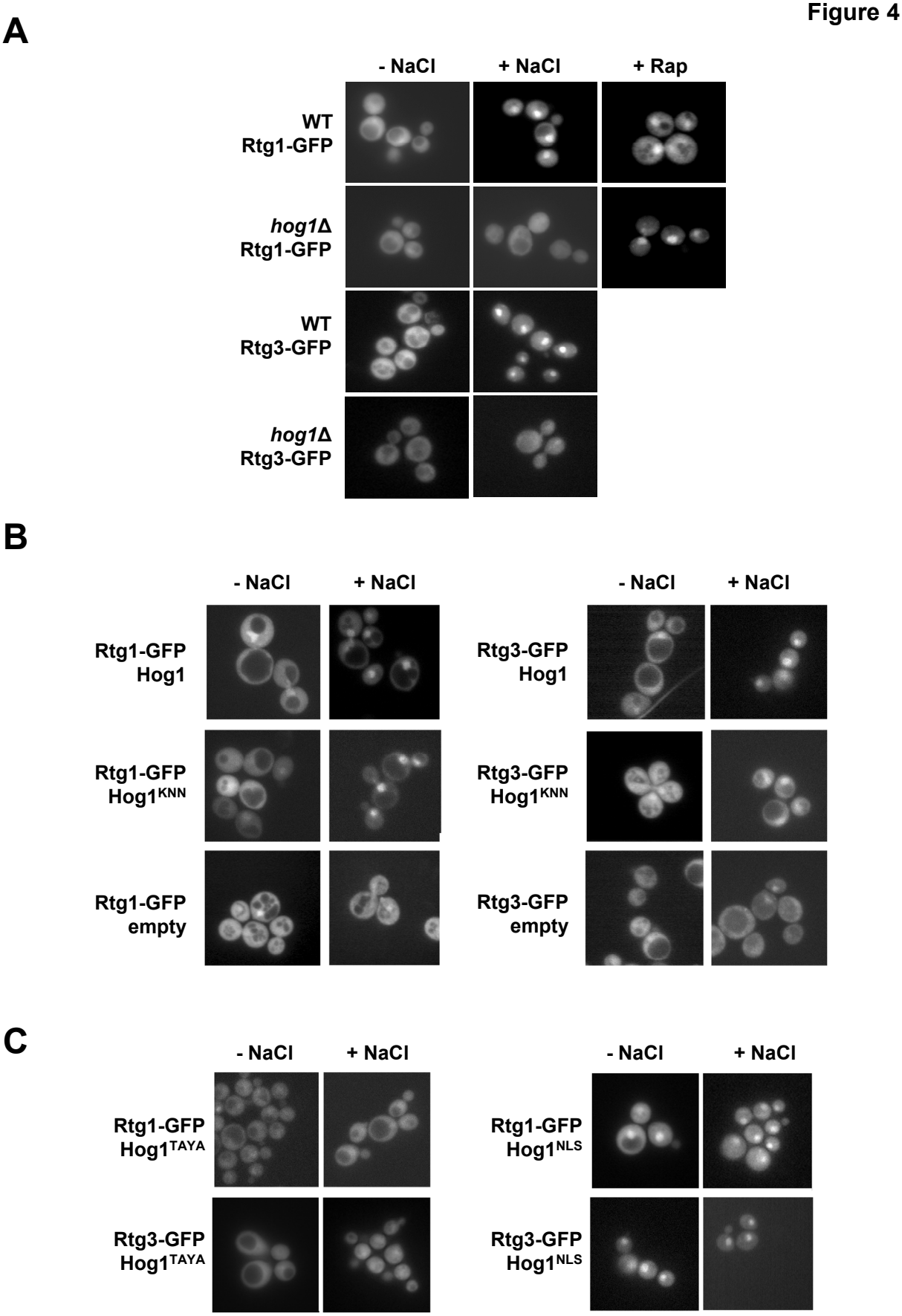
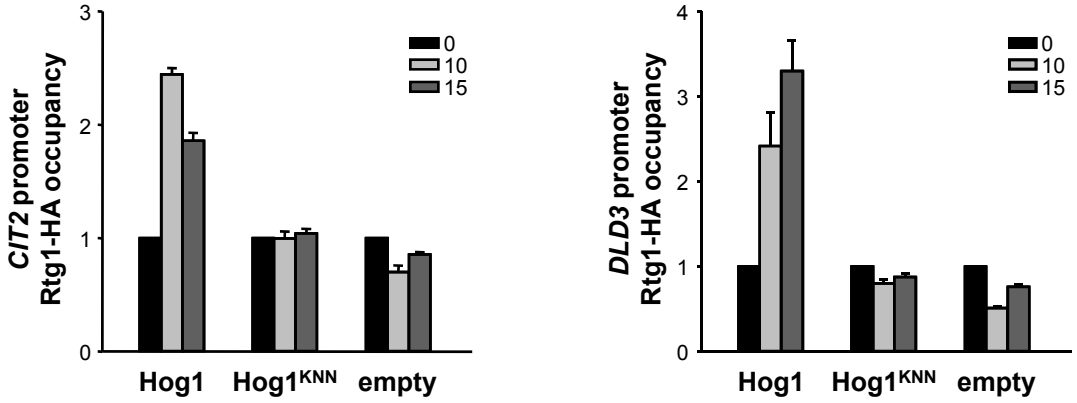

A
- NaCl
+ NaCl
+ Rap
WT Rtg1-GFP
hog1Δ Rtg1-GFP
WT Rtg3-GFP
hog1Δ Rtg3-GFP
B
- NaCl
+ NaCl
Rtg1-GFP Hog1
Rtg1-GFP Hog1KNN
Rtg1-GFP empty
Rtg3-GFP Hog1
Rtg3-GFP Hog1KNN
Rtg3-GFP empty
C
Rtg1-GFP Hog1TAYA
Rtg3-GFP Hog1TAYA
Rtg1-GFP Hog1NLS
Rtg3-GFP Hog1NLS

Figure 5

A


CIT2 promoter
Rtg1-HA occupancy
0
10
15
Hog1
Hog1KNN
empty
DLD3 promoter
Rtg1-HA occupancy
0
10
15
Hog1
Hog1KNN
empty

B

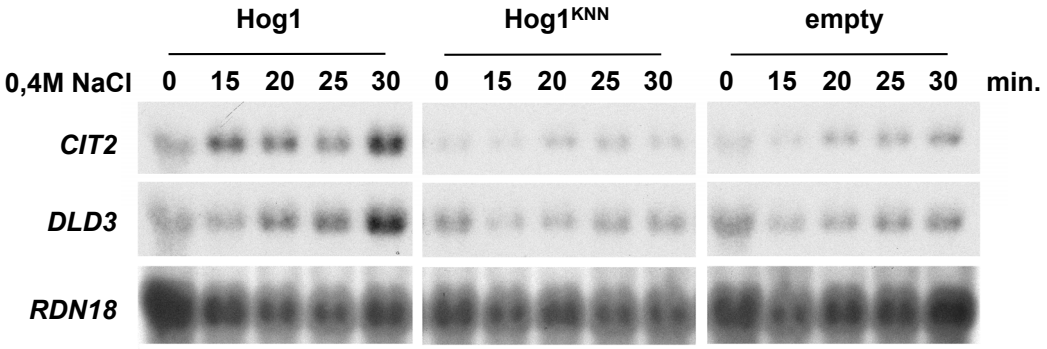
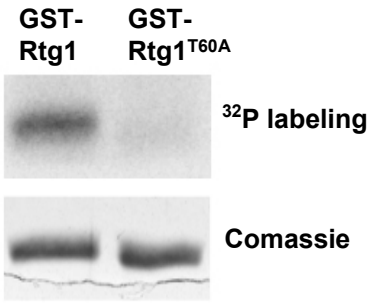
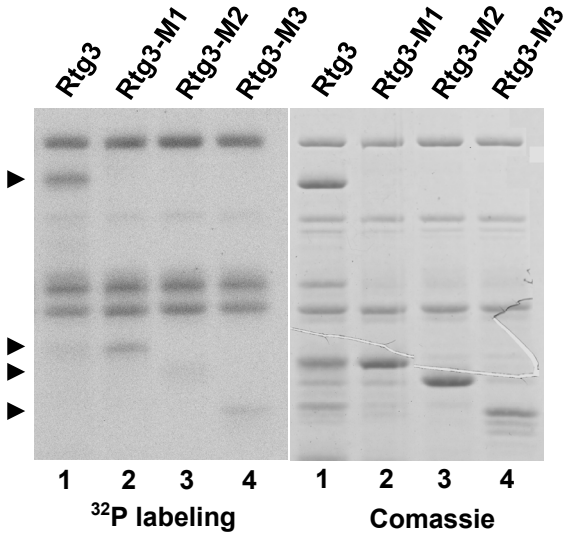

Hog1
Hog1KNN
empty
0,4M NaCl
0 15 20 25 30
0 15 20 25 30
0 15 20 25 30
min.
CIT2
DLD3
RDN18

Figure 6

A


GST-Rtg1
GST-Rtg1T60A
32P labeling
Comassie

B


Rtg3
Rtg3-M1
Rtg3-M2
Rtg3-M3
Rtg3
Rtg3-M1
Rtg3-M2
Rtg3-M3
1 2 3 4 1 2 3 4
32P labeling
Comassie

C

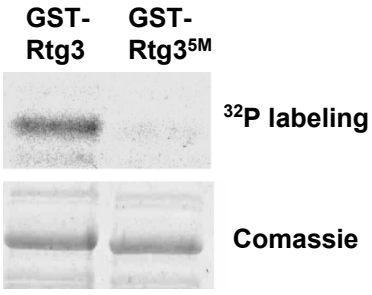

GST-Rtg3
GST-Rtg35M
32P labeling
Comassie

Figure 7

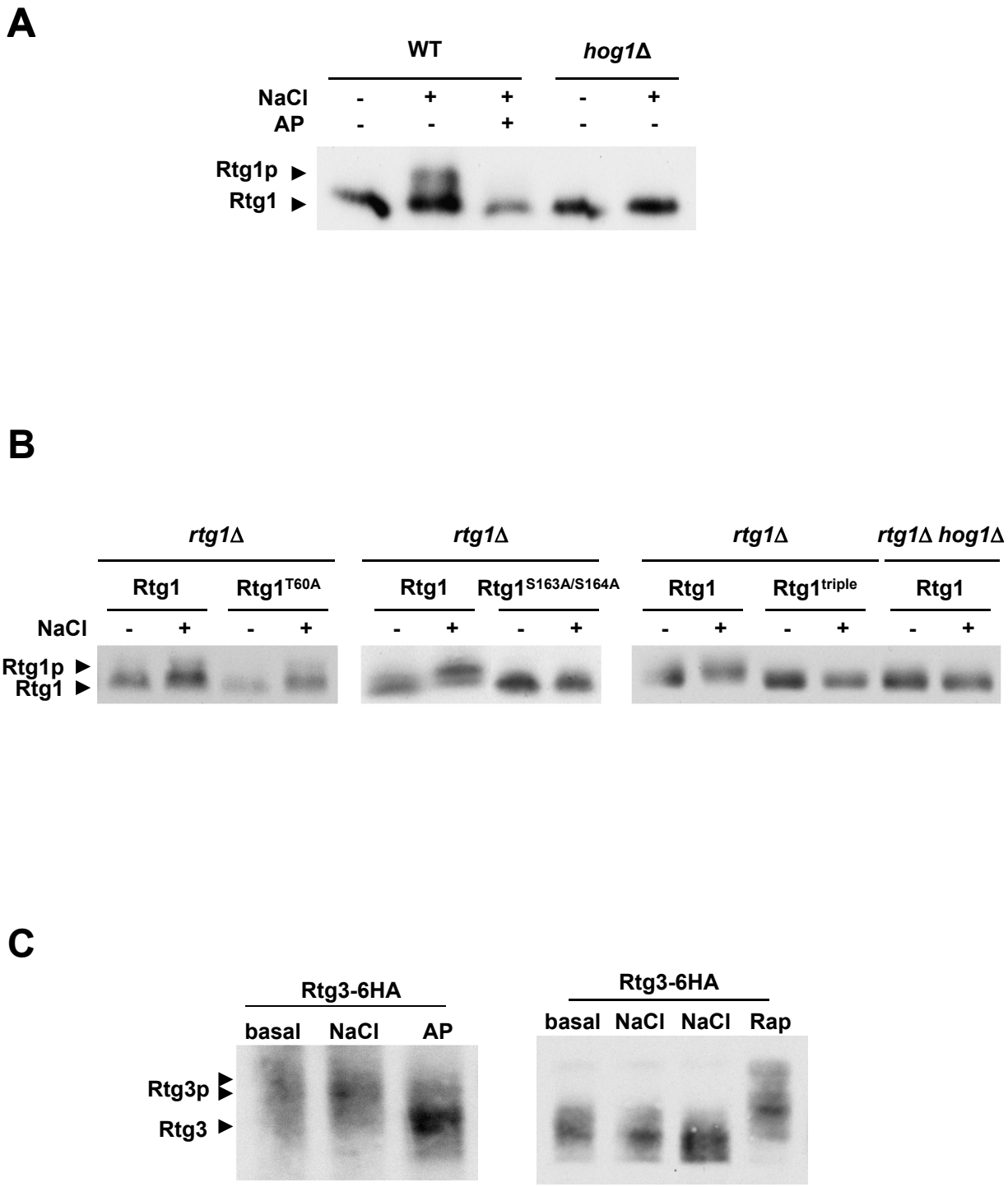

A
WT
hog1Δ
NaCl - + + - +
AP - - + - -
Rtg1p
Rtg1
B
rtg1Δ
rtg1Δ
rtg1Δ
rtg1Δ hog1Δ
Rtg1
Rtg1T60A
Rtg1
Rtg1S163A/S164A
Rtg1
Rtg1triple
Rtg1
NaCl - + - + - + - + - + - + - + - +
Rtg1p
Rtg1
C
Rtg3-6HA
basal NaCl AP
Rtg3p
Rtg3
Rtg3-6HA
basal NaCl NaCl Rap

Figure 8

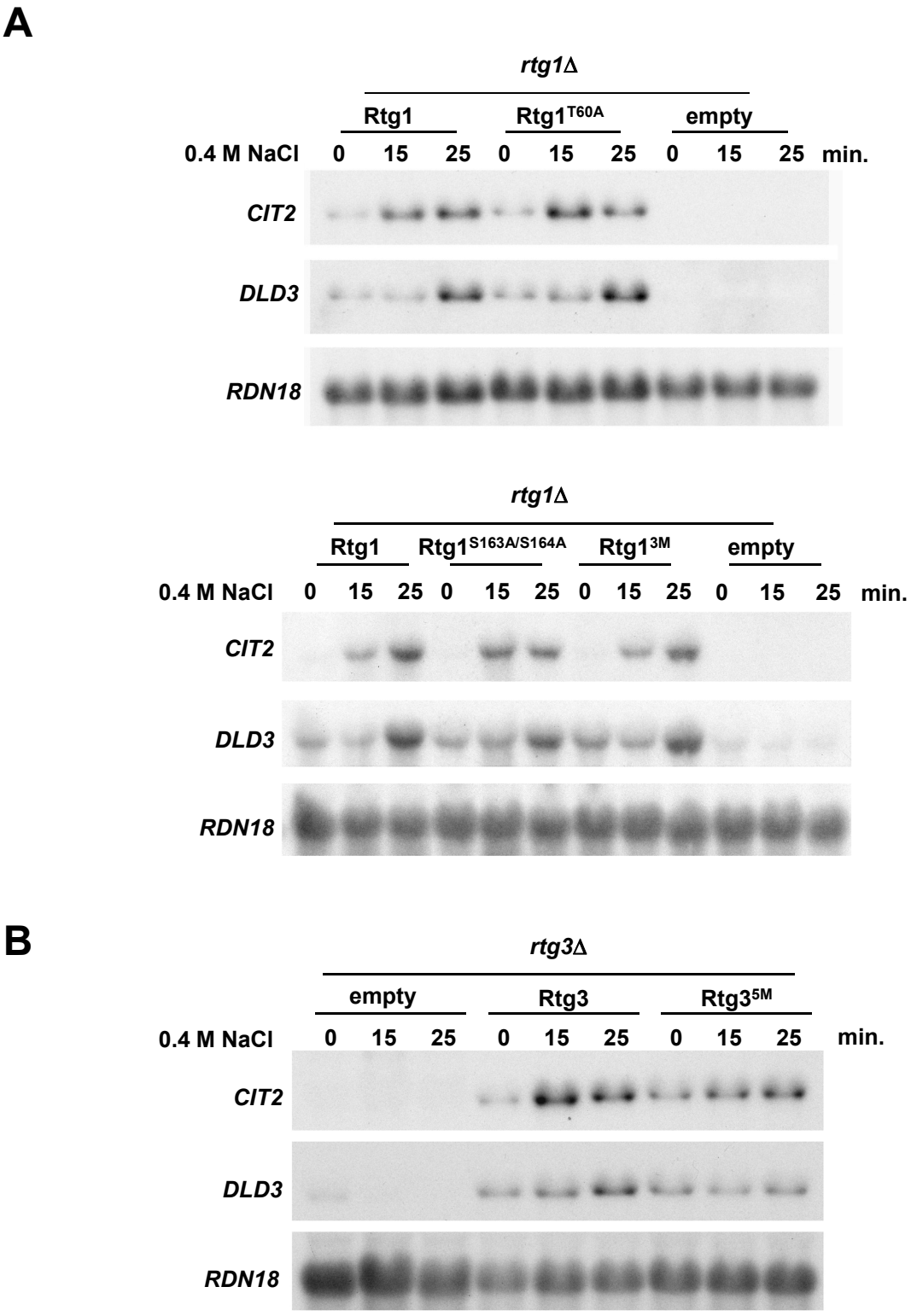
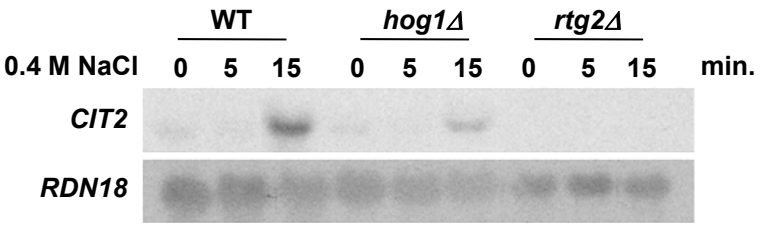
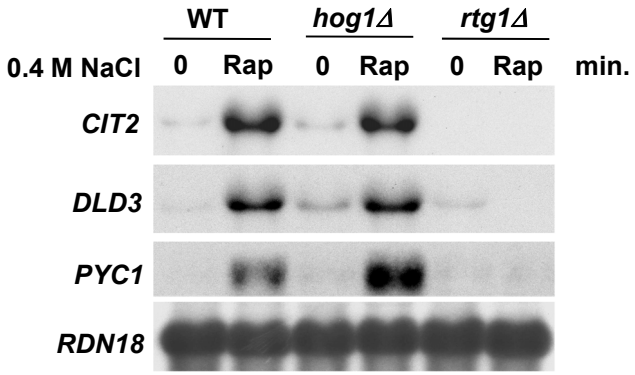

A
rtg1Δ
Rtg1
Rtg1T60A
empty
0.4 M NaCl
0 15 25 0 15 25 0 15 25 min.
CIT2
DLD3
RDN18
rtg1Δ
Rtg1
Rtg1S163A/S164A
Rtg13M
empty
0.4 M NaCl
0 15 25 0 15 25 0 15 25 0 15 25 min.
CIT2
DLD3
RDN18
B
rtg3Δ
empty
Rtg3
Rtg35M
0.4 M NaCl
0 15 25 0 15 25 0 15 25 min.
CIT2
DLD3
RDN18

Figure S1

A



B



C

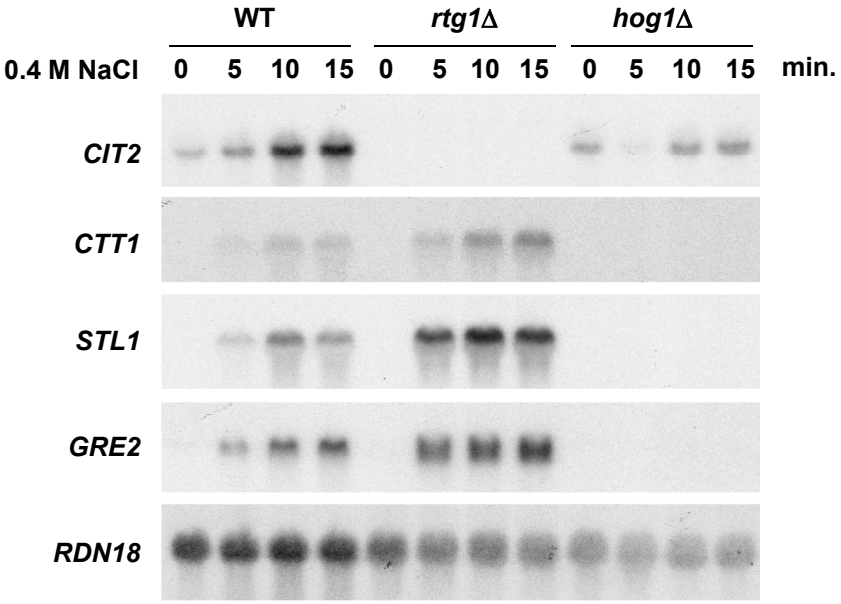


Figure S2

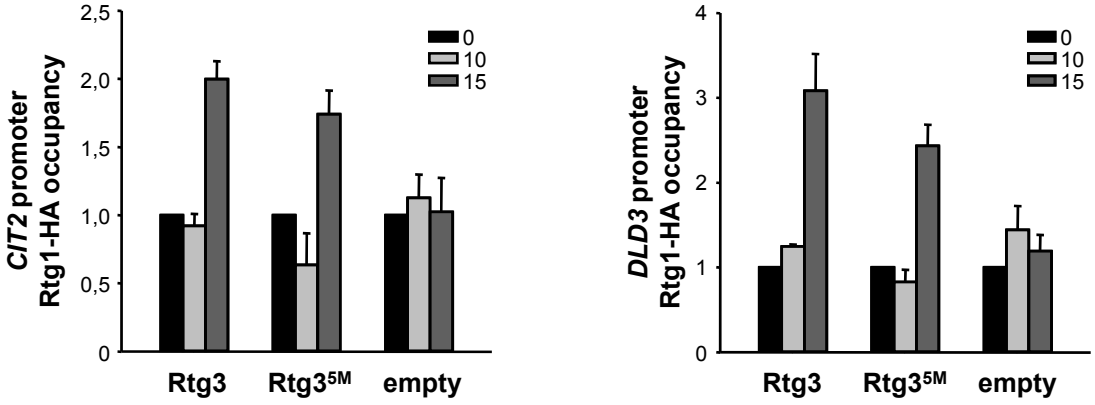
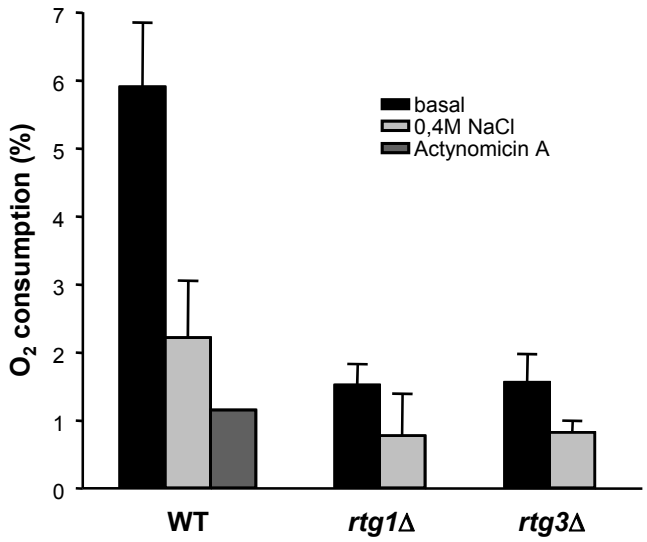

CIT2 promoter
Rtg1-HA occupancy
2,5
2,0
1,5
1,0
0,5
0
0
10
15
Rtg3
Rtg3^{5M}
empty
DLD3 promoter
Rtg1-HA occupancy
4
3
2
1
0
0
10
15
Rtg3
Rtg3^{5M}
empty

Figure S3


7
6
5
4
3
2
1
0
O2 consumption (%)
basal
0,4M NaCl
Actynomicin A
WT
rtg1Δ
rtg3Δ

Targeting the basic helix-loop-helix leucine Zipper Rtg1 and Rtg3 transcription factors by the Stress-Activated Protein Kinase Hog1

Clàudia Ruiz-Roig*, Núria Noriega*, Francesc Posas† and Eulàlia de Nadal†

(Manuscript for submission)

(1) The Rtg1 and Rtg3 transcription factors are needed for cell survival upon osmostress

To identify new factors involved in the osmoadaptive response, we used a genome-wide genetic screen that searched for gene deletion mutants of *S. cerevisiae* unable to grow at high osmolarity. This genetic screen, in addition to identifying genes already known to be important for the response to osmostress (e.g., *HOG1*, *PBS2*, and *GPD1* genes), yielded several protein complexes involved in transcription, such as Rpd3, SAGA, Mediator and RSC, which have been further characterized and reported in other studies (de Nadal *et al.*, 2004;Mas *et al.*, 2009;Zapater *et al.*, 2007). Thus, the use of this kind of genome-wide genetic screens, which have been also used for other stresses (Abe *et al.*, 2008;Giaever *et al.*, 2002;Ruiz-Roig *et al.*, 2010), represents a useful tool for the identification and characterization of genes involved in the adaptive response in front of a specific stress.

In the genetic screen we also obtained that mutations in *RTG1* and *RTG3* genes, encoding for basic helix-loop-helix leucine Zipper (bHLH-Zip) transcription factors, rendered cells unable to grow at high osmolarity. To confirm and further characterize the phenotype of osmosensitivity of these mutants, we manually spotted the mutant strains onto YPD plates with different concentrations of salt and sorbitol. The results confirmed that cells deficient for *RTG1* and *RTG3* genes are not able to grow in mild and high osmostress conditions. Thus, Rtg1 and Rtg3 transcription factors are important for osmoadaptation. Noteworthy, the fact that the *rtg1*Δ and *rtg3*Δ strains displayed a clear defect in growth not only in plates with high concentrations of salt, but also of sorbitol, indicated that these strains display not a phenotype of ion sensitivity but a clear osmosensitive phenotype. Rtg1 and Rtg3 transcription factors are the key activators of the retrograde (RTG) pathway, which is involved in responding to mitochondrial dysfunction by mediating changes in nuclear gene expression. Strikingly, the osmosensitivity of other mutants involved in mitochondrial function was reported before. Deletion mutants of *ACO1*, encoding aconitase, or *COX6*, encoding cytochrome c oxidase subunit 6, showed a clear sensitivity to osmostress (Martínez-Pastor *et al.*, 2009). Moreover, in the genetic screening used in this study for searching osmosensitive mutants, several mutants

belonging to mitochondrial processing complexes were identified as osmosensitive (Zapater *et al.*, 2007). Thus, our data together with other studies suggest that mitochondrial function is important for efficient adaptation to osmostress.

(2) RTG-dependent genes are induced in response to osmostress in a Hog1-dependent manner

The Rtg1 and Rtg3 transcription factors were required for cell viability upon osmostress. Therefore, we decided to further investigate their role in the regulation of gene expression in response to high osmolarity. *CIT2*, encoding a peroxisomal isoform of citrate syntase, *PYC1*, encoding pyrubate carboxylase, and *DLD3*, encoding a cytoplasmic isoform of D-lactate dehydrogenase, are typical Rtg1/3-target genes. Northern blot assays showed that these genes are strongly induced in response to osmostress, and induction is impaired in *rtg1* and *rtg3* mutant strains. Therefore, Rtg1 and Rtg3 transcription factors are required in osmostress to regulate gene expression. Moreover, we found that cells lacking *RTG2,* encoding another component of the RTG pathway, also displays impaired transcription of the RTG-dependent genes upon osmostress, indicating that the integrity of the RTG pathway is required for gene activation upon osmostress. Finally, we showed that Rtg1 and Rtg3 transcription factors are not required for induction of other osmoresponsive genes, such as *CTT1*, *STL1* or *GRE2,* which depend on Msn2/4, Hot1 and Sko1 transcription factors respectively, indicating that Rtg1/3 are required for induction of a specific subset of genes in osmostress.

Interestingly, the Rtg1/3-dependent genes analyzed in this work are known to be induced upon respiratory dysfunction, known as retrograde response (Chelstowska *et al.*, 1999;Epstein *et al.*, 2001;Liao *et al.*, 1991), although had not been previously characterized as osmoresponsive genes. Actually, other genes involved in mitochondrial function were previously shown to be induced upon osmostress. In particular, *CIT1*, which is also a known RTG-dependent gene, was shown to be induced in osmostress dependently on the Rtg1 and Rtg3 transcription factors (Martínez-Pastor *et al.*, 2009). Overall, the involvement of Rtg1 and Rtg3, in addition to Rtg2, for induction of these specific RTG-dependent genes in osmostress suggest that, upon osmostress, the RTG pathway might mediate a genetic response to adjust the functional state of the mitochondria (see below).

Because Hog1 plays a crucial role in the transcriptional response to osmostress by regulating the activity of several transcription factors, we wondered whether induction of the Rtg1/3-target genes in response to high osmolarity was dependent on the Hog1 MAPK. *In vivo*

gene expression analysis showed that Hog1 is required for induction of RTG-dependent genes in response to omostress. Moreover, the role of Hog1 is specific for osmostress, since activation of the RTG-dependent genes by rapamycin, a known activator of the RTG pathway, is not affected in a *hog1*Δ strain.

It is worth noting that deletion of *RTG1* and *RTG3* did not render cells as osmosensitive as deletion of *HOG1.* Nevertheless, the phenotype of osmosensitivity of the *rtg1*Δ and *rtg3*Δ strains was obvious, since a clear growth defect was observed in plates with mild and high concentrations of salt and sorbitol. This result was unexpected, since deletion of none of the transcription factors described to be involved in the transcriptional response to osmostress causes osmosensitivity. Indeed, a strain carrying deletions of several of the activators is not osmosensitive. Because we know that the Rtg1 and Rtg3 transcription factors are required for the induction of a specific subset of genes in response to osmostress, the phenotype of osmosensitivity is not likely due to a genome-wide defect of gene expression, but rather indicates that the response mediated by Rtg1/3 plays a key physiological role for adaptation to osmostress (see below).

Activation of *CIT2*, *PYC1*, and *DLD3* genes upon osmostress was completely abolished in the absence of Rtg1 or Rtg3. However, albeit it was strongly affected in the *hog1*Δ strain, it was not completely impaired, suggesting that other regulators besides Hog1 may be acting upstream of Rtg1/3 for the regulation of gene expression. In fact, Martínez-Pastor *et al.* reported that certain genes such as *CIT1*, *SDH2* or *COX6,* encoding for mitochondrial proteins, are induced upon osmostress depending on various degree on different kinases, such as Hog1 and Snf1 (Martínez-Pastor *et al.*, 2009).

(3) Hog1 and the Rtg1/3 complex interact in vivo and bind to RTG-dependent promoters in an interdependent manner

As a first approach to investigate the relationship between Hog1 and the Rtg1 and Rtg3 transcription factors, we tested whether Hog1 was able to interact with Rtg1 and Rtg3 under osmostress. I*n vivo* GST-pull down experiments showed that Hog1 is able to coprecipitate both Rtg1 and Rtg3 proteins in osmostress. It is worthy to mention that Rtg1 and Rtg3 are found as a complex both in the cytoplasm and in the nucleus (Sekito *et al*., 2000). Thus, the results of the CoIP experiment indicate that the Hog1 MAPK physically interacts with the Rtg1/3 complex. Further experiments could be done to test whether this interaction is dependent

specifically on Rtg1 or Rtg3. In any case, the results provide biochemical evidence for the relationship between the MAPK and the Rtg1/3 transcription complex.

It has been previously described that recruitment of osmoresponsive activators to specific target-promoters, such as recruitment of the Hot1 transcription factor to *STL1* promoter, depends on the presence of the Hog1 MAPK (Alepuz et al., 2001). Similarly, Chromatin Immunopreceipitation (ChIP) experiments showed that the Rtg1 transcription factor is recruited to the *CIT2* and *DLD3* promoters in response to osmostress, and it is dependent on Hog1. Rtg1 forms a heterodimer with Rtg3 to bind to the R box sites of promoters and activate gene expression. In fact, neither protein alone is able to bind to DNA (Jia *et al.*, 1997). Thus, the Rtg1/3 transcription complex is recruited at promoters upon osmostress, and this is dependent on Hog1.

On the other hand, it is known that the Hog1 MAPK itself is targeted to specific osmostress-responsive promoters in response to stress, and this recruitment is dependent on specific transcription factors (Alepuz *et al.*, 2003;Alepuz *et al.*, 2001;Pokholok *et al.*, 2006). For instance, the Hot1 transcription factor targets Hog1 to the *STL1* osmoresponsive promoter (Alepuz *et al.*, 2001). Similarly, our results also showed that Hog1 associates to the *CIT2* and *DLD3* promoters upon osmostress, and this recruitment is dependent on the presence of Rtg1. Therefore, the Rtg1/3 transcription complex targets the MAPK to the specific target promoters.

Overall, the interdependence of binding of Hog1 and Rtg1/3 to promoters of target genes reveals, as in the case of other stress-mediating transcription factors, a functional connection between the two factors for promoter recruitment and anchorage to chromatin. Moreover, the Hog1 recruitment to target promoters provides evidence that Hog1 is directly involved in the regulation of transcription of the Rtg1/3-target genes in response to osmostress.

(4) Hog1 regulates the Rtg1/3 transcription complex by controlling its nuclear accumulation in response to osmostress

The RTG pathway is regulated by the dynamic localization of the Rtg1/3 heterodimer from the cytoplasm to the nucleus. When the RTG pathway is inactive, the Rtg1/3 complex is found in the cytoplasm, whereas when the RTG pathway is active, the transcription complex concentrates in the nucleus (Sekito *et al.*, 2000). Thus, we wanted to investigate the subcellular localization of the Rtg1/3 transcription complex upon osmostress. Rtg1 and Rtg3

localize in the cytoplasm in basal conditions and concentrate rapidly into the nucleus upon an osmostress, which correlates with the transcriptional induction of *CIT2* and *DLD3*. Hog1 is required for transcription of Rtg1/3-target genes in response to osmostress. Therefore, Hog1 could be controlling the nuclear accumulation of the Rtg1/3 complex. In fact, previous data indicated that MAPK signaling pathways can regulate the localization of transcription factors from the cytoplasm to the nucleus (Yang *et al.*, 2003). Regarding to the Hog1 MAPK, it was described that subcellular localization of the Smp1 transcription factor depends on Hog1 in stationary conditions (de Nadal et al., 2003). However, a role for Hog1 in controlling the nuclear loaclization of a transcription factor upon osmostress has not been described before. Remarkably, microscopic analysis revealed that Rtg1 and Rtg3 nuclear accumulation upon osmostress clearly diminishe in *hog1*Δ cells, indicating that the Hog1 MAPK is regulating the localization of the Rtg1/3 transcription complex upon osmostress. Noteworthy, Hog1 is not required for nuclear accumulation of the Rtg1/3 transcription complex in response to other activators of the RTG pathway, such as rapamycin. These results are consistent with the previous observations that Hog1 is required for expression of RTG-dependent genes upon osmostress but not in response to rapamycin treatment, indicating that Hog1 controls the Rtg1/3 transcription complex specifically in response to osmostress.

In order to test whether Hog1 activity was required for accumulation of the Rtg1/3 complex into the nucleus upon osmostress, we used a catalyically impaired mutant of Hog1, which is unable to phosphorylate its substrates (Ferrigno *et al.*, 1998). Remarkably, the subcellular localization of Rtg1/3 by Hog1 does not depend on the Hog1 catalytic activity. Therefore, the only presence of Hog1 is sufficient to control this event. To demonstrate that only the presence of Hog1 is required for the localization of the complex, we followed the localization of Rtg1-GFP and Rtg3-GFP in cells expressing Hog1 mutants with altered subcellular localization. When Hog1 is retained in the cytoplasm, the Rtg1/3 transcription complex cannot be translocated into the nucleus in stress conditions. Conversely, when Hog1 is retained inside the nucleus, the Rtg1/3 complex is permanently localized in the nucleus independently on the presence of stress. Therefore, the localization of the transcription complex is dependent on the localization of the Hog1 MAPK, suggesting that control of the subcellular localization of Rtg1/3 by Hog1 is likely dependent on the MAPK interaction with the complex. While under normal conditions Hog1 appears to be distributed throughout the cytoplasm and the nucleus, Hog1 activation causes a rapid and marked concentration of protein in the nucleus (Ferrigno *et al.*, 1998;Reiser *et al.*, 1999). Thus, upon detection of an increase in external osmolarity and Hog1 activation, the interaction of Hog1 with the Rtg1/3

complex represents a first level of control, consisting in targetting of the complex into the nucleus (Figure 11). These results are consistent with the previous data showing that Rtg1/3 chromatin binding is dependent on Hog1.

(5) Hog1 catalytic activity is required for binding of Rtg1/3 complex at promoters and proper RTG-dependent gene expression

Although Hog1 kinase activity is not required for the control of Rtg1/3 nuclear translocation, there remained the possibility that Hog1 activity was required for regulation of the transcription complex by acting at other levels of control, such as control of chromatin anchorage or transcriptional activity. For many mammalian MAPKs, it has been proposed that modification of a DNA binding activator allows either binding of this factor to DNA or its cooperation with coactivators (Cuadrado and Nebreda, 2010;Karin *et al.*, 1995;Kyriakis *et al.*, 2001;Yang *et al.*, 2003). First, by ChIP experiments, we assessed the recruitment of Rtg1 at promoters in a Hog1 catalyically impaired mutant. Our results demonstrated that the catalytic activity of Hog1 is required for the association of the Rtg1/3 complex at chromatin. Remarkably, this represents a second level of control of the activators by the MAPK (Figure 11). Previous data have shown that Hog1 activity is required to modulate promoter association of other transcription factors, such as the Hot1 activator (Alepuz *et al.*, 2001).

Then, by performing northern blot experiments we analyzed gene expression in a Hog1 catalyically impaired mutant. Correspondingly, the Hog1 catalytic activity is essential for induction of the *CIT2* and *DLD3* osmoresponsive genes. Taken together, the data indicate that although the catalytic activity of the MAPK is not required for the first level of control of the Rtg1/3 transcription complex upon osmostress (Rtg1/3 nuclear translocation), it is essential for anchorage of the heterodimer to promoters, and for induction of transcription. Finally, the observation that the Hog1 catalytic activity is needed for transcriptional activation can be a consequence of its requirement for binding of the activators at promoters, but can also be possible that Hog1 controls the activity of the transcription factor. Thus, we aimed to determine whether Rtg1 and Rtg3 were substrates for Hog1 and the phosphorylations important for controlling these processes.

(6) Rtg1 phosphorylation by Hog1 does not affect Rtg1 function

One of the most common mechanisms by which MAPKs regulate gene expression is by modification of specific transcription factors (Cuadrado *et al.*, 2010;Karin *et al.*, 1995;Kyriakis *et al.*, 2001;Yang *et al.*, 2003). Our results showed that Rtg1 is actually a direct substrate for

the Hog1 MAPK. *In vitro* phosphorylation assays demonstrated that phosphorylation of Rtg1 by Hog1 occurs specifically at the Thr60 residue. Moreover, *in vivo* Rtg1 phosphorylation upon stress is totally dependent on Hog1. However, Rtg1 with a substitution of Thr60 to Alanine is phosphorylated *in vivo* to the same extent as the wild-type in response to osmostress, suggesting that Rtg1 might integrate inputs from different regulatory kinases in a Hog1-dependent manner. Rtg1 contains Ser163 and Ser164 residues, which match the consensus sequence for phosphorylation by cyclic AMP-dependent protein kinase (PKA) (R/KR/KXS/T). Thus, we assessed the *in vivo* phosphorylation in Rtg1 with mutation of these sites. Interestingly, a mutant form of Rtg1 containing substitutions of Ser163 and Ser164 to Alanine showed no *in vivo* phosphorylation upon osmostress. Thus, whereas Ser163 and Ser164 are phosphorylated *in vivo* upon osmostress in a Hog1-dependent manner, the *in vitro* phosphorylation of Thr60 by Hog1 could not be detected *in vivo*. Taken together, the data indicated that Hog1 directly phosphorylates *in vitro* Rtg1 at Thr60, and indirectly at Ser163 and Ser164 residues in response to osmostress, likely through an intermediate kinase.

It is worth noting that this scenario resembles that of Sko1 transcription factor, which is regulated by both Hog1 and PKA (Proft *et al.*, 2001). However, northern blot experiments showed that cells expressing $Rtg1^{T60A}$, $Rtg^{S163AS164A}$ or the triple mutant, were able to induce transcription to the same extent as cells expressing the wild-type Rtg1. Thus, the Rtg1 phosphorylation mediated directly or indirectly by Hog1 is not essential for the transcriptional activity. Notably, it is not the first time that phosphorylation of a transcription factor by Hog1 is not required for regulation of gene expression. For instance, osmostress-dependent phosphorylation of the Hot1 transcription factor by Hog1 appeared to be unnecessary for proper gene activation (Alepuz et al., 2001; Alepuz et al., 2003). However, we cannot discard that Rtg1 phosphorylation plays a role in modulating some aspect of transcription. Single cell studies should be performed to assess the relevance of these phosphorylations in gene expression.

(7) Hog1 regulates activity of Rtg1/3 transcription complex by phosphorylation of Rtg3

In vitro phosphorylation experiments showed that Rtg3 is also a direct substrate for the Hog1 MAPK. By testing *in vitro* phosphorylation of different Rtg3 fragments and Rtg3 punctual mutants, we obtained that Rtg3 *in vitro* phosphorylation was abolished when Rtg3 was mutated at five different residues, namely Thr197, Ser222, Ser227, Thr249 and Ser376. Thus, Hog1 directly phosphorylates Rtg3 at these five specific sites. However, we could not detect *in vivo* Rtg3 phosphorylation in response to osmostress. Similarly to other studies, Rtg3 appeared

as multiply phosphotylated in basal conditions. However, whereas we could detect a change in mobility of the Rtg3 protein in response to rapamycin, no change in mobility could be detected upon addition of salt. It is worth noting that the relevant kinase and phoshatase activities that mediate the phosphorylation state of Rtg3 remain to be identified. However, it is known that phosphorylation of Rtg3 is dependent on Rtg1. Correspondingly, in *rtg1*Δ cells, Rtg3 is always dephosphorylated (Sekito *et al.*, 2000). Therefore, a possible experiment in an attempt to detect *in vivo* phosphorylation of Rtg3 upon osmostress, is to analyze the mobility pattern of Rtg3 in response to osmostress, under the *RTG1* deletion background.

Remarkably, the Rtg3 non-phosphorylatable mutant ($Rtg3^{5M}$) displayed reduced transcriptional activation upon osmostress compared to the wild-type. Thus, the Rtg3 sites phosphorylated by Hog1 *in vitro* are required for activation of gene expression in response to stress. These results together with previous data indicating that Hog1 activity is required for activation of RTG-dependent gene expression, suggest that Hog1 is most probably the kinase responsible for these phosphorylations *in vivo*. Taken together, it seems that, although Rtg1 is also a direct target for Hog1, Hog1 controls the Rtg1/3 heterodimer transcriptional activity through the phosphorylation of Rtg3. This is consistent with the fact that, although both Rtg1 and Rtg3 are required for activation of transcription, only Rtg3 contains transactivating domains (Rothermel *et al.*, 1997). Phosphorylation of numerous transcription factors by MAPKs is important for modulating their transcriptional activity. In response to osmostress, the Smp1 and Sko1 transcription factors are also phosphorylated by the Hog1 MAPK and the corresponding phosphorylations are important for modulating the transcriptional activity of the activators (de Nadal *et al.*, 2003;Proft *et al.*, 2001). An interesting experiment to complement the results of this study would be to analyze whether recruitment of the polymerase is affected in the Rtg3 non-phosphorylatable mutant ($Rtg3^{5M}$).

ChIP experiments showed that, in the Rtg3 non-phosphorylatable mutant, binding of the transcription complex at promoters was not altered compared to wild-type. These results indicate that Rtg3 phosphorylation by Hog1 is important for transcriptional activation upon osmostress but is not the key mechanism that allows for anchorage of the complex. Definitely, it would be interesting to investigate which is the mechanism by which Hog1 activity controls chromatin binding of the complex. Overall, we can conclude that, in addition to control the subcellular localization of the Rtg1/3 complex and the control of Rtg1/3 chromatin anchorage, Hog1 still has a third independent layer of control of the Rtg1/3 complex, which is the control of transcriptional activity through Rtg3 phosphorylation (Figure 11).

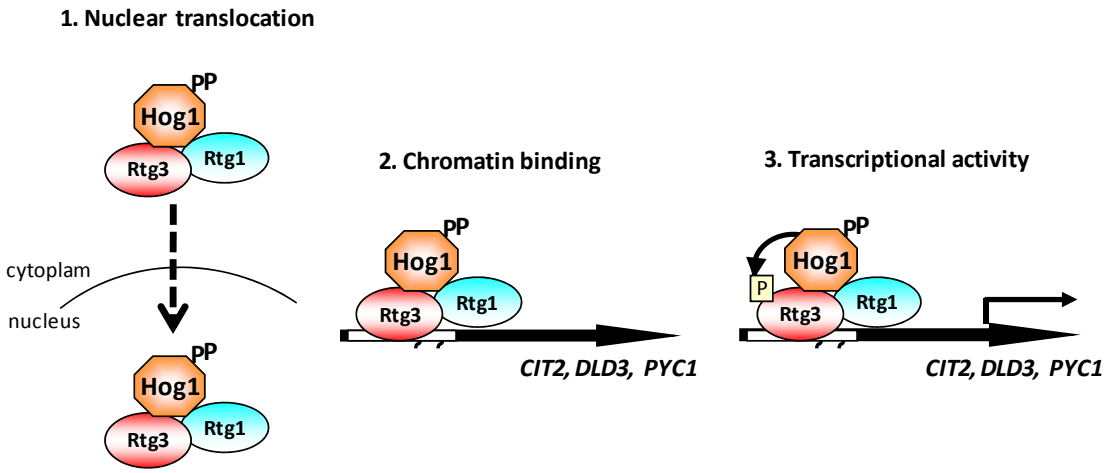


Figure 11. Hog1 controls the Rtg1/3 transcription complex through three independent mechanisms. In response to osmostress, Hog1 is required for nuclear translocation of the Rtg1/3 complex. Once in the nucleus, Hog1 activity is needed for association of the Rtg1/3 complex to chromatin. Moreover, Rtg3 phosphorylation by Hog1 is required for proper transcriptional activity.

(8) Physiological function of the RTG pathway in osmostress

Previous studies demonstrated that osmostress affects mitochondrial function by reducing the mitochondrial electron transport in plants (Hamilton and Heckathorn, 2001). Moreover, recently it was described that constitutive activation of Hog1 provokes a decrease in respiration, which leads to cell death caused by an increase in reactive oxygen species (ROS) (Vendrell *et al.*, 2011). The probably most important adaptive response in front of an osmotic shock is the increase of glycerol accumulation. Under anaerobic conditions cells accumulate glycerol faster than under aerobic conditions (Krantz *et al.*, 2004). Thus, it appears reasonable to propose that cells alter metabolic activity to increase the production of glycerol upon osmostress. Results of this study showed that a decrease in respiration rate is produced upon osmostress. Thus, an osmotic shock leads to alterations in mitochondrial function and respiration capability. Moreover, *rtg1* and *rtg3* mutants displayed affected respiration before osmostress treatment and almost impaired respiration after a brief osmotic shock. Therefore, Rtg1 and Rtg3 transcription factors are essential determinants to maintain minimal levels of mitochondrial respiration. The lack of respiration in the *rtg1*Δ and *rtg3*Δ strains could be the reason why these strains are not able to grow at high osmolarity, suggesting that although a decrease in respiration rate might be an important adaptive response to osmostress, maintaining minimum levels of mitochondrial function is essential for optimal growth in osmostress. In fact, it was reported that mitochondrial function is needed for efficient adaptation to osmostress (Martinez-Pastor *et al*. 2009). The fact that Rtg1, Rtg3 and Rtg2 are required for the induction of gene expression in osmostress is an indication that the integrity

of the RTG pathway is needed in response to such conditions. Taken together, the data suggest that alterations in the mitochondrial state are produced upon osmostress and the RTG pathway is activated to adjust gene expression, leading to a reconfiguration of cellular metabolism to accommodate cells to the new conditions.

Interestingly, Martinez-Pastor *et al*. investigated which was the possible protective function of mitochondria necessary to properly adapt to osmostress. They found that neither a defect in transcriptional activation nor a depletion of ATP levels could explain the severe growth defect of mitochondrial mutants under osmostress. However, they observed that mitochondrial dysfunction increased reactive oxygen species (ROS) production. Moreover, the growth defect in osmostress of mutants affecting mitochondrial function was rescued by supplementing the media with an antioxidant agent, indicating that ROS detoxification was deficient in mutants with impaired mitochondrial function and it was an important determinant for the adaptation to osmostress (Martínez-Pastor *et al*. 2009). Therefore, the osmosensitive phenotype of the *rtg1* and *rtg3* mutants might be explained because mitochondrial function is not maintained in these strains, therefore cells cannot counteract the oxidative stress produced by increased levels of ROS in osmostress.

Osmostress causes the rapid loss of water from the cell, which results in intracellular ion imbalances that interfere with many vital functions. In *S. cerievisiae*, the MAPK Hog1 orchestrates several adaptive responses affecting many diverse physiological functions (de Nadal *et al.*, 2002;Hohmann, 2002;Hohmann *et al.*, 2007). Although the exact physiological role of the RTG pathway in osmostress remains to be elucidated, in the present study we propose a new role of Hog1 for the control of the Rtg1/3 transcription factors and induction of mitochondrial releated genes, likely to regulate mitochondrial function in response to osmostress.

Personal contribution to this work: Together with Dra. Nuria Noriega, I have been fully involved in the design, execution and discussion of the experiments and results described in this study. Moreover, I have been fully involved in the preparation of the manuscript.

CONCLUSIONS

From the results presented in this PhD Thesis, the following conclusions can be drawn:

The Rpd3L HDAC complex is essential for the heat stress response in yeast:

• A high-throughput genetic screen reveals that a network of activities covering almost all general features of cell physiology is required for adaptation and survival to heat stress.

• Rpd3 histone deacetylase complex is required for acute transcriptional activation and repression in response to heat stress.

• Upon heat stress, Rpd3 has a differential effect depending on the transcription factor that specifically governs gene expression, being required for the activation of genes specifically under the control of Msn2/4 transcription factors.

• Upon heat stress, the large Rpd3L complex is recruited at promoters to mediate activation of gene expression and its binding is dependent on Msn2/4 transcription factors.

• Rpd3 is needed for entry of polymerase at promoters but is not required for chromatin remodeling upon heat shock.

• The function of Rpd3 in heat stress gene induction requires its histone deacetylase activity.

Targeting the basic helix-loop-helix leucine Zipper Rtg1 and Rtg3 transcription factors by the Stress-Activated Protein Kinase Hog1:

• The Rtg1 and Rtg3 transcription factors are required for cell adaptation to osmostress.

• Induction of Rtg1/3-target genes in response to osmostress depends on the Hog1 MAPK.

• Hog1 interacts with both Rtg1 and Rtg3 in osmostress.

• Hog1 and Rtg1 are recruited to the promoters of the RTG-dependent genes upon osmostress in an interdependent manner.

• Subcellular localization of the Rtg1/3 complex depends on the Hog1 MAPK.

• Hog1 activity is required for chromatin association of the Rtg1/3 transcription complex.

• Rtg3 phosphorylations by Hog1 are required for transcriptional activity.

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I'm a great believer in luck, and I find the harder I work the more I have of it.
~Thomas Jefferson