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THE Rtg1 AND Rtg3 PROTEINS ARE NOVEL TRANSCRIPTION FACTORS REGULATED BY THE YEAST Hog1 MAPK UPON STRESS

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A mi familia.

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Los recuerdos están en los besos...
Sólo queda el presente, el pasado no existe
No importa quienes somos, ni qué seremos
Sólo importa qué sentimos
No importa qué tenemos, ni qué tendremos
Maldita esta goma de borrar
Maldita condena la del olvido

In *Saccharomyces cerevisiae* the adaptation to high osmolarity is mediated by the HOG (high-osmolarity glycerol) pathway, which elicits different cellular responses required for cell survival upon osmostress. Regulation of gene expression is a major adaptative response required for cell survival in response to osmotic stress. At least five transcription factors have been reported to be controlled by the Hog1 MAPK. However, they cannot account for the regulation of all of the genes under the control of the Hog1 MAPK. Here we show that the Rtg1/3 transcriptional complex regulates the expression of specific genes upon osmostress in a Hog1-dependent manner. Hog1 phosphorylates both Rtg1 and Rtg3 proteins. However, none of these phosphorylations are essential for the transcriptional regulation upon osmostress. Here we also show that the deletion of RTG proteins leads to osmosensitivity at high osmolarity, suggesting that the RTG-pathway integrity is essential for cell survival upon stress.

La adaptación de la levadura *Saccharomyces cerevisiae* a condiciones de alta osmolaridad está mediada por la vía de HOG ((high-osmolarity glycerol). La activación de esta vía induce una serie de respuestas que van a permitir la supervivencia celular en respuesta a estrés. La regulación génica constituye una respuesta clave para dicha supervivencia. Se han descrito cinco factores de transcripción regulados por Hog1 en respuesta a estrés osmótico. Sin embargo, éstos no pueden explicar la totalidad de los genes regulados por la MAPK Hog1. En el presente trabajo describimos cómo el complejo transcripcional formado por las proteínas Rtg1 y Rtg3 regula, a través de la quinasa Hog1, la expresión de un conjunto específico de genes. Hog1 fosforila Rtg1 y Rtg3, aunque ninguna de estas fosforilaciones son esenciales para regulación transcripcional en respuesta a estrés. Este trabajo también muestra cómo la deleción de proteínas RTG provoca osmosensibilidad celular, lo que indica que la integridad de la vía de RTG es esencial para la supervivencia celular frente a un estrés osmótico.

SUMMARY

In *Saccharomyces cerevisiae*, changes in the extracellular osmotic conditions are sensed by the HOG MAPK pathway, the functional homolog of the mammalian stress-activated MAPK kinases JNK and p38. The Hog1 SAPK elicits the program for cell adaptation that includes modulation of several aspects of cell biology, such as gene expression, protein synthesis and cell-cycle progression. Genome-wide studies indicate that about 7% of the yeast genes change their pattern of expression upon osmotic stress, and the transcriptional induction of most of these genes is dependent on the presence of the Hog1 MAPK. Several transcription factors have been proposed to be controlled by Hog1. However, they cannot account for the regulation of all of the genes under the control of the kinase. In addition, a mutant strain carrying the deletion of the known transcription factors is not osmosensitive. These observations suggest that additional transcription factors are required for the osmostress-induced transcription by the Hog1 MAPK.

To identify novel transcription factors that regulate gene transcription upon osmostress, a genome-wide genetic screening was performed. By this approach, the Rtg1 transcription factor appeared as a possible regulator of gene expression upon stress. Here we show that the Rtg1/3 transcriptional complex regulates the expression of specific genes upon osmostress, which correlates with the nuclear translocation of the complex. In addition, we show that Hog1 phosphorylates directly Rtg1 at the Thr60 residue, and indirectly at Ser163 and Ser164 residues through an unknown intermediate kinase. Furthermore, Hog1 phosphorylates the Rtg3 protein at the Thr197 residue. Although the catalytic activity of the Hog1 MAPK is required for the proper induction of the RTG-regulated gene expression, none of these phosphorylations are essential for the transcriptional regulation upon osmostress. Here we also show that the deletion of RTG proteins leads to osmosensitivity, suggesting that the RTG-pathway integrity is essential for cell survival upon stress. Overall, our results define additional transcription factors that regulate gene expression upon osmostress, and have been

important to achieve a better view of how the Hog1 regulates transcription at these conditions.

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INTRODUCTION

I1. OSMOTIC STRESS

I1.1. Yeast adaptation to environment conditions

Cells living freely in the natural environment, such as the yeast *Saccharomyces cerevisiae*, are constanly exposed to a highly variable conditions with respect to the availavility and quality of nutrients, temperature, pH, radiation, acces to oxigen and concentration of solutes and water activity (Al-Fageeh and Smales, 2006; Hohmann et al., 2007). These environmental changes are commonly referred to as cell stress. In response to stress, cells are able to generate the appropriate responses that maximize their probability of survival and proliferation.

In the yeast's natural environment, water activity is of special importance. It is necessary for maintaining an appropriate cell volume and favorable conditions for biochemical reactions. For this purpose, the water activity of the cytosol and its organelles has to be lower than that of the surrounding medium. Two different situations can alter the water activity: an osmotic upshift (or hyperosmotic shock) and an osmotic downshift (or hypoosmotic shock). A hyperosmotic shock is accompanied by rapid water outflow and cell shrinking. Oppositely, a hypoosmotic shock increases the water concentration gradient and leads to a rapid influx of water, cell swelling and increased turgor pressure. In the yeast's natural environment, the water activity can change widely and rapidly. For this reason, yeast cells have developed mechanisms to ensuring survival to changes in water activity. Since passive water loss or uptake occurs very fast, the survival mechanisms need to operate within the first seconds after a sudden osmotic shift (Hohmann, 2002).

In the case of a hyperosmotic shock (in the literature, often synonymous with osmotic stress), the water outflow results in an increase of cell biomolecules concentration. This phenomenon usually leads to an arrest of cellular activity. Yeast cells have developed mechanisms to adjust to high

external osmolarity and maintain or re-establish an inside-directed driving force of water. These mechanisms imply a detection of the osmolarity change, as well as the development of the appropriate responses to maintain optimal cellular activity. In *S. cerevisiae*, the production and accumulation of chemically inert osmolytes, such as glycerol, plays a central role in the process of osmoadaptation (Hohmann, 2002; de Nadal et al., 2002; Hohmann et al., 2007).

Although far from complete, the present picture of yeast osmoregulation is both extensive and detailed. Simulations using mathematical models combined with time courses measurements have allowed elucidation of the properties of the yeast regulatory network (Klipp et al., 2005; Mettetal et al., 2008).

I1.2. Signalling pathways involved in osmoadaptation

In *S. cerevisiae* changes in medium osmolarity have been shown to affect different signalling pathways. By far the best-characterized system is the mitogen-activated protein kinase (MAP kinase) cascade known as the HOG pathway (High Osmolarity Glycerol response pathway) (de Nadal et al., 2002; Sheikh-Hamad and Gustin, 2004; Hohmann et al., 2007), whose involvement in yeast osmoadaptation was discovered in 1993 (Brewster et al., 1993). This pathway is activated in less than 1 minute by osmotic upshift. The inability of mutants with an inactive HOG pathway to adapt properly to high-osmolarity medium indicates the significant role of this pathway for cell survival upon these changing conditions.

In addition to the HOG pathway, protein kinase A (cyclic AMP [cAMP]-dependent protein kinase) pathway affects gene expression upon hyperosmotic shock (Norbeck and Blomberg, 2000). However, protein kinase A mediates a general stress response under essentially all stress conditions, such as heat shock, nutrient starvation, high ethanol levels, oxidative stress and osmotic stress (Ruis and Schuller, 1995; Kafadar and

Cyert, 2004; Slattery MG et al., 2008). For this reason, protein kinase A most probably does not respond directly to osmotic changes. It has also been observed that an osmotic stress stimulates production of phosphatidylinositol-3,5-bisphosphate, a molecule that could serve as a second messenger in an osmotic signalling system (Dove et al., 1997; Hohmann, 2002). However, its function is still to be resolved.

I2. MAPK PATHWAYS

MAP kinase pathways are highly conserved signalling units apparently occurring in all eukaryotes, where they play essential roles in the response to environmental signals or hormones, growth factors, and cytokines. MAP kinase pathways control cell growth, morphogenesis, proliferation and stress responses (Chang and Karin, 2001; Gustin et al., 1998; Kyriakis and Avruch, 2001; Hohmann et al., 2007). The high conservation of these cascades between yeast and humans is indicated by the fact that individual kinases in the yeast pathway can be replaced by the corresponding human enzymes (de Nadal et al., 2002). This degree of conservation is both exciting and very valuable, since it allows the lower eukaryotes to serve as suitable model systems for studies on the regulation and function of the stress-activated pathways.

I2.1. Components of a MAPK signalling pathway: the central core

MAPK modules have been well characterized by genetic and biochemical analysis. The MAP kinase pathways are organized in modules containing at least three types of protein kinases, which transmit signals by sequential phosphorylation events in a hierarchical way (Figure 1).

Activation of MAPKKK occurs by phosphorylation through an upstream protein kinase or through interaction with other proteins, a process that often involves small G-proteins. Once activated, MAPKKKs phosphorylate

and thereby activate MAPKKs on serine and threonine within a conserved part at the N-terminal lobe of the kinase domain. Subsequently, MAPKKs phosphorylate a MAPK on a threonine (sometimes serine) and tyrosine residue, which are located adjacent to each other separated by a single aminoacid (Thr/Ser-X-Tyr). These phosphorylation sites are located in the activation loop of the catalytic domain and are essential to reach activation of the MAPK. Typically, phosphorylation of the MAPK stimulates its translocation from the cytosol to the nucleus, where it phosphorylates targets on serine/threonine followed by a proline. However, a portion of activated MAPK remains in the cytoplasm to mediate cytoplasmic events (Reiser et al., 1999; Hohmann, 2002). Until now, the vast majority of defined substrates for MAPKs are transcription factors. However, MAPKs have the ability to phosphorylate many other substrates including other protein kinases, phospholipases, cytoskeleton-associated proteins and ionic transporters (Bilsland-Marchesan et al., 2000; Kim and Shah, 2007).

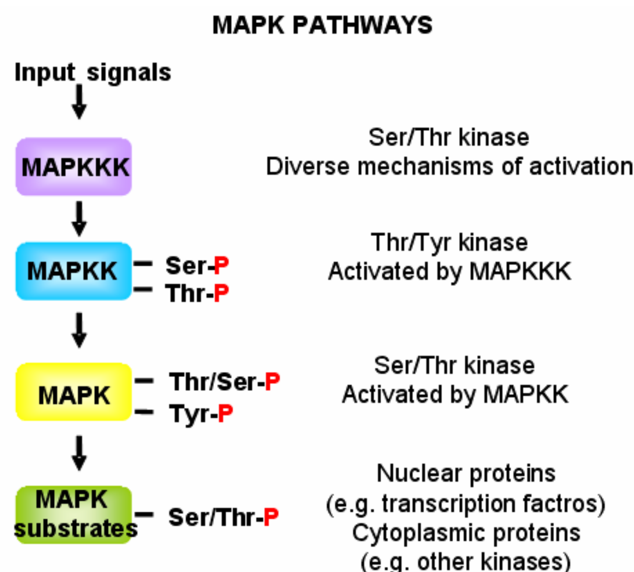

Figure 1. Schematic diagram of a MAPK pathway module. Core module of a MAPK pathway is composed of three kinases: MAPK kinase kinase, MAPK kinase and MAPK, that are sequentially activated by phosphorylation.

I2.2. Yeast MAPK pathways

Understanding of the *S. cerevisiae* MAPK pathways is more complete than that of MAPK pathways in other organisms. In *S. cerevisiae*, genetic

analysis as well as studies on the transcriptional readout upon physiological, pharmacological and genetic stimulation reveals a set of five MAPK located to five functionally distinct cascades: the mating pheromone response pathway (MAPK Fus3p), the pseudohyphal development pathway (Kss1p), the HOG pathway (Hog1p), the cell integrity pathway (Slt2p) and the spore wall assembly pathway (Smk1p) (Krisak et al., 1994; Hunter and Plowman, 1997; Chen and Thorner, 2007) (Figure 2).

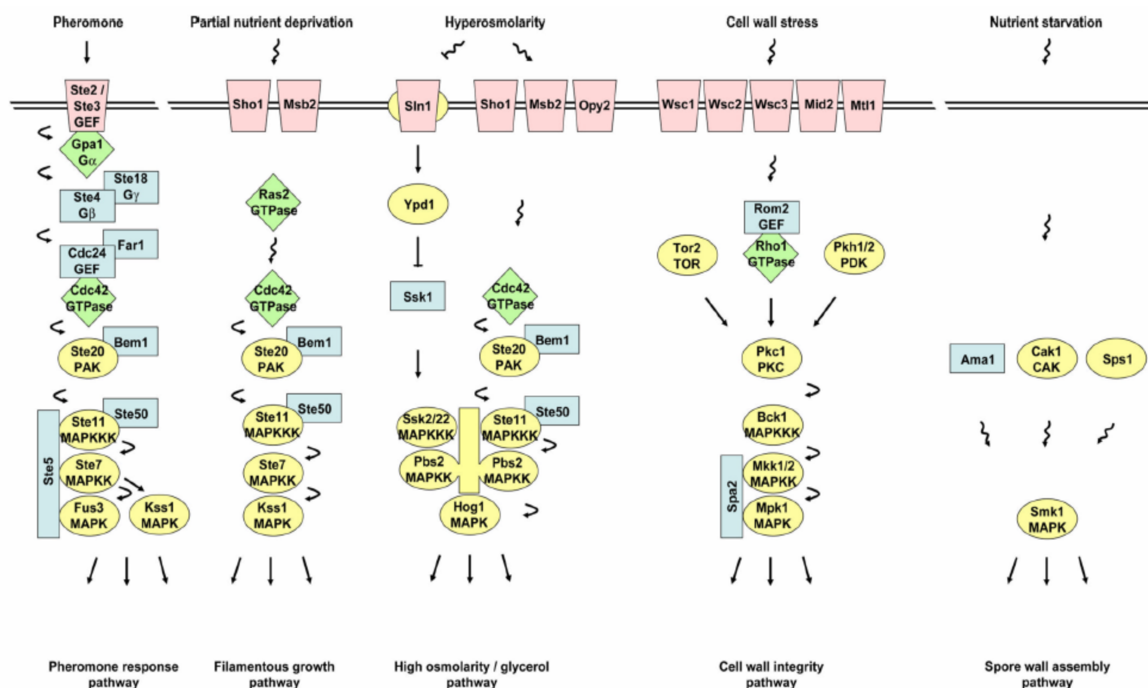

Figure 2. *S. cerevisiae* MAP kinase pathways. *S. cerevisiae* contains five MAPK pathways: pheromone response, filamentation-invasion, osmoregulation, cell wall integrity and sporulation pathways. Some of the elements are shared between pathways.

I2.3. Signalling specificity in MAPK pathways

Since different MAPK pathways within the same organism share protein kinases and phosphatases, there are numerous nodes for the crosstalk between these pathways. Given the complexity and diversity of MAPK regulation and function, cells must have evolved mechanisms to maintain

specificity in order to avoid unwanted responses to stimuli, while at the same time allowing the proper response to take place.

Scaffolding proteins play a key role in this signalling specificity. These proteins promote the interaction of the proper partners, allowing the coordinated and efficient activation and function of MAPK components in response to specific types of stimuli (Pawson and Scott, 1997). In some MAPK pathways, the signalling components themselves possess intrinsic scaffolding properties, such as the yeast MAPKK Pbs2 in the HOG pathway (see below). Alternatively, distinct proteins can act as scaffolding elements binding and segregating groups of signalling components, such as *S. cerevisiae* Ste5 protein in the mating pathway (Printen and Sprague, Jr., 1994; Schwartz and Madhani, 2004).

Despite the important role of scaffolding proteins in maintaining signalling specificity, other mechanisms must exist for this purpose, such as the MAPK substrate specificity. Although MAPKs are proline-directed kinases, substrate selectivity is often conferred by specific MAPK docking sites present on physiological substrates, often at considerable distance from the phosphorylation site in the primary sequence. This allows for a strong interaction with selected MAPK subfamilies to the exclusion of others (Tanoue et al., 2000; Sheridan et al., 2008). On the other hand, MAPK pathways are negatively controlled by protein phosphatases acting on both the MAPKK and the MAPK or only on the MAPK (Keyse, 2000).

I3. THE HOG PATHWAY

The HOG pathway is the best-characterized osmoresponsive system in eukaryotes and hence serves as a prototype osmoregulating signalling pathway. It specifically responds to increased 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 includes metabolic regulation, regulation of protein synthesis, cell cycle adaptation

and gene expression regulation. The mammalian relatives of HOG pathway are the p38 and c-jun N-terminal kinases (JNK) families of stress-activated protein kinases (SAPKs). The functional conservation of the stress MAPK cascades between yeast and humans is indicated by the fact that individual kinases in the yeast pathway can be replaced by the corresponding human enzymes (Galcheva-Gargova et al., 1994; Han et al., 1994; Sheikh-Hamad and Gustin, 2004).

I3.1. Osmosensing branches of the HOG pathway

The HOG pathway is activated predominantly by two independent mechanisms that lead to the activation of either the Ssk2 and Ssk22 or the Ste11 MAPKKKs. A signal emating from either branch converges on a common MAPK kinase (MAPKK), Pbs2, which is the specific activator of the Hog1 MAPK (Brewster et al., 1993; Maeda et al., 1994; Hohmann, 2002) (Figure 3).

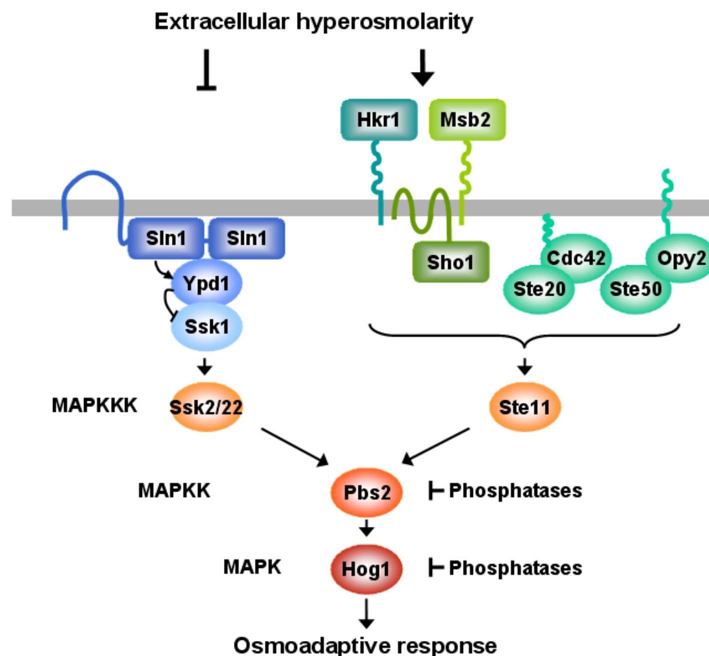

Figure 3. Schematic diagram of the yeast HOG pathway. Two major independent upstream osmosensing mechanisms lead to the activation of specific MAPKKKs and converge on a common MAPKK, Pbs2. Under osmostress activated Pbs2 activates the MAPK Hog1, which induces a set of osmoadaptative responses.

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 subsequent the Pbs2 MAPKK activation (Posas et al., 1996). At normal osmolarity, the osmosensor Sln1 is turned on and Hog MAPK is downregulated. In a high osmolarity environment, Sln1 switches off and the HOG pathway is activated (Maeda et al., 1994). Genetic disruption of the *SLN1* gene is lethal, due to the resulting constitutively activation of the HOG pathway (Maeda et al., 1994).

Pbs2 can also be achieved by a second mechanism that involves the protein Sho1 and the potential osmosensors Hkr1 and Msb2 mucin-like proteins (Maeda et al., 1995; Posas et al., 1998; Tatebayashi et al., 2007; de Nadal et al., 2007). Sho1 is a transmembrane protein whose 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). Sho1 binds to Pbs2 and thereby recruits it to the cell surface. In addition, Cdc42 binds to both the PAK-like kinase Ste20 and the Ste11-Ste50 complex. Consequently, Cdc42 brings activated Ste20 to its substrate Ste11 MAPKKK (Truckses et al., 2006; Tatebayashi et al., 2006). Once activated, Ste11 activates Pbs2, which in turn, activates Hog1 (Posas and Saito, 1997). It's interesting to mention the recent involvement of Hkr1 and Msb2 mucin-like proteins in activation of SHO1 branch (Tatebayashi et al., 2007). Since mammalian cells do not seem to have specific stress sensors similar to Sln1, determination of mucin-like proteins Msb2 and Hkr1 as a potential sensor mechanism coupled to Sho1 could open the attractive hypothesis of mucins sensing and signalling in eukaryotic cells during osmostress.

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), Sho1 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).

I3.2. Signalling through the HOG pathway

The activation of sensor branches converges on Pbs2 MAPKK phosphorylation on Ser514 and Thr518 residues. Then, Pbs2 phosphorylates Hog1MAPK on the conserved Thr174 and Tyr176 residues in the cytoplasm (Brewster et al., 1993; Schuller et al., 1994; Choi et al., 2008). Hog1 phosphorylation causes a rapid and marked concentration of protein in the nucleus, while under normal conditions Hog1 appears to be distributed between the cytosol and the nucleus (Ferrigno et al., 1998; Reiser et al., 1999). Nuclear concentration of Hog1-GFP can be observed within less than 1 min after a mild hyperosmotic shock and disappears within about 30 minutes.

Hog1 phosphorylation and activation is a transient event (Maeda et al., 1994; Jacoby et al., 1997; Tamas et al., 2000; Hohmann et al., 2007). These observations illustrate that the pathway is controlled by specific feedback mechanisms. In this regard, like any other MAPK signalling pathway, the HOG pathway is controlled negatively by protein phosphatases. Three phosphatases are physiologically relevant: Ptp2 (a nuclear phosphotyrosine phosphatase), Ptp3 (a cytosolic phosphotyrosine phosphatase) and Ptc1 (a type 2C serine/threonine phosphatase (PP2C) located in both the cytosol and the nucleus) (Saito and Tatebayashi, 2004). Simultaneous knockout of *PTP2* and *PTC1* is lethal because of overactive

Hog1 (Maeda et al., 1993), demonstrating that the phosphatases are needed to control the noise and thresholds of signalling. In addition, overexpression of any of these phosphatases suppresses the lethality caused by inappropriate activation of the pathway (Ota and Varshavsky, 1992; Maeda et al., 1994; Jacoby et al., 1997; Wurgler-Murphy et al., 1997; Mattison and Ota, 2000; Warmka et al., 2001). Protein phosphatases are critical for HOG pathway regulation for various purposes: i.e., to reduce the basal activity in order to prevent initiation of undesirable response in the absence of relevant stimuli, to prevent excessive MAPK activation upon stimuli, and to resume normal cell growth after adaptive responses.

I4. DOWNREGULATION OF Hog1 SIGNALLING

Nuclear accumulation of Hog1 suggests that an important part of Hog1 actions take place in the nucleus. However, Hog1 also mediates regulatory effects outside the nucleus. The best documented of such effects is activation of the protein kinase Rck2 (Bilsland-Marchesan et al., 2000; Teige et al., 2001), which controls translation efficiency (Teige et al., 2001) (see below), and the phosphorylation of ions transporters, which is crucial for the rapid reassociation of proteins, previously dissociated from chromatin due to the osmotic stress, with their target sites in chromatin (Proft and Struhl, 2004). Specifically, cell adaptation to osmotic stress includes several aspects of cell biology essential for cell survival, such as gene expression, cell cycle progression, protein synthesis and metabolic adaptation (Figure 4).

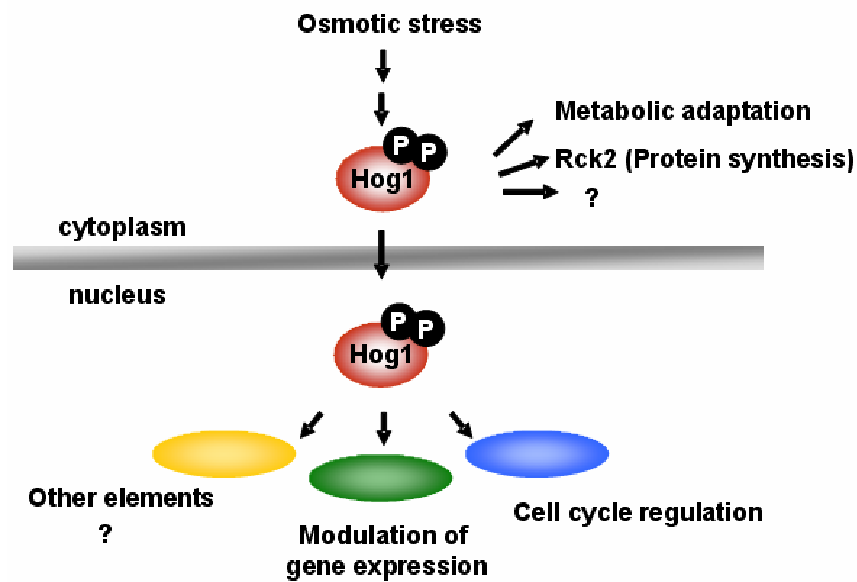

Figure 4. Functions of the MAPK Hog1. Once Hog1 is phosphorylated and activated, it controls several functions, such as cell cycle regulation, modulation of gene expression, metabolic adaptation and regulation of protein synthesis.

I4.1. Metabolic adaptation

As mentioned above, one of the roles of Hog1 upon osmotic stress is metabolic adaptation. Significant expression changes occur in genes encoding carbon metabolism proteins. The intracellular glycerol accumulation is one of the best known and well understood reactions of yeast cells on increased extracellular osmolarity (Hohmann, 2002). For glycerol synthesis during hyperosmotic stress the overexpression of the glycerol-3-phosphate dehydrogenase gene *GPD1* is known to play an important role (Blomberg, 2000; Posas et al., 2000; Remize et al., 2003). Additionally, an activation of 6-phosphofructo-2-kinase (PFK2), which catalyzes the synthesis of fructose-2,6-biphosphate (upper part of glycolysis), stimulates glycolysis for glycerol production (Dihazi et al., 2004). Yeast cells also produce and accumulate trehalose and glycogen upon osmotic stress. On the basis on the characteristics of its production and degradation, glycogen appears to have all the features of a typical storage carbohydrate. On the other hand, trehalose appears to have a role

as stress protectant. It is, however, still unclear if trehalose can serve the role of an osmolyte in *S. cerevisiae*, as it does in bacteria (Blomberg, 2000).

In addition, it has been detected a strong increase in expression of genes encoding plasma membrane sugar transporters, as well as enzymes involved in defense from oxidative damage and in redox metabolism. 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.

I4.2. Regulation of protein synthesis

In response to increases in external osmolarity, 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-E, 2002). The HOG pathway is involved in the control of 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). Similar effects have been shown in other organisms, as the yeast *Saccharomyces pombe* (Dunand-Sauthier et al., 2005; Asp et al., 2008). In mammalian cells, stress-induced regulation of protein synthesis is mediated by phosphorylation of the eukaryotic initiation factor eIF4e by the Mnk1 kinase or eEF2 by the eEF2 kinase. Both kinases have been reported to be regulated by p38 in response to stress (Knebel et al., 2001). Thus, translation control by SAPKs could be mediated by similar mechanisms in yeast and mammals.

An overall reduction of protein synthesis may well be compatible with a transient inhibition of cell growth and proliferation caused by osmotic stress. However, expression of genes encoding functions important 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).

I4.3. Regulation of cell cycle progression

Progression through the cell cycle is critically dependent on the presence of nutrients and stress stimuli. Therefore, in response to osmostress, cells transiently modulate cell-cycle progression to allow cell adaptation. Cell cycle is divided in four phases: S-phase (DNA synthesis), M-phase (mitosis), and G1 and G2 (Figure 5). At Start, yeast cells decide if begin a new cycle, conjugate with another cell, or sporulate. Transitions between G1/S and between G2/M are strongly regulated in order to provide a successful cell division. When one of these events is aborted, cells use diverse mechanisms, known as checkpoints, to monitor proper completion of each stage of the cell cycle. Thus, cell cycle progression can be delayed until the execution of an unfinished step allowing the cells to begin a new cycle.

In Clotet et al. (2006), it is proposed a novel regulatory mechanism of G2 checkpoint that allows cells to integrate stress signals to modulate cell cycle. Hog1 controls G2 progression by a dual mechanism: the downregulation of Clb2 levels, as well as the direct phosphorylation of the Hsl1 kinase. Upon osmotic stress, Hsl1 phosphorylation prevents Swe1 from being phosphorylated, which leads to Swe1 accumulation and G2 arrest (Figure 5). The Hog1 MAPK also controls the G1 transition in response to osmotic stress. Similarly to the mechanism for G2 regulation, this consists in a dual mechanism that involves regulation of cyclin expression and the targeting of the cell cycle regulatory protein Sic1 (Escoté et al., 2004) (Figure 5). Thus, the coordinated action of the MAPK Hog1, modulating cyclin transcription and targeting specific cell cycle regulators, controls cell cycle progression upon osmotic stress to prevent entry into the next cell

cycle phase without the cells being adapted to the new extracellular conditions. Because different reports and unpublished observations indicate that different types of mammalian cells arrest at several stages of the cell cycle upon osmostress (Gustin et al., 1998; Christoph et al., 2007), it has been proposed that a similar response to stress might be coordinated by SAPKs in mammals.

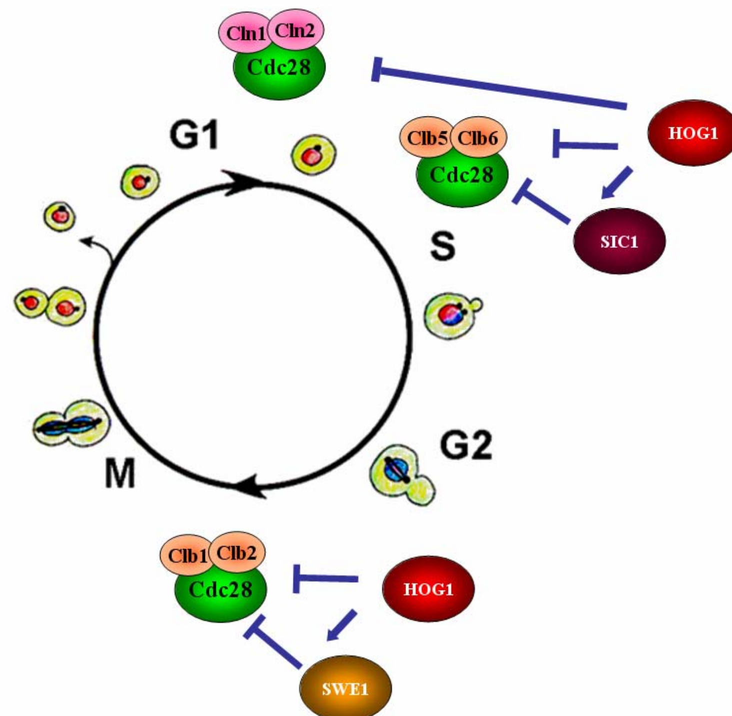

Figure 5. Cell cycle regulation by Hog1. Hog1 regulates cell cycle transitions between G1/S and G2/M by both regulating cyclin expression and targeting several cell cycle regulators, such as Sic1 and Hsl1. At both stages, cell cycle progression is delayed.

I4.4. Regulation of gene expression

One of the main functions of SAPKs in response to osmostress is the regulation of gene expression (Figure 4). In the last years, several studies have been published in which *S. cerevisiae* cells were exposed to osmotic stress and the expression of yeast genes was analyzed (Posas et al., 2000; Rep et al., 2000; Gasch et al., 2000; Causton et al., 2001; O'Rourke and

Herskowitz, 2004). From these studies some conclusions can be drawn. First, a large number of genes (~ 5-7% of the total genes) show significant changes in their expression levels after a mild osmotic shock. Second, the main groups of genes highly induced after exposure to osmotic stress for 10 minutes are genes encoding proteins involved in carbohydrate metabolism, general stress protection, protein biosynthesis, ion homeostasis, signal transduction and aminoacid metabolism (Posas et al., 2000; Gasch et al., 2000; Causton et al., 2001). And third, the Hog1-mediated signalling pathway plays a key role in global gene regulation under osmotic stress conditions. For instance, in a large number of cases the response to osmostress was fully or strongly dependent on the presence of Hog1 (Posas et al., 2000). Thus, although in some cases the HOG pathway is not the unique relevant signalling pathway involved, its central role in the global transcriptional response to osmotic stress is clearly established.

Exposure of yeast to high osmolarity results in an association of Hog1 with transcription factors for stimulation of transcriptional initiation (Alepuz et al., 2001). However, it is increasingly obvious that MAPK cascades do not modulate transcription by targeting transcription factors alone. Hog1 is recruited to chromatin upon stress and there it recruits Pol II, SAGA and Mediator complexes to promoters leading to transcriptional activation (Alepuz et al., 2003; Zapater et al., 2007). The finding that the downstream kinases MSK1 and MSK2 in mammal cells phosphorylate nucleosomal proteins (Thomson et al., 1999; Clayton and Mahadevan, 2003) provided the first direct link between a MAPK cascade and chromatin modification during gene induction. On the other hand, acetylation of histones H2B and H4 by ATF-2 is stimulated by JNK phosphorylation (Kawasaki et al., 2000), and histone deacetylation by the Rpd3 histone deacetylase complex is stimulated by Hog1 (de Nadal et al., 2004).

Moreover, the role of Hog1 in the regulation of the transcription cycle is not limited to transcription initiation but rather extends to the process of transcriptional elongation. Hog1 MAPK is a selective transcriptional

elongation factor of genes responding to osmotic stress, being part of the transcription elongation complex (TEC) (Proft el al., 2006) (see Supplementary article).

I5. REGULATION OF TRANSCRIPTION BY Hog1

As mentioned previously, one of the main functions of MAPKs in response to stress is the regulation of gene expression. There is no unifying mechanism by which MAPKs modulate gene expression under stress. The more traditional mechanism is the direct phosphorylation of promoter-specific transcription factor targets (Karin and Hunter, 1995; Treisman, 1996; Kyriakis and Avruch, 2001). This mechanism can regulate the target activity by several mechanisms, including control of protein levels, regulation of binding to DNA, nucleocytoplasmic shuttling and altering their ability to transactivate (Yang et al., 2003). Thus, kinases such as Hog1, p38 and JNK target several transcription factors directly enhancing their ability to activate transcription.

I5.1 Transcription factors downstream of Hog1

Once inside the nucleus, Hog1 MAPK regulates genes expression via at least five transcription factors: the redundant zinc finger proteins Msn2 and Msn4 (Schuller et al., 1994), the Hot1 protein (which does not belong to a known family of transcription factors) (Rep et al., 1999a), the bZIP protein Sko1 (Proft et al., 2001) and the MADS box protein Smp1 (de Nadal et al., 2003) (Figure 6). These factors are unrelated, and the mechanism by which Hog1 regulates their function may differ from one to another. Due to their DNA binding specificities and the profile of gene induction upon stress shown by DNA microarrays, they cannot account for the regulation of all of the genes under the control of Hog1. Additionally, a mutant strain carrying deletions in *hot1*, *msn1*, *msn2*, and *msn4* transcription factors is still able to grow under osmotic conditions. These observations suggest the possibility that additional transcription factors are required for gene expression upon

stress. The identification and characterization of these transcription factors is the main objective of this Thesis.

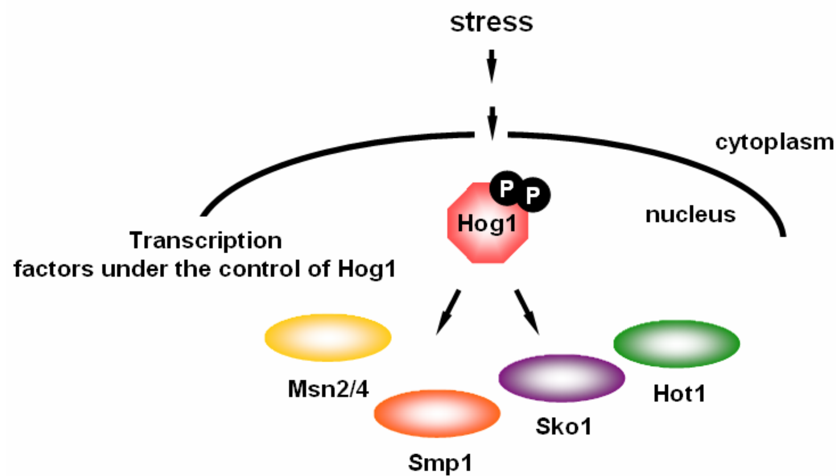

Figure 6. Transcription factors that are regulated by Hog1 upon osmostress. Hog1 regulates gene expression by controlling the following transcription factors: the redundant zinc finger proteins Msn2 and Msn4, the MAPD box Smp1, the bZip Sko1 and the Hot1 protein.

I5.1.1. Msn2/Msn4

Several stress conditions, including osmostress, induce the expression of genes controlled through the so-called Stress Response Element (STRE), via the Cys_2His_2 zinc finger proteins Msn2 and Msn4 (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996). The STRE element is characterized by the core sequence CCCCT in either orientation and it is usually found in two or more copies in front of Msn2/4 target genes (Treger et al., 1998; Moskvina et al., 1998). 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 and Werner-Washburne, 2002).

Msn2/4 activity is regulated by their subcellular localization, residing in the cytosol under standard growth conditions and translocating to the nucleus under stressful conditions. Once in the nucleus, Msn2/4 induce the

expression of genes such as *CTT1*, which encodes a cytosolic catalase, and *HSP12*, encoding for a small heat shock protein. Although the expression of these genes is totally dependent on the Hog1 MAPK upon osmostress, the localization of Msn2/4 is unaffected in a *hog1Δ* mutant (Görner et al., 1998). Interestingly, the Hog1 kinase itself becomes associated with the promoters of these genes, and this requirement is dependent on both its catalytic activity and the presence of the transcription factor (Alepuz et al., 2001). Actually, more work is needed to better understand the HOG-dependent control of STRE-driven genes and whether Hog1p controls Msn2/Msn4p directly.

It has been known for many years that the expression of STRE-dependent genes or reporters is sensitive to altered activity of protein kinase A (Bissinger et al., 1989; Marchler et al., 1993; Garreau et al., 2000). Since unregulated protein kinase A activity renders Msn2 transcription factor nuclear even under optimal conditions, nuclear localization of the transcription factor is negatively controlled by the cAMP-dependent protein kinase (PKA) (Görner et al., 1998). In addition, it has been described that Msn2 activation by sudden glucose depletion correlates with a fast but transient decrease in phosphorylation that depends on the protein phosphatase 1 (PP1). Thus, it has been proposed PP1 as a potential mediator of glucose starvation signals that target this transcription factor (De Wever et al., 2005). Further studies should be addressed to understand the mechanism by which Msn2 localizes into the nucleus upon osmotic stress.

I5.1.2. The Hot1 transcription factor

Hot1 (High-Osmolarity-induced Transcription) is a transcription factor related to Msn1 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., 1999b).

The transcription factor Hot1 is a nuclear protein both under standard growth and osmotic stress (Rep et al., 1999a). As revealed by chromatin immunoprecipitation, Hot1 is associated with the *GPD1* promoter under all conditions, although its level on the promoter increases under stress. This association is independent on Hog1. The situation seems to be different in the *STL1* promoter, where Hot1 binds only under osmotic stress and needs the Hog1 kinase activity to become associated with the promoter (Alepuz et al., 2001). Hot1 also appears to be associated with the promoters of *CTT1* and *HSP12*, although its contribution to osmotic induction of these genes is minor (Rep et al., 1999b; Alepuz et al., 2001). Interestingly, Hog1 kinase itself becomes associated with the promoters of these genes, and its recruitment is dependent on the presence of the transcription factor (Alepuz et al., 2001).

Hot1 is directly phosphorylated by Hog1. However, since Hot1 mutations in all five putative Hog1 phosphorylation sites do not affect the Hot1-mediated gene expression upon stress (Alepuz et al., 2003), this phosphorylation is not critical for regulation and activation *per se*. Thus, activation of gene expression by Hot1 must revolve around a mechanism other than phosphorylation of the activator by the MAPK. It has been described that RNA Pol II binds to Hot1-mediated promoters, and this binding depends on the Hog1 kinase activity (Alepuz et al., 2003). Then, it has been proposed a mechanism for regulation of gene expression in which Hot1 protein act as an anchor for the MAPK, and Hog1 is the key factor for inducing gene expression by directly recruiting the RNA Pol II holoenzyme. This mechanism of regulation by Hog1 is not restricted to Hot1-dependent genes, since it has been reported that binding of Hog1 and Pol II to Msn2/4-dependent promoters is also dependent on the transcription factor. Thus, different activators under the control of the Hog1 MAPK could be using a similar mechanism to induce gene expression upon stress.

I5.1.3. 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 (reviewed in De Cesare and Sassone-Corsi et al., 2000). Such factors possess a bZIP domain, i.e., a leucine zipper for dimerization, and an adjacent basic transcription activation domain.

Sko1 inhibits transcription of several genes that are inducible by osmotic stress by recruiting the general corepressor complex Ssn6-Tup1 (Proft and Serrano, 1999; Garcia-Gimeno and Struhl, 2000). These genes are *GRE2*, involved in ergosterol metabolism, and *HAL1*, involved in ion homeostasis, among others.

Release from Ssn6-Tup1 repression in response to osmotic stress requires direct phosphorylation of Sko1 by the Hog1 MAPK (Proft et al., 2001). Hence, in a *hog1Δ* mutant, expression of these genes is low or undetectable and unresponsible to osmotic shock. Interestingly, Hog1 phosphorylation switches Sko1 activity from a repressing to an activating state, which involves recruiting of SWI/SNF and SAGA complexes (Proft and Struhl, 2002). In addition, Sko1 repressor activity is further enhanced in strains with high protein kinase A (PKA) activity. PKA phosphorylates Sko1 near the bZIP domain, and mutation of these sites eliminates modulation of Sko1 responses to high PKA 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 signalling mechanism through the PKA pathway (Proft et al., 2001).

I5.1.4. The MADS box protein Smp1

Smp1 is a member of the MEF2C family of transcription factors (Yu et al., 1992) reported to act downstream of Hog1. It was identified by a genetic screen that isolated genes whose overexpression was able to induce *STL1::LacZ* reporter gene expression. *In vivo* coimmunoprecipitation and phosphorylation studies showed that Smp1 and Hog1 interacts and that Smp1 was directly phosphorylated by Hog1 on several residues within its transactivation domain upon osmostress (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 displayed impaired *STL1::LacZ* reporter gene expression (de Nadal et al., 2003). Furthermore, the same study reported that Smp1 might play an important role not only in osmostress responses, but also in a new function for the Hog1 MAPK required for cell survival in stationary phase. In mammals, regulation of MEF2A and MEF2C factors has been shown to be under the control of the p38 MAPK (Kyriakis and Avruch 2001; McKinsey et al., 2002). There is evidence indicating a crucial role for the p38 MAPK signalling via the MEF2 transcriptional regulators during early mammalian somite development and myotome formation (de Angelis et al., 2005; Berkes and Tapscott, 2005).

I5.2. Hog1 is part of the transcription initiation machinery at the promoters of osmostress genes

Transcription regulation of eukaryotic protein-coding genes is an orchestrated process that requires the concerted functions of multiple proteins. The requirement of these factors for the transcriptional regulation is reflected below.

Actually, recruitment of the MAPK to target promoters is mediated through physical interactions with specific transcription factors that function as anchors to chromatin. For instance, 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 (Alepuz et al., 2001). Recent evidence has shown that other MAP kinases such as Fus3 and Kss1 are also recruited to chromatin (Pokholok et al., 2006). The appearance of Hog1 at target promoters indicates that Hog1 itself might be part in the activation process (Alepuz et al., 2001; Chellapan, 2001). This possibility suggests a new dimension to gene regulation by signalling 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. However, phosphorylation of Hot1 or Msn2 and Msn4 by the MAPK seems not to be essential for gene expression. Recruitment of the active Hog1 MAPK by the Hot1 activator 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 activator. In fact, it has been reported that a critical step to induce gene expression upon stress is the recruitment of the RNA Polymerase II complex by Hog1 to the promoters (Alepuz et al., 2003). Similar to the MAPK and the transcriptional regulators, the RNA polymerase II machinery is recruited to osmoresponsive genes in response to stress and its association depends on both active Hog1 and the presence of specific transcription factors. In addition, Hog1 interacts with Pol II upon osmostress. It’s worth noting that the p38 MAPK also interacts with the core of the RNA Polymerase II in human cells (Alepuz et al., 2003), and a rapid hormonal activation of Erk and Msk kinases is required for recruitment of RNA polymerase II to the MMTV promoter (Vicent el al., 2006). These data suggest that a novel mechanism of gene transcription mediated by stress-activated MAPKs could exist among eukaryotic cells.

In addition, two major transcriptional coactivators have been identified as essential for cell viability in osmolarity conditions: 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).

I5.3. Chromatin remodeling and Hog1 MAPK

Generally, DNA is packaged into nucleosomes and thus inaccessible to the basal transcription machinery. However, during replication, transcription and repair, the respective cellular machineries have to work on chromatin as the native DNA template. To overcome the naturally repressive state of the chromatin structure, there are some complexes with chromatin remodelling/modifying activities to manipulate DNA-histone interactions (Uffenbeck and Krebs, 2006). In addition, histone-modifying complexes control access to DNA in chromatin. Histone-modifying complexes alter the state of chromatin through covalent modification, and involve the addition or removal of many different moieties, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination, and proline isomerization (Kouzarides, 2007).

The specific chromatin 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 cells osmosensitive at high osmolarity showed that the Rpd3 histone deacetylase complex play an important role in osmostress gene expression. Histone deacetylation has been traditionally associated with repression of gene expression (Robyr et al., 2002). However, more than 90% of genes induced in response to osmotic stress that are Hog1 dependent have a significant reduction in expression in a *RPD3* mutant strain. Actually, Hog1 binds and facilitate the recruitment of the Rpd3 deacetylase complex to stress promoters, leading to histone deacetylation, entry of RNA Polymerase II and induction of gene expression (de Nadal et al., 2004). An overview of transcription initiation mediated by Hog1 MAPK is depicted in Figure 7.

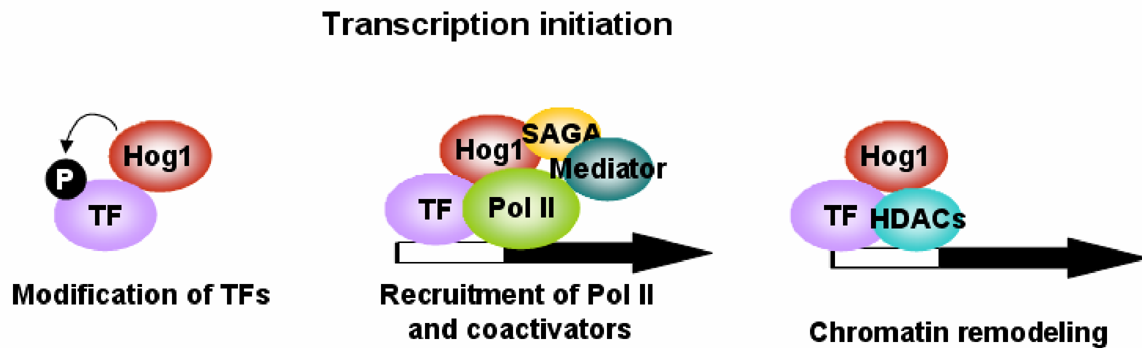

Figure 7. Hog1 regulates transcription initiation by several mechanisms upon osmostress. The role of Hog1 in transcription initiation involves several mechanisms, such as targeting transcription factors, recruitment of coactivators and Pol II to osmoresponsive promoters and chromatin remodeling.

I5.4. Hog1 MAPK and transcription elongation

Transcription is a complex process responsible for the generation of a mature mRNA molecule. The so-called transcription cycle includes preinitiation, initiation, promoter clearance, elongation and termination (Sims et al., 2004). Transcription of eukaryotic genes can be performed by three different DNA-dependent RNA polymerases: RNA polymerase I, which exclusively synthesizes ribosomal RNA genes, RNA polymerase II, used to transcribe most of protein-coding genes, and the RNA polymerase III, which is responsible for the transcription of tRNA genes, the 5S RNA genes and the snRNAs.

The RNA Polymerase 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 Rpb1, formed by multiple repeats of the heptapeptide sequence YSPTSPS. Studies using functional assays together with specific antibodies revealed the existence of hypo- and hyperphosphorylated forms of CTD. The hypophosphorylated form is recruited to promoters, whereas transcription-competent RNA Pol II is heavily phosphorylated on its CTD (Sims et al., 2004). RNA Pol II phosphorylated predominantly at Ser 5 of the

heptapeptide is associated with promoter-proximal regions of transcribed genes, whereas the amount of the enzyme phosphorylated at Ser 2 increases toward the 3’-end of genes.

In Proft et al., (2006) (see Suplementary Article), it is extensively described that Hog1 interacts with elongating RNA polymerase II and with general components of the Transcription Elongation Complex (TEC). Moreover, Hog1 is recruited to ORFs of stress genes, and this recruitment is essential for proper gene stress activation (Figure 8). The selective association of Hog1 to coding regions is mediated by the 3’ UTRs of osmoresponsive genes, and indicates that Hog1 travels through coding regions of osmostress-responsive genes. Hence, the role of Hog1 in the regulation of the transcription cycle is not limited to transcription initiation but rather extends to the process of transcriptional elongation. For detailed explanation, see Supplementary Article.

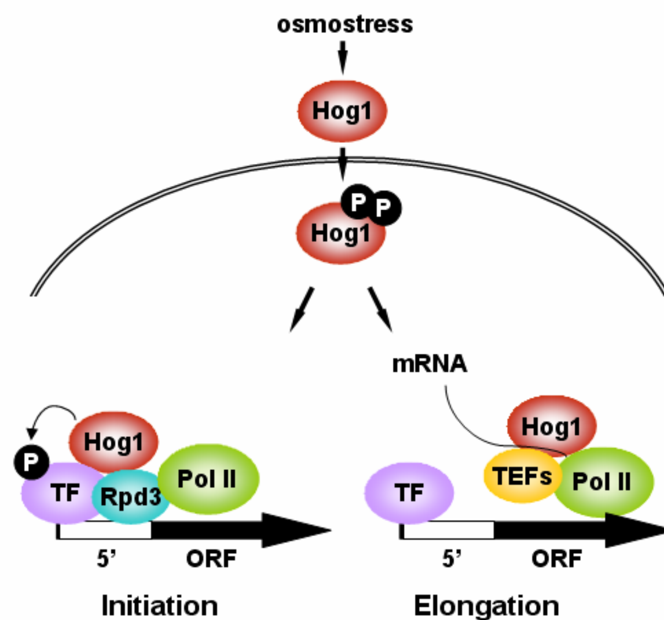

Figure 8. Activation of the Hog1 MAPK has an essential role in transcription regulation of osmostress genes. Exposure of yeast to osmolarity conditions results in activation of the SAPK Hog1, which associates with transcription factors and stimulates transcriptional initiation. Furthermore, the MAPK is recruited to the coding regions of osmostress genes behaving as a transcriptional elongator factor.

I6. MITOCHONDRIA AND ITS RELATIONSHIP WITH THE NUCLEAR TRANSCRIPTIONAL PROGRAM

I6.1. Mitochondrion: structure and organization

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 in eukaryotic cells, 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", and this function is intimately related to its structural morphology (Zhang and Qi, 2008).

From a structural perspective, the mitochondrion is unusual since it contains two membranes that separate four compartments: the outer membrane, intermembrane space, inner membrane and the matrix (McBride et al., 2006; Eisenberg et al., 2007). The inner membrane is divided into two distinct domains. One area is juxtaposed closely to the outer membrane ("inner boundary membrane"), and it makes close contacts with the outer membrane in numerous positions. The second membrane domain forms the cristae, a structure that forms lamellas or tubular structures, and that serves to increase the surface area of this membrane. Cristae houses the megadalton complexes of the electron transport chain and ATP synthase that control de basic rates of cellular aerobic metabolism.

I6.2. Mitochondria and metabolism

Despite the fact that the major respiratory pathways were elucidated decades ago, relatively little is known about their regulation and control. Respiration, a phenomenon that requires oxygen (see below), can be divided into three main pathways: glycolisis, that takes place within the

cytoplasm, mitochondrial tricarboxylic acid (TCA) cycle (also known as citric acid cycle or Krebs cycle) and mitochondrial electron transport, both of them occurring within the mitochondria. However, some organisms live in places where oxygen is not always present. Then, they use alternative ways to obtain energy. In this regard, *Sacharomyces cerevisiae* uses anaerobic respiration when glucose concentrations are high, even under aerobic conditions. This phenomenon is known as *glucose repression* or *catabolite repression* (Gancedo, 1998).

I6.2.1. Glycolisis

Since glycolisis is the common pathway used by organisms that use aerobic (respirative) and anaerobic (fermentative) metabolism, it represents the archetype of universal metabolic processes known and occurring in many types of cells in nearly all organisms.

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 ATP and NADH as cellular energy sources, and 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. Moreover, pyruvate produced during glycolisis is processed in Krebs cycle as part of aerobic metabolism.

I6.2.2. Anaerobic metabolism

In the absence of oxygen, cells make anaerobic respiration. In this case, organisms continue to carry out glycolisis, since glycolisis does not use oxygen in its chemical process. However, cells must turn NADH back to NAD^+, required for glycolisis. Then, cells oxidize pyruvate producing either

lactic acid (lactic acid fermentation) or ethanol (alcoholic fermentation). The alcoholic fermentation is the one used by yeasts and some kinds of bacteria, and converts sugars in ethyl alcohol and carbon dioxide in the cytosol.

I6.2.3. Aerobic metabolism

In cells that undergo respiration, pyruvate produced in glycolisis is oxidized to CO_2 via Krebs cycle, and the NADH produced in glycolisis and Krebs Cycle is reoxidized via the respiratory chain, with production of much additional ATP.

Krebs cycle

Pyruvate molecules are taken up into the mitochondria through specific members of the mitochondrial carrier family. Once in the matrix of mitochondrion, they are processed through the Krebs cycle. Firstly, the oxidative conversion of pyruvate into acetyl-CoA occurs. Then, acetyl-CoA enters Krebs cycle. Its main use is to convey 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 by enzymes in the inner mitochondrial membrane during the oxidative phosphorylation chain to drive more ATP synthesis.

Apart from providing energy, TCA cycle also supplies metabolic intermediates for biosynthetic process. For instance, α-ketoglutarate is the precursor of glutamate, which provides all the nitrogen used in biosynthetic reactions, and purines as well. Oxaloacetate is the precursor of some aminoacids and pirimines, and citrate generates fatty acids and sterols.

Oxidative phosphorylation chain

In this process electrons generated from NADH and $FADH_2$ 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 (Beckman and Ames, 1998; Ames et al., 2005). To avoid these processes, cells have developed a number of antioxidants defense mechanisms that include glutathione, thioredoxin, superoxide dismutase (SOD), catalase and peroxidase enzymes (Gutteridge, 1994; Valko et al., 2006).

I7. MITOCHONDRIAL SIGNALLING: THE RETROGRADE RESPONSE

The function of respiratory-competent mitochondria results from a collaboration between gene products derived from both mitochondrial and nuclear genomes (Poyton and McEwen, 1996; Chen and Butow, 2005). This data indicates that nuclear and mitochondrial genomes interact in some way. Just as the nuclear genome can affect the expression of mitochondrial genes, the mitochondrial genome can affect the expression of nuclear genes for mitochondrial proteins (Parikh et al., 1987). The molecular details that underlie this phenomenon, called retrograde response, are still being clarified. As discussed below, communication from the mitochondrion to the nucleus involves metabolic signals and some signal transduction pathways.

I7.1. The RTG-Dependent Pathway of Retrograde Signalling

The most detailed information on the retrograde response has been obtained with the budding yeast *Saccharomyces cerevisiae*. In *S. cerevisiae*, the retrograde signalling pathway functions as a homeostatic or stress response mechanism to adjust various biosynthetic and metabolic activities to the alterations in the mitochondrial state (Liao and Butow, 1993; Liu and Butow, 1999). Then, retrograde response is seen when mitochondrial respiratory function declines or is absent, for instance, in "petites" mutants, also called rho^o and ρ^o cells, that are a special class of respiratory-deficient mutants characterized by large deletions in their mtDNA or a complete lack of the mitochondrial genome.

Multiple positive and negative regulators in the retrograde response have been identified. Thus, Rtg1, Rtg2 and Rtg3 have been related to the RTG pathway activation, whereas Mks1, Bmh1 and Bmh2 have been related to the RTG pathway repression (see Figure 9).

I7.1.1. Positive regulators of the RTG pathway

Rtg1 and Rtg3 proteins are basic helix-loop-helix-leucine zipper-(bHLH/Zip) type transcription factors that heterodimerize to activate transcription. Unlike most bHLH-type transcription factors, which bind to the consensus sequence, CANNTG, called the E box (Massari and Murre, 2000), Rtg1/3 bind to an unusual site, GTCAC, termed the R box (Liao and Butow, 1993; Jia et al., 1997; Liu and Butow, 2006). Rtg3 contains transactivation domains, whereas no one has been identified in Rtg1. Activation of Rtg3 results in a change of its phosphorylation state (hyperphosphorylation or hipophosphorylation), which is influenced by strain- and nutrient- specific conditions (Komeili et al., 2000; Sekito et al., 2000; Dilova and Powers, 2006). Rtg2 is another regulator of the yeast retrograde response pathway. It has an N-terminal ATP binding motif, similar to that found in the Hsp70/actin/sugar kinase superfamily of ATP binding proteins (Koonin,

1994), whose integrity is essential for Rtg2 function (Liu et al., 2003). Although there is much to be learned about this protein, the current data suggest that Rtg2 may act as a proximal sensor of mitochondrial dysfunction by promoting nuclear accumulation of Rtg3/Rtg1 complex when the retrograde response is activated in ρ^0 cells (Sekito et al., 2000).

One key regulatory step in the regulation of the RTG pathway is nuclear translocation of Rtg1/3 proteins (Sekito et al., 2000). When the RTG pathway is off, Rtg1 and Rtg3 are sequestered together in the cytoplasm. Upon activation of the RTG pathway, the Rtg1/3 complex translocates into the nucleus, where these proteins are assembled at the R box sequences in the promoter region of target genes (Figure 9). In cells lacking Rtg2, the Rtg1/3 complex remains cytoplasmic, no longer responsive to retrograde signals, suggesting that Rtg2 dictates where the Rtg1/3 complex resides. In addition, deletion of Rtg1 results in a constitutive nuclear localization of Rtg3 even in *rtg2Δ* cells, suggesting a negative regulatory role of Rtg1 in the cytoplasm. The basic region in the bHLH domain of Rtg3 also functions as a nuclear localization signal (NLS) (Sekito et al., 2000).

Once in the nucleus, the transcriptional activation domain of the Rtg1/3 complex is located to Rtg3. Rtg3 contains both N-terminal and C-terminal transcriptional activation domains. The N-terminal transcriptional activation domain is related to a so-called activation domain 1 (AD1) (Massari et al., 1999), which is specific to type I HLH transcription factors (Quong et al., 1993; Massari et al., 1996). 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. Deletion of *CYC8* reduces RTG dependent gene expression, suggesting that besides its well-established repressor activity, Cyc8 can also function as a novel coactivator (Conlan et al., 1999).

I7.1.2. Dynamic interaction of Mks1 between Rtg2 and Bmh1/2 proteins

Genetic screen designed to identify mutants that could bypass the requirement for Rtg2 gave Mks1 protein (Sekito et al., 2002). Mks1 is a phosphoprotein that binds to Rtg2 and that negatively regulates the RTG pathway by inhibiting the nuclear translocation of Rtg1/3 complex (Dilova et al., 2002; Sekito et al., 2002; Dilova et al., 2004) (Figure 9).

When the RTG pathway is on, Mks1 is present in a largely dephosphorylated form complexed with Rtg2. When the RTG pathway is off, Mks1 becomes more phosphorylated, no longer interacts with Rtg2, and is in a complex with the 14-3-3 proteins Bmh1 and Bmh2, which function as negative regulators in the RTG pathway (Liu et al., 2003; Liu et al., 2005; Liu and Butow, 2006). The integrity of the ATP binding of Rtg2 is essential for its function, and this observation is mainly attributed to its ability to regulate the interaction with Mks1 (Liu et al., 2003).

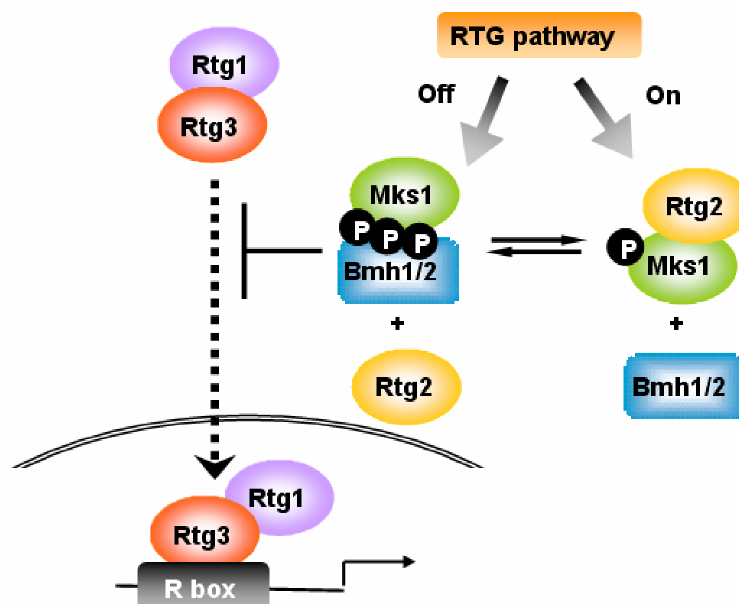

Figure 9. Regulation of *RTG*-dependent gene expression by a dynamic interaction between Rtg2p and the negative regulator Mks1p. Mks1p, when bound to Rtg2p, is inactive. Mks1p bound to the 14-3-3 proteins Bmh1p and Bmh2p is the form that prevents Rtg1p and Rtg3p from translocating from the cytoplasm to the nucleus.

I7.1.3. Genome-wide transcriptional profiling of cellular responses to mitochondrial dysfunction

Thanks to genome-wide analysis performed in rho^{o} cells it has been established that in the absence of mitochondrial DNA there is an induction of a large number of nuclear genes involved in mitochondrial biogenesis. These genes include those encoding components of the respiratory complexes (such as *CYC1*, *CYC7* and *COX5b*), assembly factors (such as *PET117*, *PET100*, *COX14*, *COX15*, *ATP11* and *ATP12*), proteins required for mitochondrial import (such as *TOM6*, *TOM20* and *TIM17*), mitochondrial ribosomal proteins, stabilization and processing of cytoplasmically synthesized mitochondrial proteins, and members of the family of mitochondrial carrier proteins. Interestingly, rho^{0} cells also induce the expression of some mitochondrial chaperones. This observation probably reflects an attempt to maintain efficient import of cytoplasmatically synthesized proteins in the impaired organelle. It's worth noting that the RTG pathway also affects mtDNA maintenance through regulation of the RTG-target gene *ACO1* (Chen et al., 2005).

It has been also described that transcripts encoding some intermediates catalyzing first steps in the TCA cycle and peroxisomal activities are elevated in rho^{o} cells (Traven et al., 2001; Epstein et al., 2001). This phenomenon ensures the production of α-ketoglutarate for glutamate biosynthesis (Figure 8). Some of these genes are *CIT1*, *ACO1*, *IDH1* and *IDH2*, which lead to the synthesis of α-ketoglutarate. *CIT2* (encoding a peroxisomal isoform of citrate synthase), which is the prototypical gene induced upon mitochondrial dysfunction and *DLD3*, (encoding a cytoplasmic isoform of D-lactate deshydrogenase) are also subjected to retrograde regulation.

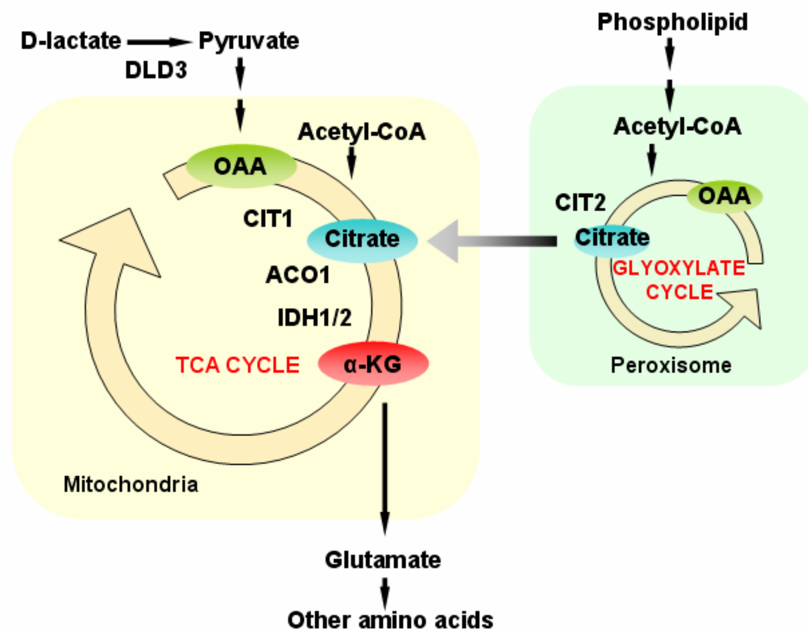

Figure 10. Metabolic reconfiguration in respiratory-deficient cells. Only genes involved in glutamate biosynthesis and anaplerotic pathways are shown. A metabolic reconfiguration of the retrograde response pathway ensures a sufficient level of alpha–ketoglutarate for glutamate synthesis to meet the demand of nitrogen for biosynthetic reactions.

Hence, mitochondrial dysfunction results in a recasting of carbohydrate and nitrogen metabolism. This metabolic reconfiguration of the retrograde pathway ensures sufficient levels of α-ketoglutarate, the direct precursor of glutamate. Glutamate, together with its downstream metabolite glutamine, provides all the nitrogen source used in biosynthetic reactions (Magasanik and Kaiser, 2002). Therefore, one function of the RTG pathway is to ensure that a sufficient level of glutamate is made to meet the demand of nitrogen supply for biosynthetic reactions in respiration-deficient cells (Figure 10). This integration of carbohydrate and nitrogen metabolism is also reflected in the finding that retrograde signalling can be activated in cells grown with urea or ammonia, which are considered poor nitrogen sources, as a sole nitrogen source (Komeili et al., 2000). However, when yeast cells use a preferred nitrogen source, including glutamine and asparagine, no activation of this pathway is observed. Remarkably, retrograde signalling can be activated in cells in which the target of rapamycin (TOR) pathway,

which is involved in nutrient sensing, is inhibited by rapamycin (Komeili et al., 2000; Shamji et al., 2000; Liu and Butow, 2006).

I7.2. Evolutionary conservation of the RTG pathway and the retrograde response in mammalian cells

Rtg2 and Mks1, two key regulatory factors in the RTG pathway, are conserved in multiple fungal species. Moreover, although there are no homolog proteins, mitochondria-to-nucleus retrograde signalling analogous to the RTG signalling pathway has also been described in mammalian cells (Amuthan et al. 2001; Butow and Avadhani, 2004; Mercy et al., 2005; Erol, 2005), and it is known as mitochondrial stress signalling. In this regard, several diseases, such as cancer and degenerative diseases have been associated with point mutations or large-scale deletions of mitochondrial DNA (Gardner et al., 2007). Partial inhibition of oxidative phosphorylation by mitochondrial gene mutations can reduce electron flux through the electron transport chain, increasing mitochondrial ROS production, and leading to DNA damage and cell dysfunction. These observations suggest that abrogation of mitochondrial stress signaling under *in vivo* diseases may offer new therapeutic intervention strategies.

OBJECTIVES

The basic objective of the research in our laboratory is to understand the mechanisms by which the stress-activated kinases regulate adaptative responses. A major response controlled by the Hog1 SAPK is the regulation of gene expression. There are several transcription factors that control gene expression upon an osmotic stress in a Hog1-dependent manner. However, the amount of genes regulated by these transcription factors is lower than the amount of genes regulated in response to osmotic stress by the Hog1 MAPK. In addition, a mutant strain carrying deletions in several of these activators is not osmosensitive. These observations suggest the action of additional transcription factors under the MAPK. Therefore, we aimed to found additional transcription factors by which Hog1 regulates gene transcription in response to osmotic stress.

The specific objectives of this PhD project were:

I. To identify novel transcription factors involved in Hog1 mediated osmostress gene expression.
II. To characterize the molecular mechanisms by which these transcription factors are regulated.

MATERIALS AND METHODS

M1. Yeast strains and media

The complete genotypes of yeast strains used in this study are listed in the following table (Table I).

Strain name	Strain genotype	Source
BY4741	MAT**a** *his3Δ1 leu2Δ met15Δ0 ura3Δ0*	EUROSCARF
BY4741*rtg1Δ*	MAT**a** *his3Δ1 leu2Δ0 met1ΔD0 ura3Δ0 rtg1Δ::kanMX4*	EUROSCARF
BY4741 *RTG1-TAP*	MAT**a** *his3Δ1 leu2Δ met15Δ0 ura3Δ RTG1-TAP-HIS3*	Open Biosystems
K699	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100*	Gustav Ammerer
YNN15	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 rtg1Δ::kanMX4*	this work
YNN21	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 RTG1-HA6-HIS3*	this work
YNN23	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 hog1Δ::kanMX4 RTG1-HA6-HIS3*	this work
YNN51	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 rtg1Δ::kanMX4 rtg3Δ::natMX4*	this work
YNN41	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 rtg2Δ::kanMX4*	this work
YNN20	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 rtg3Δ::kanMX4*	this work
YNN17	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 hog1Δ::kanMX4*	this work
YNN44	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 RTG3-HA6-HIS3*	this work
YNN45	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 RTG3-HA6-HIS3 hog1Δ::kanMX4*	this work
YNN56	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 RTG3-HA6-HIS3 rtg1Δ::kanMX4*	this work

Table I (continued)		
YNN42	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 HOG1-HA6-HIS3*	this work
YNN33	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 rtg1Δ::kanMX4 HOG1-HA6-HIS3*	this work
YNN25	*MAT***a** *leu2-3,112 ura3-1 his3-11 trp1-1 can1-100 rtg1Δ::kanMX4 hog1Δ::TRP1*	this work

YPD medium contains 10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose. MD-media contained 0.7% yeast nitrogen base, pH 5.5, 2% dextrose. Nitrogen sources (glutamine or ammonia) were added 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 (Sherman F, 1991; Dilova et al., 2004). Yeast cultures were grown at 30ºC for all experiments. Yeast transformations were performed using a DMSO-enhanced lithium acetate procedure (Hill et al., 1991).

M2. Plasmids

Full length *RTG1*, *RTG2* and *RTG3* coding regions were PCR amplified from genomic DNA using the oligonucleotides 5′-TCGCGGATCCAT GAGCAGCATTCCAGCTGG-3′ and 5′-TCGCGGATCCTTAGCTACCATTACCGTAC-3′ (Rtg1), 5′-CGCGGATCCATGTCAACACTTAGCGATAG-3′ and 5′-CGCGGATC CTTATTCTTCATAAAATTGCA-3′ (Rtg2), 5′-CGCGGATCCATGAACAATAACGA AAGTGA-3′ and 5′-CGCGGATCCCTACCCCGAACCAAATTCTA-3′ (Rtg3), 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-Rtg1-T60A and pGEX-4T-Rtg3-T197A plasmids were generated by PCR amplification with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) from the pGEX-4T plasmids containing the wild-type proteins.

Oligonucleotides used for site-directed mutagenesis are 5′-GTTAAGTGAATCCGCTCCCGGTGCGCTTG-3′ and 5′-CAAGCGCACCGGGAGCGG ATTCACTTAAC-3′ (Rtg1-T60A) and 5′-ATTCTGATATGATGGCACCAAAC ACATATT-3′ and 5′-AATATGTGTTTGGTGCC ATCATATC AGAAT-3′ (Rtg3-T197A). The mutant alleles of *RTG3* comprising amino acids 1-210 (Rtg3M1) and 1-183 (Rtg3M2) were generated by digestion of the pGEX-4T-Rtg3 construct with HindIII/EcoR1 (M1 fragment) and SacI/EcoRI (M2 fragment), treatment with Klenow and T4 DNA Polymerase, respectively, and religation. The mutant allele of *RTG3* comprising amino acids 211-387 (Rtg3M3) was generated by PCR using the oligonucleotides 5′-CGCGGATCCATGAACAATAACGAAAGTGA-3′ and 5′-CGCGGATCCCTACCCCGA ACTGGCATGGTGGT-3′ and cloned into the BamHI site of the pGEX-4T plasmid. pGEX-4T plasmid containing the constitutively activated version of Pbs2, (Pbs2[EE]), was provided by the laboratory. For the GST-fused constructs expressed in yeast, *RTG1* ORF and *RTG1* ORF containing the T60A mutation were liberated from the pGEX-4T constructs previously described and cloned into the *GST* ORF in the BamHI site of the multicopy vector pRS426-TEG (Posas and Saito, 1998). GST fusion proteins are constitutively expressed from the strong promoter P_{TEF} in pRS426-TEG. pRS426-GST-Hog1 and pRS426-GST-Hog1as constructs were provided by the laboratory.

GFP-fused constructs were generated by amplifying *RTG1*, *RTG2* and *RTG3* coding plus promoter regions (~ -800) from genomic DNA using the oligonucleotides 5′-TCCGCTCGAGGCGAGGCTGAAATGTACACA-3′ and 5′-TCCGCTCGAGCGCTACCATTACCGTACTCAC-3′ (Rtg1), 5′- TCCGCTCGAGAC CCTCCTCAATCAAGCC-3′ and 5′-TCCGCTCGAGCTTCTTCATAAAATTGCAC-3′ (Rtg2), and 5′-TCCGCTCGAGGTCCTGTCTAGATACAGGCA-3′ and 5′-TCCGCTCGAGCCCCCG AACCAAATTCTAAAAG-3′ (Rtg3), and cloned into the XhoI site of the pRS416-GFP construct (Raitt et al., 2000). The Rtg1-T60A-GFP mutant was generated by PCR using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) from the pRS416-Rtg1-GFP plasmid. Oligonucleotides used for site-directed mutagenesis are 5′-

GTTAAGTGAATCCGCTCCCGGTGCGCTTG-3′ and 5′-CAAGCGCACCGGGAGCG GATTCACTTAAC-3′. 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. 5′-CGCGGATCCGCGAGGCTGAAATGTACACA-3′ and 5′-TCGCGGATCCTT AGCTACCATTACCGTAC-3′ (Rtg1) and 5′-CGCGGATCCGTCCTGTCTAGATACAG GCA-3′ and 5′-CGCGGATCCCTACCCCGAACCAAATTCTA-3′ oligonucleotides were used. Mutated alleles described in Results section were generated as before. Then, HA 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 and the oligonucleotides 5′-AGCTCCTTCGAGTACGGAGGGTATGGTGAGTACGGTAATGGTAGCTCCGGTTCTG CTAG-3′ and 5′-GGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGT AACGCCTCGAGGCCAGAAGAC-3′ (Rtg1), and 5′-GATGACAACTCTAATCCAGC TGACTATCTTTTAGAATTTGGTTCGGGGTCCGGTTCTGCTAG-3′ and 5′-GGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCTCGAGGCCA GAAGAC-3′ (Rtg3). HA-fused constructs into the pRS414 were generated as before. YcpLac111-Hog1-HA and YcpLac111-Hog1KNN-HA vectors were provided by the laboratory.

M3. Genome-wide genetic screening

An ordered array of ~ 1000 MAT**a** viable haploid yeast gene deletion mutants (Saccharomyces Gene Deletion Project, obtained from EUROSCARF) for genes encoding proteins related to transcriptional processes (gene function was obtained from the Yeast Protein Dabatase (SGD)) was used. These mutants were crossed with strains containing the integrated osmoresponsive-promoters fused to the *LACZ* reporter gene that are indicated in Table II. After that, haploid cells containing both the deletions and the reporter genes were selected using specific selectable markers, and replica pinned onto 0.1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) yeast extract-peptone-dextrose (YPD) plates

plus 0.4 M NaCl. X-gal dye is a substrate for β-galactosidase, which is encoded by the *LACZ* gene, and turns blue when hydrolyzed by this enzyme. Thus, those mutants that were not able to activate transcription of the reporter gene (white colonies) were selected. The screen was performed by using an automated system. For automated array, yeast cells were transferred using the Biomek FX robot and a 384-floating-pin replicator (Biomek FX HDR 384-pin plate) as described previously (Tong et al., 2001). The screen was performed two times, and plates were incubated at 30ºC for 3 days before scoring.

Reporter	SGD
STL1	Member of the hexose transporter family
PUT4	High affinity proline permease, also transports alanine and glycine
YGR052 W	Serine/threonine protein kinase of unknown function
ALD3	Cytoplasmic, stress inducible aldehide deshydrogenase
SPI1	Protein induced in stationary phase, has similarity to Sed1p
PGM2	Phosphoglucomutase, major isozyme, interconverts Glc-1-P to Glc-6-P
PHM7	Protein of unknown function, transcriptionally regulated by phosphate
PNS1	Mitochondrial protein of unknown function
HXT1	Low affinity hexose transporter
ARO9	Aromatic amino acid aminotransferase II
CWP1	Mannoprotein of the cell wall, member of the seripauperin family

Table II. Integrated *LACZ* osmoresponsive-reporters tested on the genome-wide screening. On the right there is a brief description of the gene function from the Yeast Protein Database (SGD).

M4. GFP fluorescence microscopy

To determine the subcellular localization of proteins, GFP-fused constructs were generated using the yeast centromeric vector pRS416-green fluorescence protein (GFP) (Raitt et al., 2000). These constructs were introduced into the indicated strains. A background fluorescence control was performed with the corresponding strain transformed with the empty vector. Exponential growing cells (0.5-0.8 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).

M5. Total protein extraction

Cells were grown to exponential phase (0.5-0.8 OD_{660}), subjected or not to an osmotic shock (0.4 M NaCl), harvested by spinning at 3000 RPM for 3 min and frozen at -80ºC. Then, pellets were resuspended in ice-cold Buffer A (50 mM Tris-HCl pH 7.5, 15 mM EDTA, 15 mM EGTA, 0.1% Triton X-100, 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 1mM benzamidine, 5 µg/ml pepstatine, 5 µg/ml leupeptin and, when necessary, phosphatase inhibitor mixture, containing 1 mM orthovanadate, 10 mM NaF, 1 mM sodium pyrophosphate and 25 mM β-glycerophosphate) supplemented or not with 150 mM NaCl, and mixed with an equal volume of Ø 0.40-0.60 mm glass beads (Sartorius). The tubes were vigorously shaken in a bead beater (FastPrep-24; MP Biomedicals) by using 60 s mixing at a speed of 5. Tubes were centrifugated at maximum speed in a microcentrifugue for 10 min at 4ºC, and supernatant, which represents crude extracts, was transferred to a new 1.5 ml microcentrifugue tube. Protein concentration was determined by Bradford analysis (Bio-Rad Protein Assay). When necessary, yeast extracts were treated for 2h at 37ºC with 1 µl phosphatise alkaline (20U/µl; Roche). 50 µg of total protein extracts were loaded onto SDS-polyacrylamide gels. Immunoblots were developed as described above.

For trichloroacetic acid (TCA) protein extraction, yeast cultures were grown to mid-log phase (0.5-0.8 OD_{660}). When necessary, cells were either subjected to stress (0.4 M NaCl, indicated times) or untreated. 1 ml of culture per sample was collected and mixed with 300 l of TCA 85%. Cells were collected by 1 min of centrifugation at 13200 RPM, and supernatant was removed. Then, cells were resuspended in 100 l of TCA loading buffer (20% SB 5X, 20% Tris 1.5 M pH 8.8) and 300 l of Ø 0.40-0.60 mm glass beads (Sartorius) were added. The tubes were vigorously shaken in a bead beater (FastPrep-24; MP Biomedicals) by using 60 s mixing at a speed of 5. Samples were boiled 10 minutes at 100°C, and 20 l of supernatant were loaded onto SDS-polyacrylamide gels. Immunoblots were developed as described above.

M6. Western Blot Analyses

Proteins were separated on SDS-15% polyacrylamide gel electrophoresis (PAGE) (Rtg1-HA) or SDS-10% polyacrylamide gel electrophoresis (Rtg3-HA) and transferred to an Immobilon-P membrane (Millipore). The blots were blocked in TBST (Tris-buffered saline and Tween 20) containing 5% powdered milk for 30 min and then incubated overnight in 5% milk/TBST with the appropriate antibody (Ab). Rtg1-HA and Rtg3-HA fusions were detected by anti-HA monoclonal antibody 12CA5 (Roche, 1:10000). Pbs2 protein was detected by anti-Pbs2 (Santa Cruz Biotechnology), and GST fusion proteins by anti-GST monoclonal antibody (Pharmacia, 1:5000). Blots were then washed tree times in TBST and incubated with the corresponding horseradish peroxidise-conjugated Abs (donkey anti-rabbit IgG, from GE Healhtcare, in the case of the primary antibody anti-Pbs2; sheep anti-mouse IgG, from GE Healhtcare, in the case of anti-HA and anti-GST primary antibodies). Bound Abs were detected with enhanced chemiluminiscence (ECL) (GE Healhtcare). To reuse Western blots by gently stripping antibodies from proteins, membranes were incubated with a solution containing 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl pH 6.7

and SDS 2% for 30 min at 55ºC. After that, blots were raised several times in water and TBST, being ready for re-blocking and blotting again.

M7. *In vitro* kinase assays

M7.1 Expression and purification of GST proteins in *E. coli*

GST fusion proteins encoding Pbs2[EE], Hog1, and Rtg1/2/3 proteins were constructed by using pGEX-4T (Pharmacia). The Escherichia coli strain DH5α was transformed with these plasmids and cells were grow overnight at 37ºC into 25 ml of LB plus 0.1mg/ml ampicillin. Then, cells were inoculated into 500 ml LB plus ampicillin and grown until 0.4 OD_{600}. Protein expression was induced with 1mM IPTG for 5 hours at 25ºC. Cells were harvested by spinning at 6000 RPM for 10 minutes and frozen at -80ºC. Pellets were resuspended in 10 ml STET buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1mM EDTA, 5% Triton X-100) plus 0.1% β-mercaptoethanol and antiproteases, and lysated by sonication on ice at 30% amplitude for 30 seconds (x3) and at 40% amplitude for 30 seconds (Branson Digital Sonifier Model S-250D). Lysates were harvested by spinning at 6000 RPM for 10 minutes, and supernatant was collected and divided into four parts. One of these parts was incubated with glutathione-Sepharose beads (Pharmacia) for 45 minutes at 4ºC. Later on, beads were washed 4 times with STET buffer and 2 times with Tris/DTT buffer (50 mM Tris-HCl pH 8.0, 2mM DTT), and tagged proteins were eluted in 300 µl with elution buffer (glutathione 10mM, Tris-HCl 50mM pH 9.0, DTT 2mM, pH 7.5). The purified proteins were then quantified by loading onto SDS-polyacrylamide gel electrophoresis (PAGE) with known concentrations of bobine serum albumin (BSA), and visualized by comassie blue staining (7% acetic glacial, 35% methanol, 0.25% comassie). Gel destaining was performed with a mixture containing 7% acetic glacial and 35% methanol.

M7.2 *In vitro* phosphorylation assays

GST fusion proteins were constructed by using pGEX-4T (Pharmacia) and expressed and purified in *E. coli.* Then, *in vitro* phosphorylation assays of Rtg1/2/3 proteins 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 $MgCl_2$ and 2 mM DTT) and 50 M 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-^{32}P]-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.

M8. *In vivo* phosphorylation assays

Cells containing the RTG1 fused to haemagglutinin (HA) epitope were grown to mid-log phase (0.5-0.8 OD_{660}), subjected to osmotic stress treatment (0.4 M NaCl, 5 min) for the indicated times and harvested by centrifugation. Yeast extracts preparation were performed as described before. 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. Total crude extracts were loaded onto SDS-15% polyacrylamide gel, and the Rtg1-HA fusion protein was detected by immunoblotting using an anti-HA monoclonal antibody 12CA5 (Roche).

M9. *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).

M10. Northern blot analysis

Yeast cultures were grown to early log phase (0.5-0.8 OD_{660}). When necessary, cells were either subjected to stress (0.4 M NaCl, indicated times) or untreated. 20 ml of culture per sample was collected by 3 min of centrifugation at 13200 RPM. Supernatant was removed. Samples were resuspended in 400 µl of AE buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA pH 8.0) plus 40 µl of SDS 10% and 300 µl of phenol (Sigma). Immediately, samples were boiled for 5 min at 65°C. Then, samples were put on ice for 1 min, and 300 µl of chloroform were added. After 2 min of centrifugation at 13200 RPM, the aqueous phase was recovered. Then, 400 µl of chloroform were added to the aqueous phase. After 2 min of centrifugation at 13200 RPM, the aqueous phase was recovered again. Then, 40 µl of sodium acetate 3 M and 1 ml of ethanol were added to the aqueous phase. The RNA was extracted by precipitation for 2 h at -20°C and a centrifugation for 20 min at 13200 RPM at -4ºC. The pellet was washed with ethanol 70%, centrifuged 5 min at 13200 RPM, and resuspended in 50 µl of H_20 DEPC. 20µg of total RNA per sample were run in a 1% agarose gel 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 were probed by using radiolabeled PCR fragments containing the ORF region of *CIT2* (910 bp), *DLD3* (720 bp), *STL1* (1.7 kbp), *CTT1* (1.7 kbp) or the noncoding exon of

RDN18-1 (1.8 kbp) as a loading control. Signals were quantified by using a storage phosphor screen, a Typhoon 8600 phosphorimager.

M11. Chromatin immunoprecipitation

Yeast cultures were grown to early log phase (0.5-0.8 OD_{660}) before aliquots of the culture were exposed to osmotic stress treatment (0.4 MNaCl) for the indicated times. For crosslinking, yeast cells were treated with 1% formaldehyde for 20 min at room temperature. Reaction was quenched by the addition of 125 mM glycine for 15 min at room temperature, and cells were washed with TBS 1X (20 mM Tris-HCl pH 7.5 and 150 mM NaCl) and frozen at -20ºC. Then, pellets were resuspended in 300 µL of lysis buffer (50 mM HepesKOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deocycholate, 1mM PMSF, 10 µg/ml aprotinin, 1µg/ml leupeptin, 1µg/ml pepstatin and 1 mM benzamidine) and 300 µL of Ø 0.40-0.60 mm glass beads (Sartorius) were added. Cells were broken by vortexing for 13 min at 4ºC (Vortex-GENIE2, Scientific Industries). Extracts were transferred to a new 1.5 ml microcentrifugue tube, sonicated on ice at 20% amplitude for 1 minute and 30 seconds (Branson Digital Sonifier Model S-250D) and harvested by spinning at 3000 RPM for 2 min at 4ºC. At this time, 10 µl of extracts were taken as whole cell extract (WCE) samples. 50 µl of DynaBeads Protein G (Dynal Biotech, Invitrogen), previously blocked with PBS/BSA 5mg/ml and incubated with 4 µg of anti-HA antibody overnight at 4ºC, were added to samples. Then, samples were incubated for 2 h while rotating at 4ºC, and washed with lysis buffer (x2), lysis buffer plus 360 mM NaCl (x2), washing buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deocycholate and 1 mM EDTA) (x2), and once with TE 1X (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). Samples were eluted with elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% SDS) and crosslinkings were reversed by placing eppendorfs at 65ºC overnight. After that, proteins were digested with Proteinase K (15 µl of 7 mg/ml) and Glycogen (6 µL of 20 mg/ml) for 2 h at 37ºC, and eliminated using phenol:chloroform extraction. DNA was precipitated with 4 µl NaCl 5M/100

µl sample plus one volume of isopropanol for 1 h at -20ºC, harvested and resuspended in TE 1X. Quantitative PCR analysis of osmoresponsive genes and constitutively expressed genes were performed. The following primers, with locations indicated by the distance from the respect ATG initiation codon, were used:

Oligo name	Sequence	Position
CIT2-1	CAAATCCGTCCTTGGGTCAT	-460
CIT2-2	AACGTCGAGAATAATGGGCC	-141
DLD3-1	CGGGATATTGTCCACAGTCA	-253
DLD3-2	TGACTTTAGGGTATGCCTCG	+46
TEL-2a	AGTGCAAGCGTAACAAAGCC	269480 (coordinates of the right arm of chromosome VI)
TEL-2b	GCCTCACTGGTTTTTACCCT	269970 (coordinates of the right arm of chromosome VI)

M12. Cell viability assays

Yeast cells were grown to an optical density of 0.5-0.8 and diluted to a 0.05 OD_{660}. Serial dilutions of cultures were spotted directly onto MD plates containing glutamine and the indicated concentrations of salt and sorbitol, and incubated at 30ºC. Growth was assayed after 2-5 days.

M13. Mass spectrometry experiments

Exponential growing wild-type and hog1Δ cells transformed with the pRS426-GST-Rtg1 plasmid were untreated or treated by osmostress (5 min, 0.4 M NaCl), and collected by centrifugation. Yeast extracts in a mixture of buffer A and phosphatase inhibitors were prepared and incubated with glutathione-Sepharose beads (Pharmacia) for 2 h at 4ºC. Beads were

washed extensively with buffer A, resuspended in loading buffer, and separated on SDS-10% polyacrylamide gel electrophoresis. After that, protein detection was done by silver staining. Then, gel was fixed in 40% EtOH and 10% HAc for 1 hour, washed in 30% EtOH twice for 20 min and once in water for 20 min, and sensitize in 0.02% $Na_2S_2O_3$ for 1 min. After that, gel was washed in water 3 times for 20 min. Silver reaction was done by incubation in cold 0.1% $AgNO_3$ for 20 min at 4ºC, and then gel was washed in water 3 times for 20 seconds. Developing was done by incubating gel with 3% Na_2CO_3 and 0.05% formalin. After that, gel was washed in water for 20 seconds, and staining was stopped by adding 5% hydrogen acetate (HAc) for 5 min. Successive washings were done in water and gel was leaved in 1% HAc. Excised bands containing the Rtg1-GST fusion protein were sent to the Mass Spectrometry Facility of the Max F. Perutz Laboratories in Vienna where potential phosphorylation sites were analyzed.

M14. Microarray analysis

400 ml of logarithmically growing wild-type, rtg1Δ and hog1Δ cells were untreated or treated by osmostress (10 min, 0.4 M NaCl), in triplicate, collected by centrifugation, and resuspended in 10 ml of sodium acetate buffer (50 mM sodium acetate, 10mM EDTA, adjusted to pH 5.0 with acetic acid). Then, 1.5 ml of glass beads (Sartorius), 1 ml of 10% SDS and 12 ml of hot phenol were added to the cell suspension and immediately transferred to a water bath shaker at maximum speed for 4 min at 65ºC. Samples were then centrifugated at 4000 RPM for 10 min. The lower organic phase was removed, 12 ml of hot phenol were added to the remaining aqueous phase and the extraction procedure was repeated as described above. After that, 10 ml of chloropane (50% liquefied phenol, 50% clorophorm, 0.8% 8-hydroxychinoline, equilibrated with ANE buffer (ANE buffer: 10 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, pH 6.0) were added. Samples were vortexed for 2 min and the two phases were separated by centrifugation. The aqueous phase was transferred to a new tube and extracted once with 10 ml of chloroform-isoamyl alcohol (24:1).

After phase separation, the upper aqueous phase was transferred to a new tube and 1ml of 3 M sodium acetate pH 5.3 and 30 ml of ethanol were added. RNA was precipitated at -20ºC overnight. The precipitated RNA was centrifugated at 3000 RPM for 20 min, and the pellets were washed with 70% ethanol. After centrifugation, pellets were dissolved in 1200 µl of DEPC-treated. One aliquot of 200 µl was sent to the Microarrays Unit of the Centre de Regulació Gènica(CRG), where two-colour microarray hybridizations were carried out using a yeast oligo microarray kit (V2) (Agilent Technologies).

RESULTS

R1. A genome-wide genetic screening reveals Rtg1 as a transcription factor required for gene expression upon osmotic stress

The ability of cells to survive in high osmolarity depends on the HOG signalling pathway and the control of gene expression exerted by the Hog1 MAPK (see Introduction). Markedly, five transcription factors have been reported to be controlled by the Hog1 MAPK: the redundant zinc finger proteins Msn2 and Msn4, the Hot1 protein, the bZIP protein Sko1 and the MADS box protein Smp1. Each factor seems to be controlling a small subset of the osmostress inducible genes. Actually, deletion of a particular transcription factor has a very limited effect on general osmostress cell survival. In fact, a strain deleted for various of these transcription factors is still able to grow under osmotic conditions (Rep et al., 1999a). These observations strongly suggest the possibility that additional transcription factors are participating in the regulation of gene expression upon osmotic stress. To identify novel transcription factors involved in Hog1-mediated gene expression systematically, we performed an exhaustive genome-wide genetic screening searching for mutations that rendered cells unable to activate transcription of osmoresponsive genes.

The screening consisted in monitoring several transcriptional readouts by fusing a set of osmoresponsive-promoters (represented in Table 1) to the *LACZ* reporter gene and integrated into the yeast genome. Then, we analyzed the ability to activate transcription of ~ 1000 haploid deletion mutants related to transcriptional processes and containing the reporter genes. These mutants were automatically pinned onto X-gal rich medium plates plus 0.4 M NaCl (described in Material and Methods). Yeast mutants that were unable to produce β-galactosidase were selected (Figure 1). The screen was performed in duplicate and a total of 149 mutants were scored as transcription deficient mutants. An evidence that the screen was highly reliable is indicated by the repetitive identification of some mutants in upstream components of the HOG pathway, as such *PBS2* and *HOG1*, or

some elements defined previously as transcriptional regulators of osmostress-responsive genes. For instance, we identified members of the Rpd3 histone deacetylase complex and the SAGA and Mediator complexes, which are essential for the transcriptional activation upon osmostress (de Nadal et al., 2004; Zapater et al., 2007). Interestingly, the genetic screening yielded a novel transcription factor, the Rtg1 protein, not related previously to the osmostress response. A mutant strain deleted for the *RTG1* gene was not unable to activate transcription of the osmoresponsive promoters indicated in Table I. Thus, we studied the role of *RTG1* on the osmostress-induced gene expression.

Reporter	SGD
STL1	Member of the hexose transporter family
PUT4	High affinity proline permease, also transports alanine and glycine
YGR052W	Serine/threonine protein kinase of unknown function
ALD3	Cytoplasmic, stress inducible aldehide deshydrogenase
SPI1	Protein induced in stationary phase, has similarity to Sed1p
PGM2	Phosphoglucomutase, major isozyme, interconverts Glc-1-P to Glc-6-P
PHM7	Protein of unknown function, transcriptionally regulated by phosphate
PNS1	Mitochondrial protein of unknown function
HXT1	Low affinity hexose transporter
ARO9	Aromatic amino acid aminotransferase II
CWP1	Mannoprotein of the cell wall, member of the seripauperin family

Table I. Integrated osmoresponsive-promoters tested on the genome-wide screening. Osmoresponsive promoters were fused to the *LACZ* reporter gene. On the right there is a brief description of the gene function from the Yeast Protein Database (SGD). Bold letters refer to the reporters that were transcription deficient in the *rtg1Δ* strain.

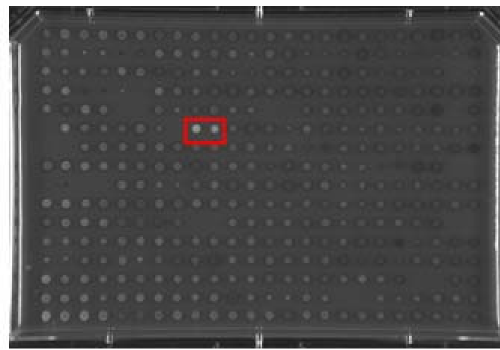

Figure 1. The screening yielded 149 mutant strains unable to induce proper gene expression on osmolarity plates (0.4M NaCl). Several osmoresponsive-promoters fused to the *LACZ* reporter gene were integrated into mutant strains deleted for genes related to transcription. β-galactosidase activity was measured qualitatively by plating cells on X-Gal plates plus salt. White colonies (red box) are mutants unable to activate transcription.

R2. The K699 strain is a good model to study the role of Rtg1 in response to osmostress

Rtg1 and its partner Rtg3 protein are transcription factors with a regulated nuclear transport. It has been described that the Rtg1/3 complex senses nitrogen quality, and localizes to the cytoplasm when cells are grown in glutamine, a preferred nitrogen source. In contrast, both proteins concentrate in the nucleus when cells are grown in the presence of ammonium, a poor nitrogen source (Komeili et al., 2000). In addition, the Rtg1/3 complex concentrates in the nucleus when cells are exposed to rapamycin, an inhibitor of the TOR kinase signalling pathway (Komeili et al., 2000) (see Introduction).

It has been shown some strain-specific differences during RTG pathway activation caused by specific mutations in *RTG3* (Dilova and Powers, 2006). Thus, we tested the activation of the RTG pathway in the K699 strain by following the subcellular localization of Rtg1 on several nitrogen sources. We constructed a plasmid encoding in-frame fusion protein between the C terminus of Rtg1 expressed from its native promoter and the GFP (green fluorescence protein). When K699 cells were grown in MD-glutamine (Gln),

Rtg1-GFP fusion protein was predominant in the cytoplasm. However, when cells were grown in MD-ammonium (NH_4^+), Rtg1-GFP fusion protein was concentrated predominantly in the nucleus (Figure 2). Additionally, the Rtg1-GFP fusion protein appeared predominantly nuclear when cells were treated with rapamycin (Figure 2, right panels). In contrast, BY4741 strain displayed a constitutive nuclear localization of Rtg1 in any growth condition (data not shown). Together, these results indicate that the K699 strain maintains the RTG pathway inactivated in the presence of a rich nitrogen source, whereas activation of the RTG pathway is observed when cells are both grown upon a poor nitrogen source or treated with rapamycin. Thus, we used the K699 strain and the MD-Gln as growth medium to study the role of the Rtg1/3 complex upon osmostress.

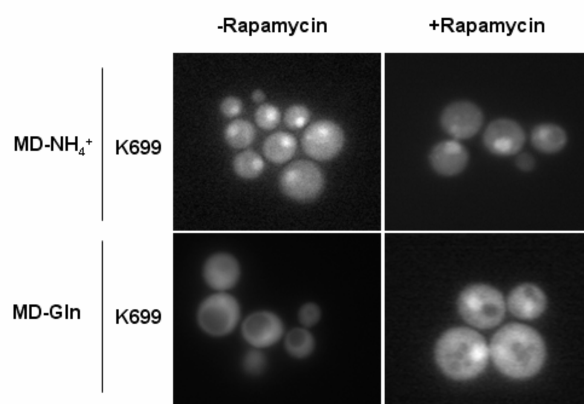

Figure 2. **Rtg1 concentrates in the nucleus in the presence of ammonium or rapamycin**. K699 cells expressing Rtg1-GFP protein were grown to 0.5-0.8 OD_{660} under different mediums. Localization of the GFP fusion protein was determined by fluorescence microscopy as described in Materials and Methods. Rtg1 localizes in the cytoplasm in cells grown in MD-Gln (left bottom panel). Nuclear localization of Rtg1 is observed in MD-$NH4^+$ medium (left upper panel) and rapamycin treatment (1µg/ml for 5 min) (right panels).

R3. The Hog1 MAPK is required for proper gene expression of RTG-pathway dependent genes in response to osmostress

Genome-wide genetic screening showed that cells deleted for *RTG1* were transcription deficient in several of the osmostress reporter genes. To further explore the role of the Rtg1 transcription factor in regulation of gene

expression upon osmostress, we analyzed gene expression in wild type cells and in cells deleted for the *RTG1* gene. The fact that *CIT2* and *DLD3* genes were known to be regulated by Rtg1 under mitochondrial dysfunction and nitrogen source (see Introduction) prompted us to test the expression of these genes in response to osmostress. Yeast cells were subjected to osmotic shock (0.4M NaCl at the indicated times) and *CIT2* and *DLD3* mRNA levels were analyzed by Northern blot. As shown in Figure 3A, yeast cells induced expression of *CIT2* upon an osmotic stress, whereas no transcription of this gene was observed in *rtg1Δ* cells. Similar results were obtained for *DLD3*. Thus, these results strongly indicate the importance of Rtg1 transcription factor for gene induction upon osmostress.

Since the Hog1 MAP kinase plays a crucial role in the response to osmostress by regulating the activity of specific transcription factors (e.g. Sko1 and Smp1), we tested whether the induction of *CIT2* and *DLD3* was dependent on the Hog1 MAPK. As depicted in Figure 3A, expression of *CIT2* and *DLD3* genes was strongly reduced upon stress in a *hog1Δ* strain. To analyze the role of Hog1 kinase activity in this transcriptional response, we transformed a *hog1Δ* strain with an empty vector, a vector expressing the wild-type Hog1 protein or a vector expressing a catalytically inactive Hog1 enzyme, named Hog1-K/N. This mutant contains the Lys52-Asn mutation in the ATP binding site, and is not able to phosphorylate its substrates (Wurgler-Murphy SM. et al., 1997). Figure 3B shows that cells containing the Hog1-K/N were not able to activate transcription of *CIT2*. Thus, the Hog1 catalytic activity is essential for the induction of *CIT2* gene expression upon osmostress. Furthermore, because the RTG pathway is composed by additional components besides Rtg1, we tested the requirement of them for the activation of gene expression upon stress conditions. As depicted in Figure 3C, both Rtg2 and Rtg3 proteins were required for gene expression, indicating that the integrity of the RTG-pathway is required for gene expression upon osmostress.

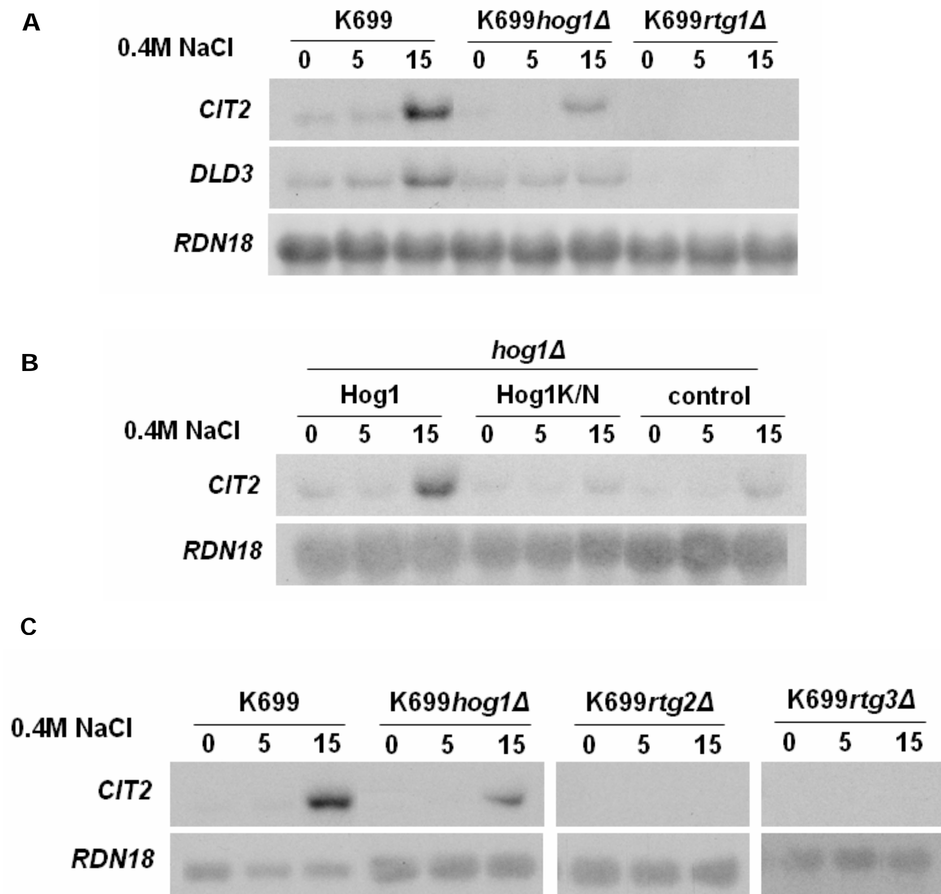

Figure 3. The Hog1 MAPK is required for a properly transcriptional induction of RTG-dependent target genes in response to osmostress. (A) Rtg1 and the Hog1 MAPK are required for the induction of *CIT2* and *DLD3* in response to osmostress. Indicated yeast strains were grown on MD-Gln to 0.5-0.8 OD_{660} and treated with 0.4M NaCl at the indicated times. Total RNA was analyzed by northern blot analysis for transcript levels of *CIT2*, *DLD3* and *RDN18* as a loading control. (B) The catalytic activity of Hog1 is needed for the transcriptional induction of *CIT2*. Cells deleted for *HOG1* were transformed with the empty vector YCPLac11 (control), the wild-type Hog1 or the Hog1 mutant allele in ATP binding site (Hog1K/N). Cells were grown and treated as before. Total RNA was assayed by northern blot analysis for transcript levels of *CIT2* and *RDN18*. (C) The integrity of the RTG pathway is required for gene expression upon osmostress. Indicated yeast strains were grown and treated with salt. Total RNA was assayed by northern blot analysis as before.

R4. Nuclear localization of Rtg1 and Rtg3 proteins is regulated by the Hog1 MAPK upon osmostress

The RTG pathway is regulated by the dynamic localization of the Rtg1 transcription factor and its partner Rtg3 from the cytoplasm to the nucleus (see Introduction). Thus, we decided to investigate the subcellular

localization of several components of this pathway upon osmostress. We constructed plasmids containing in-frame GFP fusion protein at the C terminus of full-length Rtg1 and Rtg3 proteins under their native promoters. Plasmids were transformed into wild-type and *HOG1* deleted cells. As expected, Rtg1-GFP and Rtg3-GFP were present in the cytoplasm under non stress conditions, whereas they showed a predominant nuclear localization when cells were subjected to a brief osmotic shock (Figure4A and 4B, upper panels). Thus, one level of control of the Rtg1-Rtg3 transcriptional complex upon an osmotic stress is the regulation of the subcellular localization of the complex. To test whether the subcellular localization of the Rtg1-Rtg3 complex was dependent on Hog1, we repeated the above experiments expressing Rtg1-GFP and Rtg3-GFP fusion proteins in a *hog1Δ* background. As depicted in Figure 4A and 4B (bottom panels), the nuclear accumulation of Rtg1 and Rtg3 was abolished upon osmotic stress in *hog1Δ* cells, indicating that the Hog1 protein is regulating the subcellular localization of these proteins upon stress.

The third member of the RTG family, *RTG2*, encodes a cytoplasmic protein that contains an HSP70-like ATP binding protein domain. The current data suggest that Rtg2 may act as a proximal sensor of mitochondrial dysfunction (see Introduction). To investigate the subcellular localization of Rtg2 upon osmostress, we constructed a plasmid encoding an Rtg2-GFP fusion protein as before. As shown in Figure 4C, no translocation of Rtg2-GFP protein to the nucleus was observed when cells were subjected to a brief osmotic stress. These results are consistent with those that showed that Rtg2 does not change its localization upon RTG pathway activation (see Introduction).

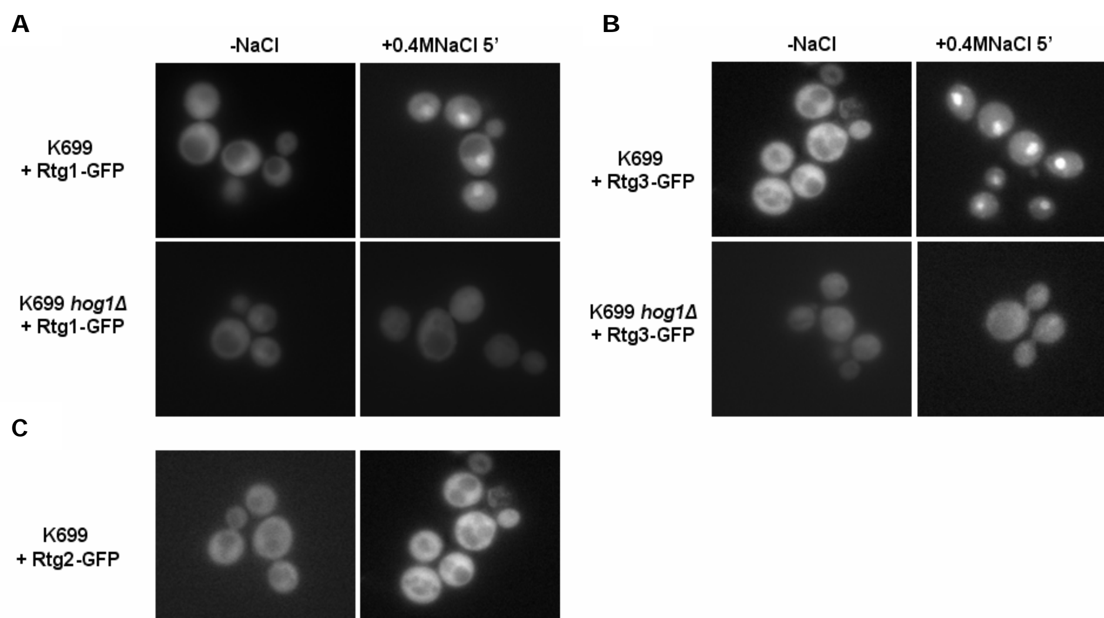

Figure 4. Rtg1 and Rtg3 proteins localize into the nucleus upon stress in a Hog1 dependent manner. Rtg1 (A) and Rtg3 (B) proteins localize in the nucleus under osmostress conditions in a Hog1 dependent manner. Wild-type and *hog1Δ* strains were transformed with Rtg1-GFP and Rtg3-GFP plasmids, grown to 0.5-0.8 OD_{660} and stressed for 5 min with 0.4M NaCl. (C) Rtg2 is localized in the cytoplasm under both stress and non-stress conditions. The experiment was repeated using cells that carried the plasmid encoding the Rtg2-GFP fusion protein. Localization of the GFP fusion proteins was determined by GFP fluorescence microscopy as described in Materials and Methods.

Previous published studies demonstrated that subcellular localization of Rtg1/Rtg3 complex is regulated by both proteins (see Introduction). Then, we studied whether both Rtg1 and Rtg3 proteins were required for osmostress-regulated nuclear transport of Rtg1/Rtg3 complex by monitoring the localization of Rtg1-GFP in *rtg3Δ* cells and, alternatively, Rtg3-GFP cells in *rtg1Δ* cells, before or after salt treatment. We observed that Rtg1-GFP remained exclusively cytoplasmic in *rtg3Δ* cells after salt addition (Figure 5, upper panel). In contrast, Rtg3-GFP was localized in the nucleus in *rtg1Δ* cells in non-stress conditions (Figure 5, bottom panel), as previously described (see Introduction).

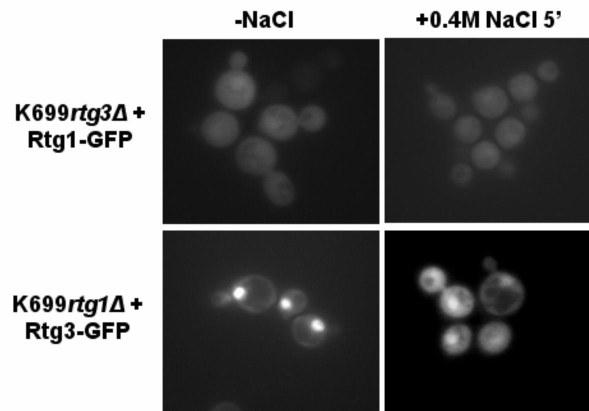

Figure 5. Regulated localization of Rtg1/Rtg3 complex requires the presence of both proteins. Rtg1-GFP was visualized in *rtg3Δ* cells (top) and Rtg3-GFP was visualized in *rtg1Δ* cells (bottom). Cells were grown to 0.5-0.8 OD_{660} and stressed for 5 min by addition of NaCl to a final concentration of 0.4M. Localization of the GFP fusion proteins was determined by GFP fluorescence microscopy.

R5. Rtg1 and Rtg3 co-immunoprecipitates with Hog1

To obtain biochemical evidence for the interaction of Rtg1 and Rtg3 transcriptional complex and Hog1 *in vivo*, we tested the Rtg1-HA and Rtg3-HA tag proteins for immunoprecipitation with the fused GST-Hog1. Cells expressing Rtg1-HA or Rtg3-HA from their own genomic locus were transformed with a plasmid that expressed GST-Hog1 or empty GST under the P_{TEF1} promoter. Cells were subjected to a brief osmotic shock (0.4M NaCl for 10 min) and Hog1 was pulled down using glutathione-Sepharose beads. The presence of Rtg1-HA and Rtg3-HA was probed by a specific monoclonal antibody against HA. As shown in Figure 6, Hog1 was able to precipitate both Rtg1 and Rtg3 proteins upon osmotic conditions, indicating that Rtg1 and Rtg3 proteins physically interact with the Hog1 MAP kinase.

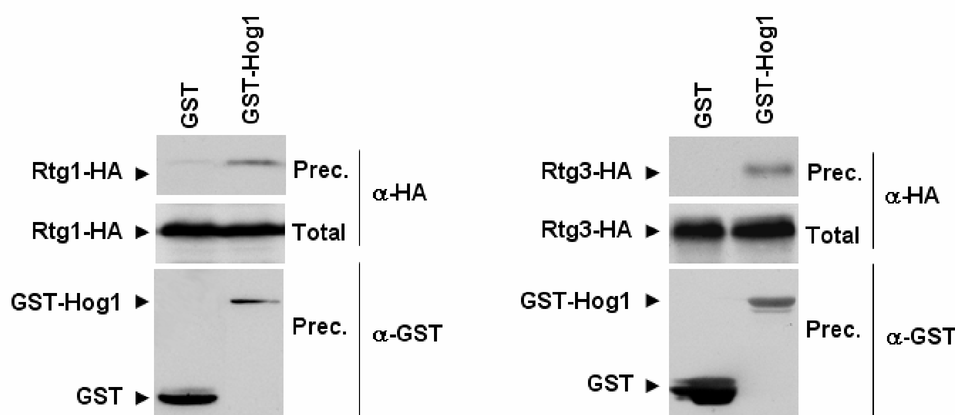

Figure 6. The Hog1 MAPK interacts with Rtg1 and Rtg3 proteins. HA-tagged Rtg1 and Rtg3 strains expressing GST or GST-Hog1 under the P_{TEF1} promoter were grown and samples were taken 10 min after treatment with 0.4M NaCl. GST proteins were pulled-down by glutathione-Sepharose 4B and the presence of Rtg3-HA/Rtg1-HA was probed by immunobloting using anti-HA (upper panel). Total extracts are presented in the middle panel. The amount of precipitated GST proteins was detected using anti-GST (lower panel).

R6. The Rtg1/Rtg3 complex binds to the *CIT2* and *DLD3* promoters depending on the Hog1 MAPK in response to stress

Previous data established that osmoresponsive transcription factors bind to the promoters to regulate gene expression (Alepuz et al., 2001; Alepuz et al., 2003). To answer the question whether Rtg1/Rtg3-complex binds to the promoters of the corresponding genes under osmotic conditions, we made use of Chromatin Immunoprecipitation Assays, a method that measures the extent to which certain genomic DNA regions can be cross-linked to a specific protein under *in vivo* conditions (see Materials and Methods). Rtg1 and Rtg3 are bHLH/Zip transcription factors that bind to specific DNA sequences, GTCAC, called the R box. Then, we analyzed the binding of Rtg1 to these promoter sequences before and after stress. The results for the binding of Rtg1-HA to *CIT2* and *DLD3* genes are shown in Figure 7A. A comparison between normal and stressed cells revealed that Rtg1-HA was engaged with these region promoters to a much higher degree in stressed cells than in non-stressed cells. Interestingly, the stress-induced increase in Rtg1-HA recruitment upon osmostress was sensitive to the presence of the

Hog1 MAP kinase. Hence, we conclude that the Rtg1/Rtg3-complex binds to specific promoter regions of some stress-responsive genes upon hyperosmotic stress in a Hog1-dependent manner.

It is known that the Hog1 MAPK is targeted to specific osmostress-responsive promoters in response to stress, and this recruitment is dependent on specific transcription factors. Figure 7B shows that Hog1 is recruited to the promoter of *CIT2* gene, and this recruitment is dependent on the presence of the Rtg1 transcription factor. Thus, an interdependence of binding of Hog1 and Rtg1 to specific osmostress promoters occurs upon osmotic stress.

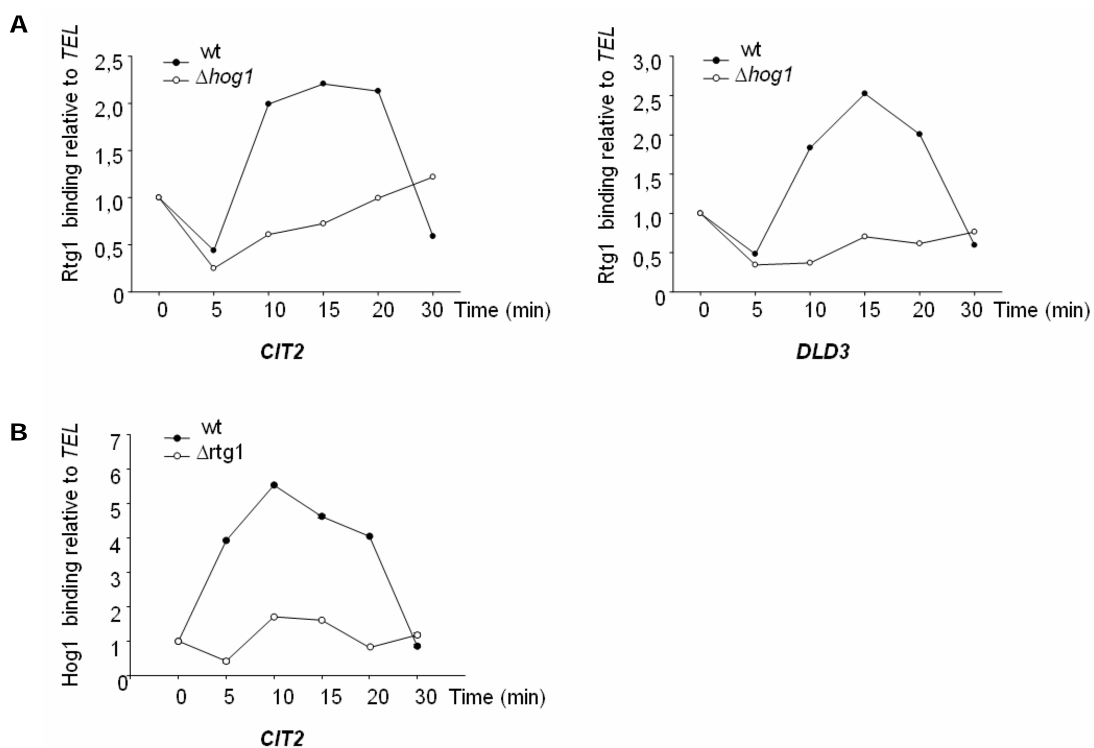

Figure 7. Kinetics of occupancy of Rtg1 and Hog1 to the promoter sequences of RTG-dependent genes. (A) Association of Rtg1 to *CIT2* and *DLD3* promoter regions in wild-type or *hog1Δ* strains. (B) Recruitment of the Hog1 MAPK to *CIT2* promoter region in wild-type and *rtg1Δ* strains. Cells were or were not exposed to hyperosmotic stress for the indicated times. Proteins were immunoprecipitated with anti-HA. Binding to *CIT2* and *DLD3* was determined by ChIP. Quantification is depicted as fold binding over *TEL1*.

R7. Hog1 phosphorylates Rtg1 *in vitro* and *in vivo*

R7.1. The Hog1MAPK phosphorylates *in vitro* the Rtg1 transcription factor

The HOG pathway controls gene expression through several mechanisms, such as direct regulation of transcription factor activities (see Discussion). We then tested whether Hog1 was able to phosphorylate specifically Rtg1 *in vitro*. Thus, we performed *in vitro* kinase assays using GST-tagged proteins purified from *E. coli*. In the first step of the reaction, Hog1 was activated by phosphorylation in the presence of Pbs2[EE] and ATP. After that, Rtg1 was incubated with both [γ-^{32}P]-ATP and the activated Hog1 protein. As shown in Figure 8, the *in vitro* kinase assay revealed that the Rtg1 protein was phosphorylated by Hog1, suggesting that the MAPK Hog1 could be controlling the Rtg1 transcription factor by direct phosphorylation.

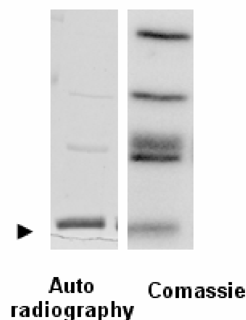

Figure 8. Hog1 phosphorylates Rtg1 *in vitro*. Recombinant tagged proteins were purified from *E. coli* as described in Materials and Methods. Hog1 and the constitutively active Pbs2 mutant (Pbs2[EE]) were incubated in the presence of kinase buffer and ATP. Purified Rtg1 was then incubated with [γ-^{32}P]-ATP and the Hog1 protein previously activated with Pbs2[EE]. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography (left panel). The GST-tagged Rtg1 protein was detected by comassie staining (right panel). The position of tagged Rtg1 is indicated on the left.

R7.2. Rtg1 protein is phosphorylated upon osmotic stress in a Hog1-dependent manner

We next investigated whether Rtg1 is phosphorylated *in vivo* during hyperosmotic stress. We fused six haemagglutinin (HA) epitopes at the C-

terminus of the *RTG1* gene at its chromosomal locus. The Rtg1-HA fusion protein was detected by Western Blot from wild type and *hog1Δ* yeast extracts resolved by SDS-PAGE using the specific 12CA5 monoclonal antibody against HA. Upon a brief shock (0.4M NaCl for 5 min), Rtg1 rapidly changed its mobility in SDS-polyacrylamide gels (Figure 9, lanes 1 and 2). To test whether the mobility shift of Rtg1 was due to phosphorylation, protein extracts were treated with alkaline phosphatase before western blot analysis. As expected for a phosphorylation event, the mobility shift of Rtg1 was eliminated (Figure 9, lane 3). Notably, phosphorylation of Rtg1 in response to osmotic stress depended completely on Hog1, since Rtg1-HA expressed from *hog1Δ* cells did not undergo the mobility shift observed in wild-type cells (Figure 9, lanes 4 and 5). Taken together, these results show that Rtg1 is rapidly phosphorylated upon hyperosmotic shock *in vivo* and that this modification depends on the Hog1 MAPK.

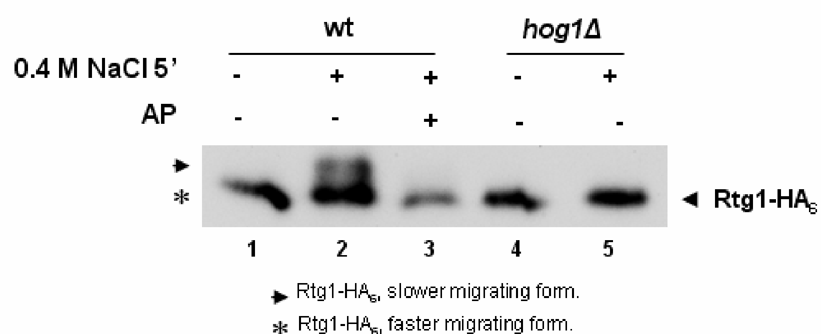

Figure 9. Rtg1 is phosphorylated upon osmostress in a Hog1 dependent manner. Wild-type and *hog1Δ* cells were grown to 0.6-1 OD_{660}. Samples were taken before (-) or 5 min after (+) the addition of NaCl to a final concentration of 0.4M NaCl, and the extracts were treated (+) or not (-) with 10U of alkaline phosphatase (AP). Rtg1-HA was detected by immunoblotting using anti-HA monoclonal antibody.

R8. Analysis of Thr60 phosphorylation by Hog1

R8.1 Hog1 phosphorylates Rtg1 at the Thr60 residue *in vitro*

Because Hog1 catalytic activity is required for transcriptional response and Rtg1 is phosphorylated by Hog1, we next wanted to determine the role of

Rtg1 phosphorylation(s) by Hog1 in response to osmostress. As it is indicated in Figure 10A, Rtg1 contains one putative phosphorylation site for MAPKs (serine-proline or threonine-proline), namely Thr60, that is located within the loop region that separates the two amphipathic helices in the bHLH/Zip region of the transcription factor. Then, we created a point mutant version of Rtg1 which contained a Thr replacement with Ala (Rtg1^{T60A}). This mutant was fused to the GST tag and expressed in *E. coli* as described before. As shown in Figure 10B, phosphorylation of Rtg1^{T60A} by Hog1 was completely abolished, indicating that Hog1 phosphorylates *in vitro* Rtg1 protein specifically at the Thr60 residue.

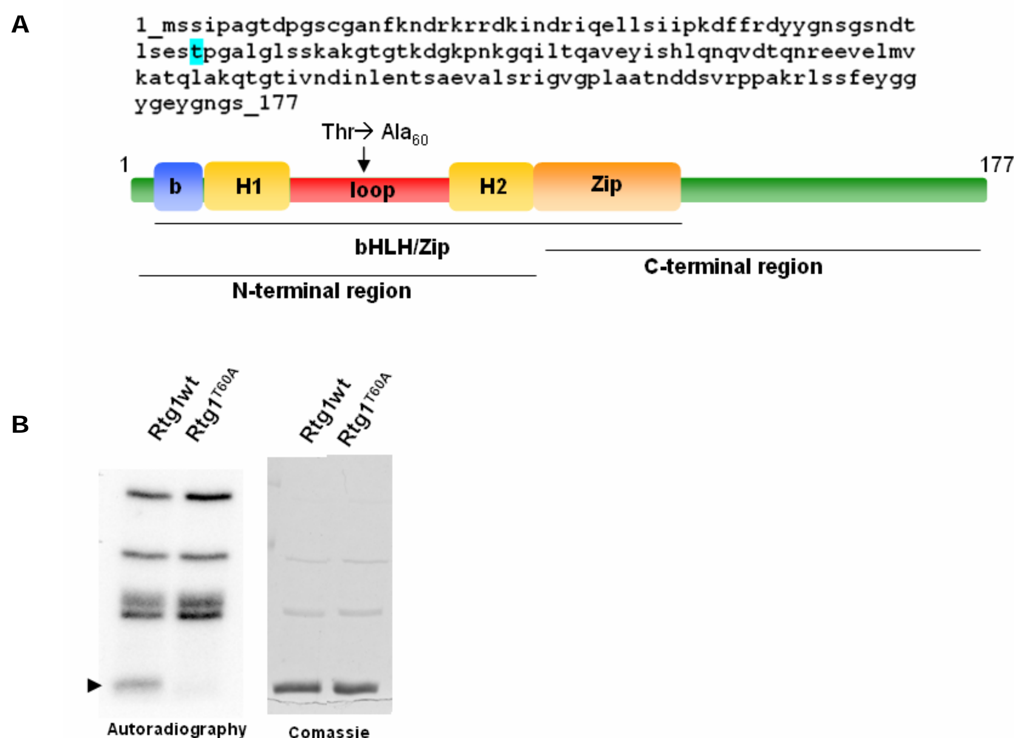

Figure 10. Rtg1 is phosphorylated at the Thr60 by Hog1 *in vitro*. (A) Sequence and structural domains of Rtg1 including the localization of the point mutation at Thr60. The basic (b), HLH (H1, loop, and H2), and C-terminal domains of Rtg1 are indicated. (B) Rtg1 is phosphorylated at Thr60 by Hog1 *in vitro*. Recombinant GST proteins were purified from *E. coli* and subjected to phosphorylation by activated Hog1 as described in Materials and Methods. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography (left panel). GST-tagged Rtg1 proteins were detected by comassie staining (right panel). The position of tagged Rtg1 is indicated on the left.

R8.2. Rtg1^{T60A} mutant is still phosphorylated in response to osmostress in a Hog1-dependent manner

Since Hog1 phosphorylates Rtg1 at the residue Thr60 *in vitro*, we tested the ability of this mutant to be phosphorylated upon an osmotic stress. We fused the Rtg1 wild-type or the Rtg1^{T60A} mutant with the haemagglutinin (HA) epitope at the C-terminus and expressed them under its own promoter. *rtg1Δ* and *rtg1Δ hog1Δ* cells were transformed with these plasmids and subjected or not to a brief shock (0.4M NaCl for 5 min). Unexpectedly, Rtg1^{T60A} mutation did not eliminate the Rtg1 mobility shift due to the phosphorylation by Hog1 in response to osmotic stress (Figure 11, 3 and 4 lanes), indicating that additional residues in Rtg1 might be the responsible for this mobility shift. Because Rtg1 phosphorylation depends on Hog1 and no other Hog1 sites are present in Rtg1^{T60A} mutant, alternatively, the MAPK could also act indirectly over the transcriptional regulator though an intermediate kinase (see below).

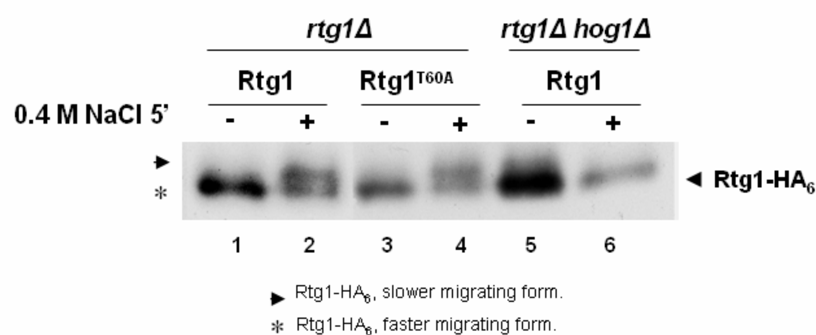

Figure 11. Thr60A mutation on Rtg1 does not eliminate the Rtg1 mobility shift due to phosphorylation in response to osmotic stress. *rtg1Δ* and *rtg1Δ hog1Δ* strains were transformed with HA-tagged wild-type Rtg1 and Rtg1^{T60A} mutant. Cells were grown and samples were taken before (-) or 5 min after (+) the addition of 0.4M NaCl. Rtg1-HA was probed using anti-HA monoclonal antibody.

R8.3. Rtg1^{T60A} mutant localizes into the nucleus and activates transcription upon osmotic stress

Because Hog1 regulates the subcellular localization of Rtg1 and the Rtg1-mediated gene expression, we tested the effect of Thr60 phosphorylation by Hog1 on these processes. Thus, we constructed a plasmid containing the Rtg1^{Thr60} mutant fused to the GFP and expressed under its own promoter. Cells deficient for *RTG1* were transformed with plasmids containing the Rtg1-GFP and Rtg1^{Thr60A}-GFP mutants. As shown in Figure 12A, Rtg1^{Thr60A} mutant was able to localize into the nucleus after a brief osmotic shock, suggesting that the phosphorylation of Rtg1 by Hog1 at the Thr60 is not essential for nuclear localization upon stress. Once in the nucleus, Rtg1 activates the expression of several genes upon osmostress. Then, we tested the ability of Rtg1^{Thr60A} mutant to activate gene expression. For this purpose, *rtg1Δ* strain was transformed with plasmids containing the empty vector and Rtg1 wild-type and Rtg1^{Thr60A} mutant. While no expression of *CIT2* was observed in cells lacking *RTG1*, no effect was observed in cells containing the Rtg1^{T60A} mutant. These results indicate that Thr60 phosphorylation by Hog1 is not a key determinant for the nuclear localization neither for Rtg1-mediated gene expression in response to osmostress.

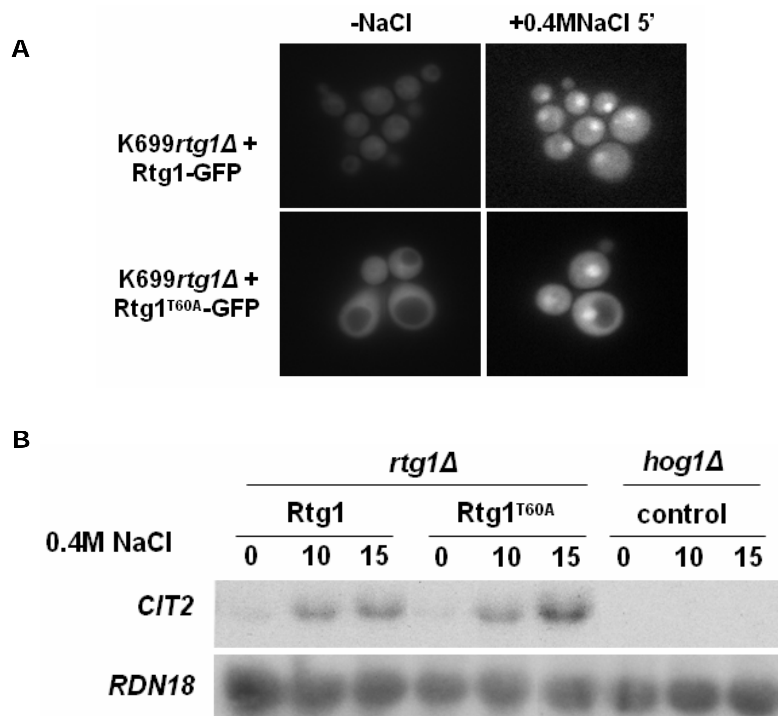

Figure 12. Rtg1 phosphorylation by Hog1 at Thr60 residue is not essential for nuclear localization of Rtg1 and *CIT2* activation upon osmostress. (A) Rtg1^{T60A} mutant localizes into the nucleus after osmotic stress. *rtg1Δ* strain was transformed with Rtg1-GFP and Rtg1^{T60A}-GFP plasmids, grown and stressed for 5 min by addition of NaCl to a final concentration of 0.4M. (B) Rtg1^{T60A} mutant is able to induce gene expression as the wild-type Rtg1. Cells deficient for *RTG1* were transformed with the empty vector (pRS415), the wild-type Rtg1 or the Rtg1^{T60A} mutant, grown and treated with 0.4M NaCl at the indicated times. Total RNA was assayed by northern blot analysis as described in Materials and Methods.

R9. Analysis of the phosphorylation on Ser163 and Ser164 residues in Rtg1 upon osmotic stress

R9.1. Rtg1 protein is phosphorylated at residues Thr60, Ser163 and Ser164 upon osmotic stress in a Hog1-dependent manner *in vivo*

The fact that Rtg1^{T60A} mutant was still able to be phosphorylated in response to osmotic stress suggested the existence of additional phosphorylation sites that depended indirectly on the Hog1 MAPK. To obtain a comprehensive map of potential phosphorylation sites in Rtg1 protein we used mass spectrometry. For that purpose, we constructed plasmids

encoding GST-Rtg1 and GST-Rtg1^{Thr60A} fusion proteins. Wild-type and *hog1Δ* strains were transformed, and cells were subjected or not to a brief osmotic shock (0.4M NaCl for 5 min). Proteins were purified using glutathione-Sepharose beads, resolved by SDS-PAGE, and visualized by silver staining (Figure 13). At this point, excised bands were sent to the Mass Spectrometry Facility of the Max F. Perutz Laboratories in Vienna where potential phosphorylation sites were analyzed. Mass spectrometry analysis, which is summarized in Table II, revealed that Thr60 becomes phosphorylated upon an osmotic shock, and this phosphorylation was dependent on the Hog1 MAPK. This data is consistent with our previous results. Interestingly, two alternative sites, the Ser163 and Ser164 residues, which match the consensus sequence for phosphorylation by protein kinase A (PKA) (R/KR/KXS/T), could be candidates for phosphorylation. Our results indicated that these sites are also phosphorylated in yeast cells although they also seemed to be phosphorylated in the absence of stress (see Discussion) and in a Hog1-independent manner. Because PKA pathway affects gene expression upon hyperosmotic stress, and indeed Msn2/4 and Sko1 transcription factors are regulated by PKA activity, we focused on these sites as possible sites to be phosphorylated upon osmostress.

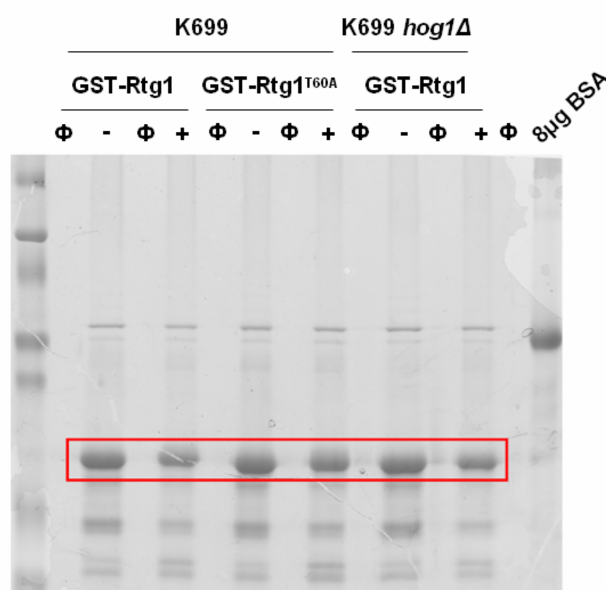

Figure 13. Silver staining of polyacrylamide gel electrophoresis. GST-Rtg1 and GST-Rtg1^{T60A} fusion proteins purified from yeast extracts from cells subjected (+) or not (-) to osmostress were separated on SDS-10% polyacrylamide gel electrophoresis. Detection of proteins was done by silver staining (see Material and Methods). Purified proteins (red box) were excised and sent to be analyzed by mass spectrometry.

	K699				K699*hog1Δ*	
	Rtg1		Rtg-T60A		Rtg1	
	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl
Thr60	-	+	-	-	-	-
Thr55	+	-	-	-	+	+
Ser52	+	+	+	+	+	+
Ser163	+	+	+	+	+	+
Ser164	+	+	+	+	+	-

Table II. Diagram of the phosphorylated residues identified by mass spectrometry upon stress (+) and non stress (-) conditions.

To analyze the ability of Ser163 and Ser164 residues to be phosphorylated in response to osmotic stress, we created a plasmid expressing Rtg1-HA fused protein containing the Ser163A and Ser164A mutations and a plasmid

expressing the Thr60A mutation together with the previous ones. Then, *rtg1Δ* and *rtg1Δ hog1Δ* cells were transformed with the plasmids carrying wild-type and mutant Rtg1 alleles. Cells were grown and subjected or not to a brief shock (0.4M NaCl for 5 min). While both Rtg1 wild-type and Rtg1^{S163AS164A} underwent a mobility shift due to phosphorylation in response to osmotic stress (Figure 14A), Rtg1 protein containing mutations in T60A together with S163A and S164A residues did not (Figure 14B). Taken together, these results indicate that Hog1 directly phosphorylates Rtg1 at Thr60, and indirectly at Ser163 and Ser164 residues through an unknown kinase likely to be PKA upon osmotic stress (see Discussion).

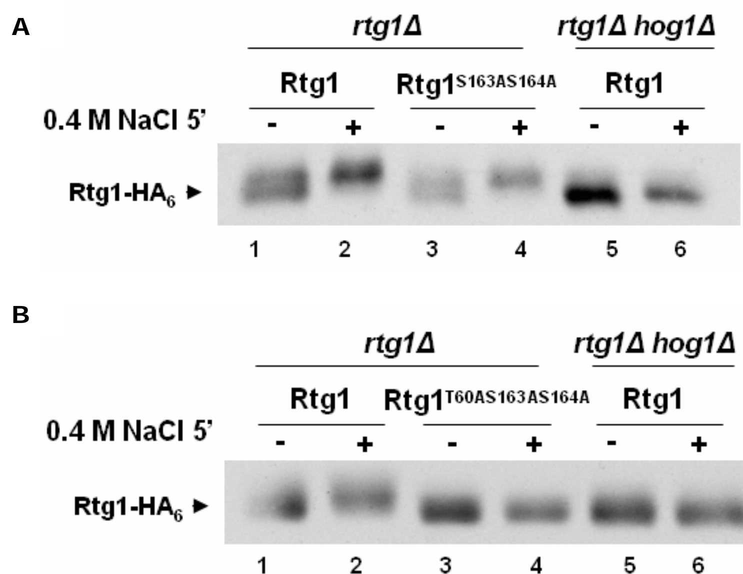

Figure 14. Rtg1^{T60AS163AS164A} mutant is not phosphorylable upon osmotic stress. *rtg1Δ* and *rtg1Δ hog1Δ* strains were transformed with a plasmid expressing HA-tagged wild-type Rtg1, Rtg1^{S163AS164A} or Rtg1 Rtg1^{T60AS163AS164A} mutants. Cells were grown and subjected to a brief osmotic shock as described in Materials and Methods. Rtg1 protein was probed with anti-HA monoclonal antibody.

R9.2. Phosphorylation of Thr60, Ser163 and Ser164 in Rtg1 is not essential for transcriptional activation upon stress.

Having found the Rtg1 phosphorylation sites that depend on the Hog1 MAPK after osmotic shock, we aimed at identifying the role of these phosphorylations in Rtg1-mediated transcriptional expression. Therefore,

Rtg1-HA and Rtg1^{T60AS163AS164A}–HA fusion proteins were expressed in *rtg1Δ* cells and the effect on transcription was measured by northern analysis following the expression of the Rtg1-dependent *CIT2* gene. As shown in Figure 15, the expression of *CIT2* was induced upon stress in wild-type and in cells containing the Rtg1^{T60AS163A164A} mutant at the same extent. Thus, the phosphorylation sites in Rtg1 that are dependent on the Hog1 protein don't play a functional role in regulating Rtg1 transcriptional activity in these specific growth conditions.

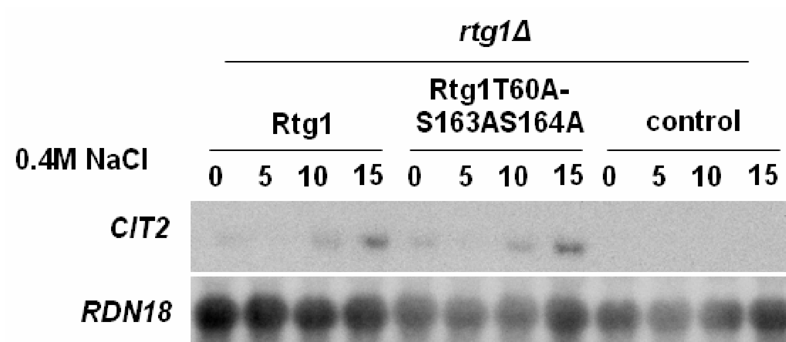

Figure 15. Rtg1 phosphorylation on Thr60, Ser163 and Ser164 residues is not required for *CIT2* activation. Cell cultures of a *rtg1Δ* mutant strain transformed with the pRS415 plasmid containing Rtg1 wild-type, Rtg1^{T60AS163AS164A} mutant or empty vector were incubated with 0.4M NaCl at the indicated times. Total RNA was assayed by northern blot for transcript levels of *CIT2* and *RDN18* as a loading control.

R10. Rtg3 is phosphorylated by Hog1 *in vitro*

Previously, we demonstrated that the Hog1 kinase activity is necessary for the transcriptional regulation of Rtg1-dependent genes upon osmostress. Since Rtg1 phosphorylation by Hog1 is not required for induction of *CIT2* upon osmostress, we focused on testing whether additional components of the RTG-pathway, such as Rtg2 and Rtg3 proteins, could be phosphorylated by the Hog1 MAPK *in vitro*. We fused Rtg2 and Rtg3 proteins to the glutathione S-transferase at the N-terminal region. Then, GST-tagged proteins were expressed in *E. coli*, purified and subjected to *in vitro* phosphorylation assays by Hog1 as described before. As shown in Figure 16, while GST-Rtg2 fusion protein was not phosphorylated by Hog1,

phosphorylation of GST-Rtg3 was detected. Therefore, Hog1 phosphorylates *in vitro* both Rtg1 and Rtg3 proteins.

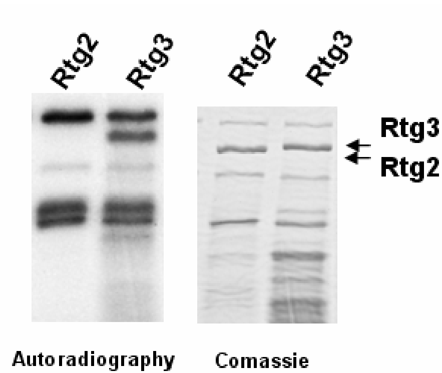

Figure 16. Rtg3 protein is phosphorylated by Hog1 *in vitro*. Recombinant GST proteins were purified from *E. coli* for *in vitro* kinase assays as in Figure 8. After phosphorylation, the proteins were resolved by SDS-PAGE and phosphorylated proteins were detected by autoradiography (left panel). GST-tagged proteins were detected by comassie staining (right panel). The position of tagged Rtg2 and Rtg3 is indicated on the right.

R11. Identification and analysis of Rtg3 residues that are phosphorylated upon osmotic stress in a Hog1-dependent manner

R11.1. Hog1 phosphorylates Rtg3 at the Thr197 residue *in vitro*

Rtg3 contains 11 putative phosphorylation sites for MAPKs (serine-proline or threonine-proline). To know which of them was phosphorylated by Hog1, we created several truncated *RTG3* alleles (Figure 17A), expressed as GST-tagged proteins in *E.coli* and analyzed by *in vitro* phosphorylation assays as before. As shown in Figure 17B, whereas M1 fragment was phosphorylated by Hog1, Rtg3M2 was not phosphorylated. These results indicate that the region comprised between amino acids 183 to 210 in Rtg3 contains at least some of the putative phosphorylation site(s) by the Hog1 MAPK. As it is

depicted in Figure 17B the C-terminus of Rtg3 was also phosphorylated by Hog1 *in vitro*, although to a lesser extent than the M1 fragment.

We then analyzed the phosphorylation sites for MAPKs present in Rtg3M1 fragment and absent on Rtg3M2 fragment. Rtg3M1 contains a putative phosphorylation site (Thr197) for MAPKs that is not present in the Rtg3M2 fragment, suggesting that this residue could be a Hog1 phosphorylation site in Rtg3. Thus, we generated an Ala substitution at Thr197 in the full-length protein. While Rtg3 wild-type was phosphorylated by Hog1, a strong decrease in phosphorylation was observed in the $Rtg3^{T197A}$ mutant (Figure 17C). It is worth noting that this mutation did not completely abolished phosphorylation by the Hog1 MAPK, indicating alternative phosphorylation sites within Rtg3. However, our results indicate that Thr197 residue in Rtg3 is a major site of phosphorylation by Hog1 *in vitro*.

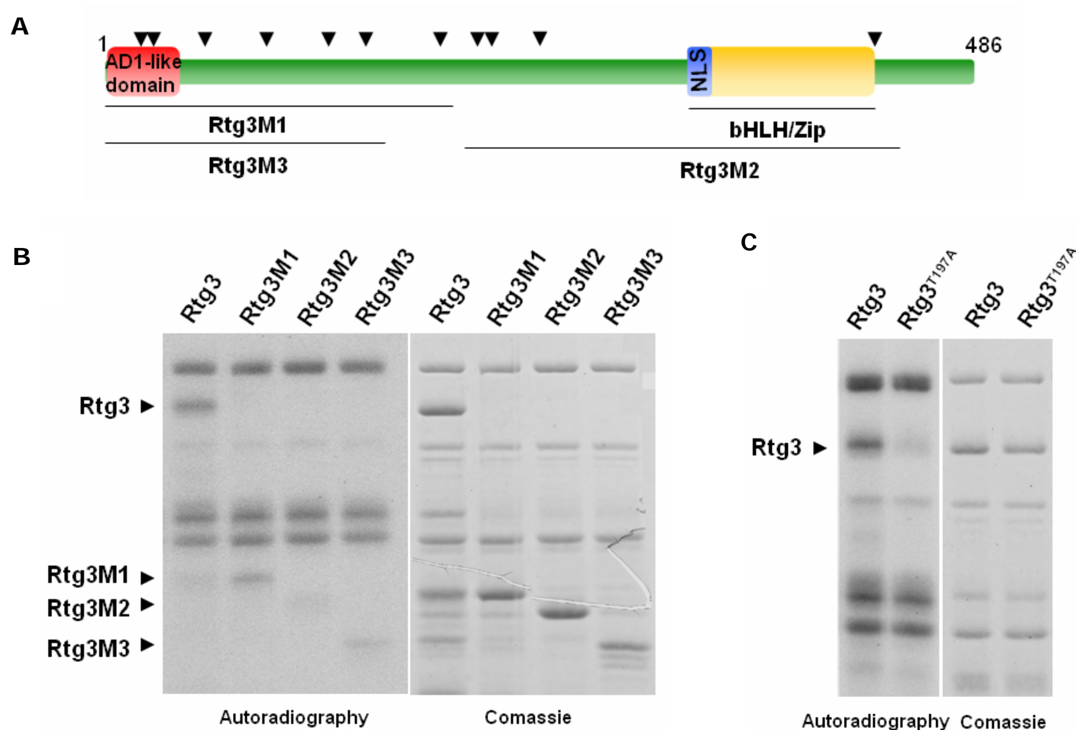

Figure 17. *In vitro* phosphorylation of Rtg3 by Hog1. (A) Schematic overview of the putative phosphorylations sites by MAPKs in the Rtg3 protein. (B) The N-terminal region is phosphorylated by the Hog1 MAPK. Full-length or various Rtg3 fragments were tested by their ability to be phosphorylated by Hog1. *In vitro* phosphorylation assays were carried out as in Figure 8. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography (left panel). GST-tagged Rtg3 proteins were detected by comassie staining (right panel). (C) Rtg3 is phosphorylated at Thr197 residue by Hog1. *In vitro* kinase assays were performed as described in Material and Methods.

R11.2. Thr197 residue in Rtg3 is not essential for *CIT2* activation upon stress.

To examine the effects of Rtg3 phosphorylation by Hog1 on transcriptional regulation, we transformed *rtg3Δ* cells with plasmids encoding the wild-type Rtg3 and the mutant Rtg3^{T197A} fused to haemagglutinin (HA) epitope and the empty vector as control. Then, cells were grown and subjected or not to an osmotic stress at the indicated times. The effect on transcription of the Rtg3-dependent *CIT2* was measured by northern analysis. As shown in Figure 18A, while *rtg3Δ* mutant was not able to induce *CIT2*, expression of *CIT2* upon stress was restored when *rtg3Δ* cells were transformed with plasmids containing wild-type Rtg3 and Rtg3^{T197A} mutant. These results indicate that Thr197 phosphorylation by Hog1 does not play a crucial role in transcriptional regulation.

As Hog1 phosphorylates both Rtg1 and Rtg3 proteins, we tested the possibility that phosphorylations of these proteins were redundant for RTG-mediated transcriptional regulation upon osmostress. Then, both Rtg1^{T60AS163AS164A} (NP) and Rtg3^{T197A} mutants were expressed in *rtg1Δ rtg3Δ* cells and the effect on transcription was measured by northern analysis as before. As depicted in Figure 18B, cells containing these mutants were able to activate transcription at the same extent than wild-type proteins, demonstrating that Hog1-dependent phosphorylation at these residues is not playing a crucial role in regulating the RTG-mediated transcriptional activity upon osmostress. At present we are analyzing the effect on transcription of alternative Rtg3 phosphorylations at the C-terminal region by the Hog1 MAPK. However, we can’t exclude the possibility that transcriptional activity is not dependent on the phosphorylation state of the Rtg1 and Rtg3 activators (see Discussion).

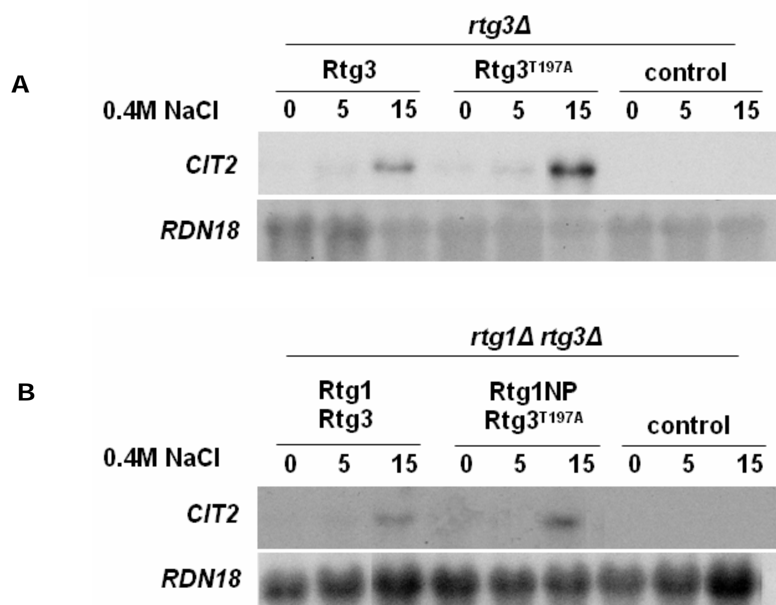

Figure 18. Thr197A point mutation in Rtg3 does not affect *CIT2* activation upon osmotic stress. (A) (B) Mutant strains containing the indicated plasmids were subjected or not to 0.4M NaCl at the indicated times. Total RNA was assayed by northern blot for transcript levels of *CIT2* and *RDN18* as a loading control.

R12. RTG-pathway activation is required for cell survival at high osmolarity

The requirement of RTG-pathway components for regulation of gene expression upon osmostress prompted us to analyze the relevance of these proteins for cell survival upon stress conditions. Thus, cells deleted for *RTG1* and *RTG3* genes were spotted onto plates containing different concentrations of salt and sorbitol. As depicted in Figure 19, *rtg1Δ* and *rtg3Δ* strains showed a resulted in a reduced cell survival at high osmolarity. It is worth noting that the deletion of these transcription factors yields cells osmosensitive, whereas a *hot1Δ msn1Δ msn2Δ msn4Δ* strain, deficient on several osmoresponsive transcription factors, had no strong effect on the sensibility to high osmolarity (see Introduction). Altogether, these results suggest that RTG-pathway integrity is essential for cell survival upon stress.

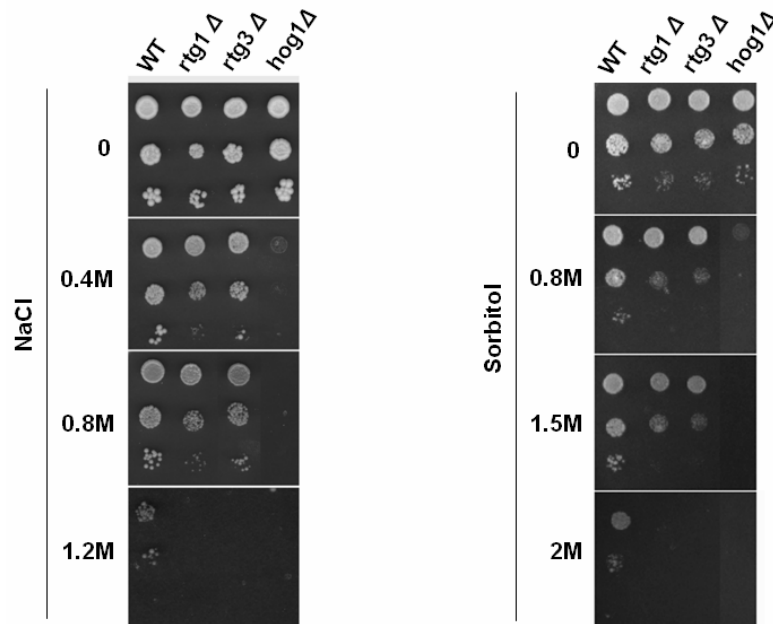

Figure 19. Mutations on RTG-pathway components render cells osmosensitive. Wild-type and the indicated mutant strains were grown to logarithmic phase and diluted to an OD_{660} of 0.05. A 10-fold serial dilution series was plated on MD-Gln plates without or with NaCl/sorbitol at the indicated concentrations. Growth was scored after 2-5 days.

R13. Effects of *RTG1* mutation on genome-wide responses to hyperosmotic stress

To characterize the role of Rtg1 in osmostress gene expression, we performed genome-wide DNA by microarrays analysis. Thus, wild-type and *rtg1Δ* cells were subjected to a brief osmotic stress (0.4M NaCl for 10 minutes), and mRNA was extracted for the subsequent analysis. In addition, the role of the Hog1 MAPK was also investigated by testing the transcriptional response in a *hog1Δ* mutant strain grown under the same conditions.

A total of 6256 genes were scored in the screening. Results revealed that the mRNA level of 365 genes (5.8%) increased at least 3-fold in response to stress. These data are consistent with previous results showing that exposure of yeast to osmostress results in a substantial transcriptional

response (see Introduction). Then, we analyzed the dependence of these genes on both *RTG1* and *HOG1*. According to the degree of reduction of the genes induced by osmostress observed in the absence of *RTG1*, we grouped the osmoresponsive genes into three categories (Figure 20). The first category includes the genes strongly affected by the *RTG1* deletion (0-50% of the total induction observed in wild type cells), and comprises 24 genes (6.6% of the total of genes induced upon osmostress). The second category includes genes weakly dependent on *RTG1* (50-75%), which represent 74 genes (20.3% of the total of genes induced upon osmostress). The third category includes genes that are independent of *RTG1* (75-100%), which includes 267 genes (73.1% of the total of genes induced upon osmostress). Interestingly, all genes that were strongly dependent on Rtg1 were also strongly dependent on the Hog1 protein. Thus, the strong correlation between the genes regulated by Rtg1 and Hog1 proteins suggest that the MAP kinase pathway plays an important role in the Rtg1 transcription factor gene regulation.

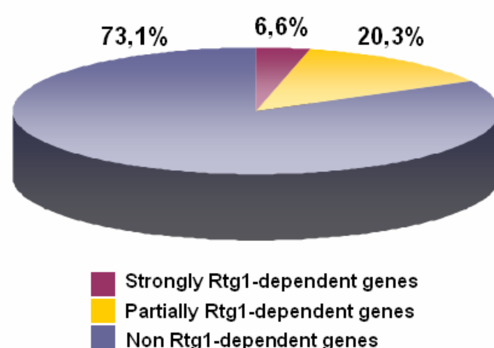

Figure 20. Role of the Rtg1 transcription factor on gene induction after exposure to NaCl. Rtg1 is participating in gene regulation of yeast cells subjected to an osmotic shock. Genes induced in wild-type versus *rtg1Δ* cells were grouped within the following three categories: strongly Rtg1-depedent genes (0-50% of the induction observed in wild-type cells), partially Rtg1-dependent genes (50-75%) and non Rtg1-dependent genes (75-100%).

Among the genes that are highly induced upon osmostress and that depends on both *RTG1* and *HOG1* (Table III), we found genes involved in carbon metabolism, such as *STL1* and *HXT5*, and redox metabolism and protection from oxidative and chemical damage, such as *CTT1*. Rtg1 also controls the induction of genes involved in signal transduction and control of

gene expression, such as *PRR2* and *CIN5,* and several genes involved in diverse functions such as *MSC1*, *SPS100*, *HSP12* and *BDH2*. In addition, when we considered an induction of 2-fold upon osmostress, an induction of genes involved in respiratory complexes (*COX5b*, *COX13*, and *PET54*), mitochondrial transport and mitochondrial DNA maintenance (SSA4, *PET18* and *RRM3*) was observed. The role of the Rtg1 transcription factor upon stress is described in the Discussion section.

Gene name	Gene description
STL1	Sugar transporter like 1
HXT5	Hexose transporter 5
YBR116C	Protein of unknown function
PHM7	Protein transcriptionally regulated by phosphate
ALD3	Stress inducible aldehyde dehydrogenase. Coenzyme A biosynthetic process
CTT1	Catalase T1, important for detoxification of superoxide radicals and hydrogen peroxide
TKL2	Transketolase 2. Monosaccharide metabolic and aromatic amino acid family biosynthetic processes
YGR043C	May be involved in signal transduction
YAL061W	Membrer of the zinc-containg alcohol deshydrogenase family. Transcription in response to mitochondrial dysfunction
YNL195C	Unknown function. Mitochondrial localization
YGR052W	Serine/threonine protein kinase of unknown function
YGR243W	May be involved in energy derivation by oxidation of organic compounds. Mitochondria localization
YHR087W	Protein possibly involved in RNA metabolism
YNL194C	Protein possibly involved in sphingolipid metabolism
HSP12	Heat shock protein of 12 kDa
CIN5	Chromosome instability 5, a transcription factor of bZIP family
MSC1	Protein that affects meiotic homologous chromatid recombination
SPS100	Sporulation specific protein involved in spore wall formation
HOR2	DL-glycerol phosphate phosphatase
PRR2	Serine/threonine protein kinase
YHR033W	Protein with similarity to Pro1p
YOR062C	Protein of unknown function
YMR107W	Protein of unknown function
CIT2	Citrate synthase 2

Table III. Classification into functional families of ORFs whose transcripts are induced more than 3-fold after 10 min of NaCl treatment in a Rtg1 and Hog1 dependent manner. Information was obtained from the Yeast Proteome Dabatase

Previous studies revealed that Msn2/4 transcription factors regulate genes such as *CTT1* and *HSP12*. The Hot1 transcription factor controls a specific subset of genes, including *STL1*, *GPD1* and *GPP2* (see introduction). Unexpectedly, the *rtg1Δ* strain showed a decrease in the induction of *STL1*::LacZ reporter upon osmostress. In addition, genome-wide DNA analysis described here revealed that the expression of *CTT1* and *STL1* genes was dependent on the Rtg1 transcription factor. To confirm these data, we measured by northern blot analysis the effect on transcription of the *CTT1* and *STL1* genes in wild type cells and in cells deleted for *RTG1*.

As shown in Figure 21, wild-type cells induced expression of *CTT1* and *STL1* genes upon an osmotic stress, whereas a strong decrease in transcription was observed when *RTG1* gene was deleted. Thus, Rtg1 transcription factor is also regulating the expression of these genes, previously described to be activated by other transcription factors. Altogether, these results contribute to the emerging view of the complexity of osmostress-induced transcription, and could explain the osmosensitive phenotype of strains deleted for the *RTG1* and *RTG3* genes (see Discussion).

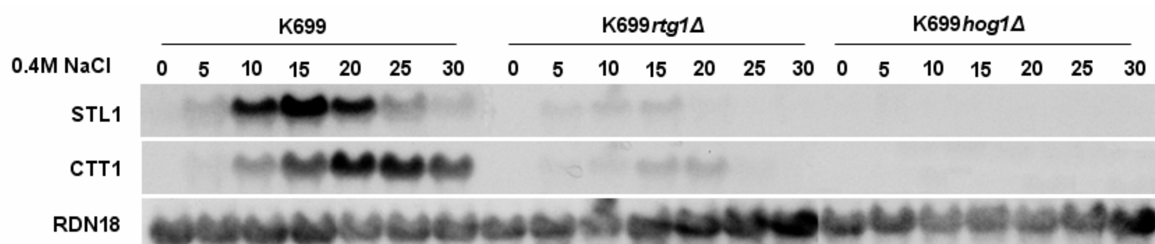

Figure 21. Rtg1 regulates the induction of *STL1* and *CTT1* genes upon osmostress conditions. Total RNA was isolated from the indicated strains treated with 0.4M NaCl at the indicated times. Then, RNA was assayed by northern blot analysis for transcript levels of *STL1* and *RDN18* as a loading control (see Material and Methods).

DISCUSSION

D1. Rtg1 is a novel transcription factor regulated upon osmotic stress

Exposure of cells to an increase in extracellular osmolarity results in a rapid activation of the stress-activated Hog1 MAPK. A major outcome of the activation of the Hog1 MAPK is the regulation of gene expression. One of the most common mechanisms by which SAPKs regulates gene expression is by modification of specific transcription factors (Karin and Hunter, 1995; Treisman, 1996; Kyriakis and Avruch, 2001), and several transcription regulators have been proposed to be controlled by the Hog1 MAPK. These transcription regulators include the redundant zinc finger proteins Msn2 and Msn4 (Schuller et al., 1994), the Hot1 protein (which does not belong to a known family of transcription factors) (Rep et al., 2000), the bZIP protein Sko1 (Proft et al., 2001) and the MADS box protein Smp1 (de Nadal et al., 2003). However, due to their DNA binding specificities and the profile of gene induction upon stress shown by DNA microarrays, they cannot account for the regulation of all of the genes under the control of Hog1. In addition, a mutant strain carrying deletions in *hot1*, *msn1*, *msn2*, and *msn4* is not osmosensitive (Rep et al., 1999a).

From these observations, we suspected that additional transcription factors were required for the osmostress-induced regulation of gene expression by the Hog1 MAPK. Thus, we performed an exhaustive genome-wide genetic screening, searching for deletion mutants that rendered cells unable to activate transcription of several osmoresponsive-promoters fused to the *LACZ* reporter gene. In this study we identified the basic helix-loop-helix-leucine zipper (bHLH/Zip) transcription factor encoded by the *RTG1* gene as a regulator of gene induction upon osmostress. Previous data indicated that Rtg1 transcription factor is activated when mitochondrial respiratory function declines or is absent, for instance, in “petites” mutants, that are a special class of respiratory-deficient mutants characterized by large deletions of mtDNA or a complete lack of mitochondrial genome. In these cases, yeast modulates the expression of a subset of nuclear genes that

enable those cells to adapt to their respiratory state via a mitochondria-to-nucleus signalling pathway called retrograde (RTG) response.

Actually, when we analysed the transcript levels of *CIT2* and *DLD3*, the prototypical genes regulated by Rtg1 (Butow and Adhavani, 2004; Liu and Butow, 2006), they were induced upon osmostress. Interestingly, their expression was totally dependent on the Rtg1 transcription factor, and partially dependent on the catalytic activity of the Hog1 MAPK. Thus, the Rtg1 transcription factor is regulating gene expression upon osmostress, and the Hog1 MAPK plays a key role in this regulation. Remarkably, induction of the *CIT2* gene was also dependent on additional components of the RTG-pathway, as such Rtg2 and Rtg3, indicating that integrity of the RTG-pathway is required for gene expression upon osmostress.

D2. Rtg1 and Rtg3 transcription factors localizes into the nucleus upon osmostress in a Hog1-dependent manner

When the RTG pathway is off, the Rtg1 transcription factor localizes to the cytoplasm. Upon activation of the RTG pathway, Rtg1 translocates into the nucleus (Sekito et al., 2000). Thus, we investigated whether this transcription factor changed its subcellular localization upon osmostress. Our results show that the Rtg1-GFP fusion protein concentrates rapidly into the nucleus after osmotic stress, and this localization correlates with the transcriptional induction of both *CIT2* and *DLD3*. Previous data indicated that MAPK signalling pathways can stimulate the translocation of transcription factors from the cytoplasm to the nucleus (Yang et al., 2003). For instance, the Smp1 transcription factor localizes to the nucleus upon osmostress, and this localization depends on the Hog1 MAPK (de Nadal et al., 2003). Interestingly, Rtg1 nuclear accumulation clearly diminished in *hog1Δ* cells, indicating that Hog1 MAPK is regulating the localization of Rtg1 upon osmostress, and thus this could impede the normal function of the transcription factor.

Rtg1 forms a heterodimer with the Rtg3 basic helix-loop-helix leucine zipper (bHLH/Zip) transcription factor and both localize together into the nucleus and bind to the promoter region of target genes for the induction of gene expression (Liao and Butow, 1993; Jia et al., 1997; Sekito et al., 2000). The nuclear translocation of the Rtg1/3 complex correlates with a change in the phosphorylation state of Rtg3 (Komeili et al., 2000; Sekito et al., 2000; Dilova and Powers, 2006). It is worth noting that the relevant kinase and phoshatase activities affecting the phosphorylation state of Rtg3, as well as the phosphorylation sites critical for its regulated subcellular localization, remain to be identified. Similarly to Rtg1, Rtg3 concentrated rapidly into the nucleus after osmostress, and its localization was dependent on the Hog1 MAPK. Rtg2, an additional regulator of the yeast retrograde response pathway, remained cytoplasmic upon osmotic stress, as previously described for "petites" mutants and cells treated with rapamycin (Komeili et al., 2000; Sekito et al., 2000).

The subcellular localization of Rtg1/Rtg3 complex is regulated by both proteins (Sekito et al., 2000). In order to study whether both Rtg1 and Rtg3 proteins were required for osmostress-regulated nuclear transport of Rtg1/Rtg3 complex, we monitored the localization of Rtg1-GFP in *rtg3Δ* cells and, alternatively, Rtg3-GFP cells in *rtg1Δ* cells, before or after salt treatment. Our results show that the Rtg1-GFP remains exclusively cytoplasmic in *rtg3Δ* cells after salt addition. In contrast, Rtg3-GFP localizes in the nucleus in *rtg1Δ* cells in non-stress conditions, as previously described (Sekito et al., 2000). The basic domain of the bHLH motif of Rtg3 contains a functional nuclear import signal (Sekito et al., 2000). Then, one potential explanation to account for the constitutive cytoplasmic localization of Rtg1 in a *rtg3Δ* strain upon osmostress is that Rtg3 is the identity that localizes the complex into the nucleus upon osmostress. Moreover, Rtg1 contains a nuclear export signal that could explain the constitutive nuclear localization of Rtg3 in a *rtg1Δ* strain at basal conditions.

D3. Rtg1 is phosphorylated after osmotic stress in a Hog1-dependent manner

Numerous transcription factors have been identified as targets for the different MAPKs. Therefore, in mammalian cells, c-Jun is regulated by JNK, and ATF2 can be regulated by both JNK and p38. As a result, these phosphorylations regulate the activity of the activators (Yang et al., 2003). In *Saccharomyces cerevisiae*, phosphorylation of the activator Smp1 by Hog1 in response to osmostress is required for transactivation (de Nadal et al., 2003). By contrast, osmotic-stress-dependent phosphorylation of the Hot1 transcription factor by Hog1 appears to be unnecessary for proper gene expression (Alepuz et al., 2001; Alepuz et al., 2003). To determine whether Rtg1 was phosphorylated upon osmostress, *in vivo* phosphorylation assays were performed. Since Rtg1 rapidly changes its mobility upon stress, and this mobility shift disappears when cell extracts were treated with alkaline phosphatase, we conclude that Rtg1 transcription factor is phosphorylated upon osmostress. Interestingly, the mobility shift does not occur in a *hog1Δ* strain, indicating that the Hog1 MAPK phosphorylates Rtg1 upon osmostress. Biochemical evidence for the interaction of the Rtg1/3 transcriptional complex and Hog1 *in vivo* is supported by the *vivo* binding assays. In addition, the relationship of Rtg1 and Hog1 is further supported by the *in vitro* evidence that Hog1 phosphorylates Rtg1 directly.

D4. Phosphorylation of Rtg1 by the Hog1 MAPK is not required for transcriptional activity

Previous results suggested that Rtg1 is actually a direct substrate for the Hog1 MAPK. Rtg1 contains a unique putative site to be phosphorylated by the Hog1 MAPK (serine-proline or threonine-proline), the Thr60, which is located within the loop region that separates the two helices in the bHLH/Zip domain. *In vitro* phosphorylation assays demonstrate that phosphorylation of Rtg1 by Hog1 occurs at this residue. However, mutation of this residue to Ala results in a Rtg1 mutant that is phosphorylated *in vivo*

to the same extent as the wild-type in response to osmostress. Since Rtg1 phosphorylation upon stress is totally dependent on the MAPK, it may be speculated that Hog1, in addition to phosphorylate Rtg1 directly at Thr60, also acts indirectly on Rtg1, perhaps through an intermediate kinase. This scenario resembles the Sko1 transcription factor, which is regulated by both the Hog1 and PKA kinases (Proft et al., 2001). Because Rtg1 mutated at the Thr60 is able to induce *CIT2* gene upon stress, and in accordance, localizes within the nucleus at these conditions, we conclude that phosphorylation of Rtg1 by Hog1 at Thr60 is not essential for Rtg1 function *per se*.

The fact that $Rtg1^{T60A}$ mutant is still phosphorylated *in vivo* suggests that Rtg1 transcription factor might integrate inputs from several different regulatory kinases in a Hog1-dependent manner. To identify additional residues that are the responsible for the phosphorylation state of $Rtg1^{T60A}$ mutant upon stress, we conducted mass spectrometry analysis. This analysis revealed Ser163 and Ser164 residues in Rtg1 as possible phosphorylation sites upon osmostress. Interestingly, a mutant form containing the Thr60A, Ser163A and Ser164A mutations showed no *in vivo* phosphorylation upon osmostress. To analyze the role of Rtg1 phosphorylation, we tested the ability of this mutant to induce gene expression. The Rtg1 non phosphorylatable mutant was able to induce transcription to the same extent as the wild-type, and thus the Rtg1 phosphorylation mediated by Hog1 is not essetial for the transcriptional activity.

It is worth noting that Ser164 and Ser164 residues match the consensus sequence for phosphorylation by PKA ((R/KR/KXS/T). The cyclic AMP-dependent protein kinase (PKA) is a serine/theonine protein kinase that contains a regulatory subunit, encoded by the *BCY1* gene, and the partially redundant catalytic subunits, encoded by the *TPK1*, *TPK2* and *TPK3* genes (Wilson and Roach, 2000). Several osmoresponsive transcription factors, such as Sko1 and the general stress response transcriptional activators Msn2 and Msn4, have been shown to be phosphorylated by the protein

kinase A. Moreover, this kinase affects gene expression upon hyperosmotic shock (Norbeck and Blomberg, 2000). To test the possibility that Ser163 and Ser164 residues in Rtg1 are being phosphorylated by PKA kinase upon osmotic stress, we are currently testing the phosphorylation state of the Rtg1^{T60A} mutant upon stress in a strain lacking all tree catalytic subunits of PKA. Altogether, Hog1 phosphorylates directly Rtg1 at Thr60 residue, and indirectly at Ser163 and Ser164 residues through an intermediate kinase, likely to be PKA, upon osmostress.

D5. Rtg3 phosphorylation on Thr196 by Hog1 is not relevant for the transcriptional response upon osmostress

Because the Hog1 MAPK plays a key role in the transcriptional regulation of RTG-dependent genes upon osmostress, we focused on testing whether additional component of the pathway were able to be phosphorylated by Hog1. *In vitro* phosphorylation assays indicate that Hog1 is able to phosphorylate both Rtg1 and its partner Rtg3 protein. However, no phosphorylation was detected on Rtg2, the cytoplasmic protein required for the Rtg1 and Rtg3 transactivation function. The relationship between Rtg3 and Hog1 is further supported by the fact that Hog1 is able to interact with Rtg3. These data indicate that Hog1 specifically phosphorylates several components of the RTG pathway, getting a new perspective for RTG-mediated gene regulation upon osmostress. It is worth noting that in response to impaired mitochondrial function Rtg3 localizes to the nucleus and this localization correlates with a change in the phosphorylation state of Rtg3 (Komeili et al., 2000; Sekito et al., 2000; Dilova and Powers, 2006). At present, the identity of the kinases and phosphatases that act upon Rtg3p are unknown.

In vitro kinase assays showed that Rtg3 is mainly phosphorylated by Hog1 at the N-terminal region, which comprises the first 210 amino acids and contains most of the potential phosphorylation sites. Remarkably, this N-terminal region contains a LDFS motif, a domain that has been suggested to

function in transactivation by interacting with components of the SAGA complex (Massari et al., 1999; Jazwinski, 2005). Interestingly, Rtg3^{T197A} mutant showed a marked decrease in phosphorylation in comparison to the wild-type. To analyze the relevance of this phosphorylation for the gene activation upon stress, northern blot analyses were done. Since our results indicate that phosphorylation on Thr197 does not seem to be essential for gene induction, future work will aim to identify novel phosphorylated residues upon osmostress. In addition, it could be that phosphorylations of both Rtg1 and Rtg3 were redundant for RTG-mediated transcriptional regulation upon osmostress. However, cells containing both the non-phosphorylable mutant of Rtg1 and the Rtg3Thr196A mutant are able to activate transcription as the wild-type.

Currently, we can not exclude the possibility that phosphorylation of RTG components by Hog1 does not play a role in transcriptional regulation. For instance, osmotic-stress-dependent phosphorylation of the Hot1 transcription factor by Hog1 appears to be unnecessary to induce expression of the *STL1* gene upon stress (Alepuz et al., 2001; Alepuz et al., 2003). Since binding of RNA polymerase II to Hot1-mediated promoters depends on the catalytic activity of the Hog1 MAPK, it has been proposed that the kinase is the entity that recruits the RNA polymerase II holoenzyme to targeted promoters. The functional relevance of the interaction of Hog1 with RNA Pol II was further exemplified by the fact that artificial recruitment of Hog1 to DNA is able to induce gene expression upon stress (Alepuz et al., 2003). In the case of Rtg1, because catalytic activity of Hog1 is essential for the transcriptional induction of *CIT2*, it is possible that the role of Hog1 in *CIT2* transcriptional regulation involves its association with, and the recruitment of transcriptional regulators to, the promoters of target genes.

D6. Interdependent binding of Hog1 and Rtg1 to *RTG*-regulated promoters after osmostress

In response to hyperosmotic environments, Hog1 becomes intimately linked with some promoter regions, and this binding is dependent on the presence of stress-mediating transcriptional activators (Alepuz et al., 2001). In this regard, Hog1 is recruited to *STL1* promoter, and this recruitment is dependent on the presence of the Hot1 transcription factor. The seemingly reversed situation has been observed upon stress. The recruitment of the activators to some promoters of target genes, such as the Hot1 transcription factor to *STL1* promoter, depends on the presence of the Hog1 MAPK (Alepuz et al., 2001),

Our results also show that Rtg1 transcription factor becomes recruited to the *CIT2* and *DLD3* promoters during osmostress, and this recruitment is strongly reduced in a *hog1Δ* strain. These results are in agreement with the subcellular localization experiments indicating that Hog1 regulates the localization of the activator upon osmostress. Moreover, Hog1 appears to be recruited to the *CIT2* and *DLD3* promoters upon osmostress, and this recruitment depends on the presence of the activator. 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 promoter recruitment and anchorage.

D7. Microarray analyses reveal that a specific subset of stress genes are regulated by the Rtg1 transcription factor

Even though glycerol production and internal glycerol accumulation, which is a key response to osmotic stress, are clearly decreased in a strain deleted for *hot1*, *msn1*, *msn2* and *msn4* transcription factors, none of the single or multiple mutations causes a pronounced defect in growth upon osmostress (Rep et al., 1999a). Remarkably, our results show that deletion of *RTG1* or

RTG3 strongly affect cell growth at high osmolarity. Taken together, these results support the hypothesis that the RTG pathway plays a key role in stress responses.

Genome-wide transcriptional profiling has provided information on both the mode of gene regulation by the Rtg1/3 complex and the physiological functions of the RTG pathway upon osmostress. Considering a 3-fold induction upon osmostress, microarray analysis shows that 24 genes of the 365 genes induced upon osmostress (6.6%) are strongly affected by the *RTG1* deletion. Interestingly, a relationship between the Rtg1 transcription factor and the Hog1 MAPK can be drawn from the observation that genes whose induction is highly dependent on Rtg1 also depend on the Hog1 MAPK. Some of these genes encode proteins involved in sugar transport and involved in different aspects of carbohydrate metabolism (such as *HXT5*, *STL1 and CIT2*), previously described to be induced by osmotic as well as other stresses (Posas et al., 2000; Rep et al., 2000). Genes encoding for some intermediates catalyzing the initial steps of the TCA cycle appear to be regulated by both the Rtg1 and Hog1 proteins. We include in this category *CIT2* (encoding a peroxisomal isoform of citrate synthase), the prototypical target of the RTG pathway (see before).

Another set of genes (>2-fold induction upon osmostress) are those related to redox metabolism and protection from oxidative and chemical damage (*CTT1, PRX1*, *GRX7*, *DAK1* and *TRX3*). Moreover, genes involved in mitochondrial biogenesis are also induced upon osmostress. These genes include those encoding components of the respiratory complexes (*COX5b* and *COX13*), proteins required for mitochondrial import (*PIL1*), stabilization and processing of mitochondrial proteins (*PET54* and *SUE1*), mitochondrial chaperones (*SSA4* and *JID1*) and mitochondrial DNA maintenance (*PET18* and *RRM3*). Moreover, many of the genes that are activated upon osmostress in a Rtg1-dependent manner are also induced in genome-wide analysis in ρ^{o} cells. Then, Rtg1 transcription factor activates a very similar pool of genes in both mitochondrial dysfunction and osmotic stress. These

data, together with the observation that cells defective in oxidative phosphorylation and other mitochondrial functions show significantly greater sensitivity to osmotic stress (data not shown; Rodríguez-Manzaneque et al., 1999), suggests that mitochondria plays a role in the osmotic response, and RTG proteins are participating in this phenomenon.

Rtg1 and Rtg3 bind to the core binding site 5'-GTCAC-3' (R box) at the gene promoters (Liao and Butow, 1993; Jia et al. 1997). Some of the genes induced upon osmostress contain several R boxes, many of them in close vicinity. However, some of the regulated genes do not appear to contain these elements at all. These results contribute to the emerging view that the relationship between protein binding *in vivo*, DNA sequence motifs and transcriptional function are complex. Similarly, although ATF/CREB motifs are likely to be important for Sko1 binding, these motifs are not overrepresented among Sko1 targets (Proft et al., 2005).

Both genome-wide genetic screening and genome-wide DNA analysis revealed that the expression of genes previously described to be regulated by other transcription factors was partially dependent on the Rtg1 activator. Northern blot analysis revealed that induction of *STL1*, whose expression is dependent on Hot1, and *CTT1*, whose expression is dependent on Msn2/4, was strongly reduced in a *rtg1Δ* strain. Whereas *STL1* contains one putative R boxes in front of the gene, no R boxes are contained in the region promoter of *CTT1*. When we analyzed the recruitment of Rtg1 to the promoter sequence of *STL1* containing the putative R box, no Rtg1 binding was observed (data not shown). These data suggest an indirect effect of the lack of *RTG1* towards several stress-regulated genes.

D8. Physiological role of RTG-pathway activation after osmotic shock

Preliminary data shows a decrease in respiration rate upon osmotic stress. Because under anaerobic conditions cells accumulate glycerol faster than

under aerobic conditions (Krantz et al., 2004), it appears reasonable to propose that cells show altered metabolic activity to increase the production of glycerol upon osmostress. In this regard, previous studies demonstrate that osmotic stress affects mitochondrial function by reducing the mitochondrial electron transport in plants (Hamilton and Heckathorn, 2001).

Both the use of oxygen for mitochondrial respiration and a decrease of the electron transport chain enhance the production of reactive oxygen species (ROS) (Pelicano et al., 2004), which appears to be the major contributor of mtDNA mutagenesis. To avoid these processes, cells have developed a number of antioxidants defense mechanisms that include glutathione, thioredoxin, superoxide dismutase (SOD), catalase and peroxidase enzymes (Gutteridge, 1994; Valko et al., 2007). Although there is no a clear connection between oxidative and osmotic responses, links between them do exist. Indeed, the transcriptional response to osmotic shock encompasses genes whose products are implicated in protection from oxidative damage (this work; Gasch et al., 2000; Posas et al., 2000; Rep et al., 2000; Yale and Bohnert, 2001; Causton et al., 2001), and it is worth noting that a *hog1Δ* mutant, as well as mutants deficient in glycerol production, are sensitive to oxidative stress (Pâhlman et al., 2001; Bilsland et al., 2004). In addition, high osmolarity treatment in yeast cells is accompanied by ROS production and mitochondrial ultratestructural alterations (Silva et al., 2005).

Because microarray analysis shows an induction of genes encoding proteins related to mitochondrial DNA maintenance, among other mitochondrial functions (see before), and oxidative protection, and this induction depends on Rtg1, it is conceivable that a respiratory deficiency induced by hyperosmotic stress, accompanied by the increased ROS production, could be determinant for the activation of retrograde response as a protective mechanism at these non-favourable conditions.

CONCLUSIONS

The following main conclusions can be drawn from the results of the work presented in this PhD Thesis:

- The Rtg1/3 transcriptional complex regulates transcription of a specific set of genes upon osmostress.
- The proper induction of RTG-dependent target genes depends on the Hog1 MAPK, and correlates with the nuclear accumulation of Rtg1 and Rtg3.
- Rtg1 is recruited to the promoters of the RTG-dependent target genes upon osmostress in a Hog1-dependent manner.
- Both Rtg1 and Rtg3 co-immunoprecipitate with Hog1 upon osmotic stress.
- Rtg1 protein is phosphorylated upon osmotic stress in a Hog1-dependent manner.
- Hog1 phosphorylates both Rtg1 and Rtg3 *in vitro*. However, neither phosphorylations in Rtg1 and Rtg3 are required for the RTG-mediated transcriptional regulation.
- The Rtg1/3 complex is required for cell survival at high osmolarity.
- Genome-wide DNA microarray analysis indicates that ~ 7% of the total genes induced upon osmostress are highly Rtg1-dependent. Interestingly, the expression of these genes are also dependent on the Hog1 MAPK.

SUPPLEMENTARY ARTICLE

The main topic of this PhD Thesis is the identification of additional transcription factors that are regulated by the Hog1 MAPK in response to osmotic stress. However, in order to understand further the roles of Hog1 MAPK in gene expression, I have also been interested in the role of the Hog1 MAPK during elongation. Since this does not cover the objectives of this work, these results have not been included in the discussion. However, a summary is included (see below).

The Stress-Activated Hog1 Kinase is a Selective Transcriptional Elongation Factor for Genes Responding to Osmotic Stress

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SUMMARY

In response to osmotic stress, the Hog1 MAPK elicits the program for cell adaptation that includes modulation of several aspects of cell biology, such as gene expression (reviewed in Hohmann 2002) (see Introduction). There is not a unique mechanism by which Hog1 regulates gene induction. Hog1 physically associates with regulatory regions of target promoters by interacting with substrates, including the transcription factors Hot1, Msn2/4 and Sko1 (Alepuz et al., 2001; Proft and Struhl, 2002). This binding seems to be important to stimulate Pol II recruitment (Alepuz et al., 2003). Moreover, these interactions promote the recruitment of other proteins, such as coactivators like the histone acethylase SAGA, the histone deacetylase Rpd3, and the SWI/SNF chromatin remodeling complex (Zapater et al., 2007; De Nadal et al., 2004; Proft and Struhl, 2002; Chow and Davis, 2006). Here, we show that Hog1 also associates with the entire transcribed region of target genes, suggesting that SAPKs may have a more

general role as chromatin-associated enzyme than previously anticipated (Chow and Davis, 2006).
By using a photocrosslinking label transfer strategy (Brown et al., 2001), in Proft et al., (2006) it is showed that Rpb1 and a lower-migrating band, possibly corresponding to Rpb2, were interacting partners of Hog1. Since the CTD of the Rpb1 subunit of RNAP II is extensively phosphorylated during different stages of transcription acting as a "docking site" for factors required for productive elongation and mRNA maturation, we studied whether Hog1 associated with phosphorylated Rpb1. Our results showed that Hog1 coprecipitated more efficiently with Rpb1 upon osmostress induction when Rpb1 CTD is phosphorylated. In addition, ChIP-on-chip analysis (combining chromatin immunoprecipitation with DNA microarray technology) revealed that many of the detected Hog1-interacting regions corresponded to 3'-end regions or both promoter and 3'-end regions of osmoresponse genes. Altogether, these results prompted us to analyze whether Hog1 association was occurring along the coding regions of target genes together with elongating RNAP II.

For this purpose, we utilized chromatin immunoprecipitation (ChIP) experiments. In agreement with previous data, our results showed that distribution of Hog1 was not confined to promoters, but displayed an occupancy profile more similar to that of RNAP II, spanning the open-reading frame (ORF) of osmoresponsive genes under stress conditions. Similarity, it has been shown that other MAPK, such as Fus3, Kss1 and Tpk1/2/3, are genomically localized at promoter and coding regions of their corresponding target genes upon inducible treatments (Pokholok et al., 2006). In addition, kinetics of occupancy of Hog1 and Pol II at the prototypical osmoresponsive gene *STL1* ORF showed that while Pol II association was maintained up to 20 minutes after salt addition, Hog1 occupancy was reduced shortly after 10 minutes. This temporal restriction of Hog1 binding suggested a role at the early stages of the transcription elongation process, but not at the subsequent rounds of transcription.

To further investigate the role of Hog1 during elongation process, we tested whether Hog1 was able to interact with different well-defined transcription elongation factors. Indeed, Hog1 was able to interact with Spt4, Dst1, Thp1 and Paf1 upon osmostress. Our results also showed that cells deleted for these elongation factors did not have compromised cell viability under non-stress conditions. However, when these cells were subjected to high osmolarity they were osmosensitive, as well as showed a significantly impaired osmostress gene expression. These results indicated that intact transcription elongation machinery was necessary for proper transcriptional activation upon osmotic shock as well as to guarantee cell survival under such conditions.

To analyze the role of Hog1 within the Pol II elongation complex, we uncoupled the process of initiation from elongation by creating constructs in which the *STL1* or *CTT1* open-reading frames (prototypical Hog1-dependent osmoresponsive genes) were fused to the LexA promoter. Then, the expression of these genes became constitutive, as it was driven by the LexA-VP16 transcriptional activator. Hog1 association at this artificial system was observed only at the coding region, and not at the LexA promoter, which only contains binding sites for the LexA binding domain, and resulted in proper Hog1-dependent mRNA expression of the target gene upon osmostress treatment. Interestingly, the catalytically inactive Hog1 mutant was not able to bind to the coding region of the *LexA-STL1* construct upon osmostress. Together, these data indicate both that the recruitment of Hog1 to promoters is neither sufficient nor necessary for its recruitment to the coding regions of target genes, and that the kinase activity is important for the functional role of Hog1 in elongation.

Pol II association at the *STL1* ORF was ~2-fold higher in osmostress-challenged cells compared to nontressed cells, and this higher density of the polymerase was dependent on the presence of Hog1. Similarly, the presence of Spt4 elongation factor at the coding region of *STL1* required osmostress induction as well as the MAPK. Thus, these data indicated that

Hog1 is important for the increased association of the RNA Pol II and the Spt4 elongation factor at coding regions of target genes, and therefore, the subsequent stimulation of mRNA production during the elongation process.
To identify the determinants required for the recruitment of Hog1 within coding regions of osmotically induced genes, we performed deletion analysis of the 3'UTR region of the *STL1* gene in the *LexA-STL1* construct. Chromatin IP of Hog1 to these new construct revealed that the 3'UTR was required for Hog1 binding to the coding region of *STL1*. Remarkably, addition of the *STL1* 3' non-coding region to a plasmid containing its wild-type promoter fused to a normally non-osmoresponsive ORF (*STL1::LacZ*) conferred osmotic-stress induced recruitment of Hog1 to the transcribed region of the heterologous gene. Hence, the 3' non-coding region of the osmoresponsive gene was necessary and sufficient for the specific association of the SAPK to coding regions. It is tempting to propose that some feature of the 3' UTR regions (i.e, sequence motifs or structural motifs) specific of osmoresponse inducible genes confers Hog1 association to the corresponding coding regions. Among the possible functions of 3'UTR sequences of osmoresponse inducible genes, there could be that some factors associated with them, and related to mRNA processing, export, translation or other processes, might be helping to stabilize the association of Hog1 with the entire transcribed region during the first rounds of transcription elongation.

My personal contribution to this work was focused on the immunoprecipitation assays that led to the first figure of the article. However, I followed closely the whole work.

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