NOVEL MECHANISMS OF TRANSCRIPTIONAL REGULATION BY THE YEAST Hog1 MAPK

Glòria Mas i Martín

PhD Thesis

Barcelona, 2007

Departament de Ciències Experimentals i de la Salut (CEXS)
Programa de Doctorat en Ciències de la Salut i de la Vida
de la Universitat Pompeu Fabra

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A la meva família

Mare, Pare, Joan i Arnau

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SUMMARY

Adaptation to environmental stress requires changes in many aspects of the cell biology. In eukaryotic cells, stress-activated protein kinases (SAPKs) play an essential role for proper cell adaptation to extracellular stimuli. In *Saccharomyces cerevisiae*, changes in the extracellular osmotic conditions are sensed by the HOG MAPK pathway, which elicits the program for cell adaptation that includes modulation of metabolism, gene expression, translation and cell-cycle progression.

Genome-wide transcriptional studies revealed that control of gene expression in response to stress is a major adaptative response controlled by SAPKs. This work is aimed at understanding the molecular mechanisms by which Hog1 is controlling gene transcription upon osmostress. Previous studies showed that Hog1 regulates transcriptional induction by direct phosphorylation of transcription factors, both activators and repressors modulate factors involved in chromatin remodeling and structure as well as RNA Polymerase II recruitment. However, eukaryotic transcription is a complex process responsible for the production of a pre-mRNA molecule that is subjected to several modifications before being transported to the cytoplasm. This process of transcription by PolII includes preinitiation, initiation, promoter clearance, elongation, and termination. Particularly, the elongation stage of transcription is a highly regulated and dynamic process, as it coordinates multiple steps in mRNA biogenesis and maturation. This work shows for the first time that the Hog1 MAPK interacts with the transcription elongation machinery, selectively travels with elongating PolII through the coding region of stress-responsive genes, and is important for full transcriptional output.

The chromatin remodeling complex RSC plays a significant role in the Hog1-mediated osmostress gene expression, and gives some insights in the functions of Hog1 during elongation. During transcription elongation, one of the roles of Hog1 is to target chromatin remodelling activities to modify chromatin. Here, we show that during osmostress, several histone modifications take place and nucleosome eviction has to occur at promoter and coding regions of osmoresponse genes. Hog1 targets the RSC complex to osmoresponsive genes to mediate nucleosome eviction, which in turn allows for rapid and transient transcriptional induction of osmoresponse genes. Thus, RSC is important for the Hog1-dependent transcriptional response required for cell survival under stress conditions.

INTRODUCTION

1. OSMOTIC STRESS

1.1. Yeasts and their environment

Cells constantly evaluate and respond to their external environment in order to maximize the probability of survival and proliferation. Of particular importance are the mechanisms cells use to respond to sudden and adverse changes in environmental conditions, commonly referred to as cell stresses.

Yeasts such as *Saccharomyces cerevisiae*, are ubiquitous unicellular fungi and hence eukaryotic microorganisms. Living as saprophytes on substrates such as fruits and flowers, these single cell organisms are exposed to a highly variable environment with respect to the availability and quality of nutrients, temperature, pH, radiation, access to oxygen, and water activity (Hohmann and Mager, 1997; Hohmann, 2002). Water activity is of special importance, as it is necessary for maintaining an appropriate cell volume and favorable conditions for biochemical reactions. If yeast cells experience a hyperosmotic shock (or osmotic upshift), there is a rapid water outflow and cell shrinking. Oppositely, a hypoosmotic shock (or osmotic downshift) leads to a rapid water influx, cell swelling and hence increased turgor pressure. Survival mechanisms need to operate within the first seconds after a sudden osmotic shift because passive water loss or uptake occurs very fast (Brown, 1976; Blomberg and Adler, 1992). In the case of a hyperosmotic shock, water loss leads to an increased concentration of biomolecules and ions in the cell, which eventually results in an arrest of cellular activity. However, yeast cells have developed an active mechanism to adapt to high external osmolarity by sensing the osmotic changes and performing the appropriate responses to maintain optimal cellular activity. The mechanisms of osmoadaptation can vary depending on the strength of the stress signals. Accumulation of chemically inert osmolytes, such as glycerol, allows the cells to balance the internal osmolarity with that of the external environment and plays a central role in the process of osmoadaptation (Gustin et al., 1998; Hohmann, 2002; de Nadal et al., 2002). As a consequence, yeast cells can be metabolically active and proliferate over a wide range of external water activity.

1.2. Signalling Pathways Involved in Osmoadaptation

The field of yeast osmoadaptation has received much wider scientific interest with the discovery in 1993 of a mitogen-activated protein kinase (MAP kinase) cascade, known as the HOG pathway (**H**igh **O**smolarity **G**lycerol response pathway). Nonetheless, changes in medium osmolarity also have been shown to activate other signaling pathways in yeast: the protein kinase A pathway and the phosphatidylinositol-3,5-bisphosphate.

The influence of cAMP-dependent protein kinase (PKA) on protein expression during exponential growth under osmotic stress was studied by Norbeck and Blomberg (Norbeck and Blomberg, 2000). Reports suggest that low PKA activity cause a protein expression resembling that of osmotically stressed cells, and thus, PKA is a major determinant of osmotic shock tolerance. However, this pathway mediates a general stress response observed under essentially all stress conditions, such as heat shock, nutrient starvation, high ethanol levels, oxidative stress and osmotic stress (Marchler et al., 1993; Ruis and Schuller, 1995). Therefore, protein kinase A most probably does not respond directly to osmotic changes. In fact, it is not fully understood how the activity of protein kinase A is regulated by stress.

On another hand, the production of phosphatidylinositol-3,5-bisphosphate seems to be stimulated by an osmotic upshift. This molecule could serve as a second messenger in an osmotic signalling (Dove et al., 1997), although its clear function is still to be resolved.

By far the best-characterized osmoresponsive system in eukaryotes is the HOG pathway. It is a MAP kinase cascade consisting of a well-conserved eukaryotic signal transduction module and involved in the process of osmoadaptation (Gustin et al., 1998; Hohmann, 2002; de Nadal et al., 2002). The HOG pathway is activated within less than one minute by osmotic upshift, being essential for cells to rapidly adapt and survive in high-osmolarity medium (Brewster et al., 1993). Thus, the cellular role of the HOG pathway is indeed to orchestrate a significant part of the response of yeast cells to high osmolarity.

2. MAP KINASE PATHWAYS

MAP Kinase pathways are highly conserved signaling units in both higher and lower eukaryotes, where they allow cells to quickly response and adapt to environmental stresses, hormones, growth factors, and cytokines (Kyriakis and Avruch, 2001). MAP kinase pathways control cell growth, morphogenesis, proliferation and stress responses, and they are involved in many disease processes.

1.2. Modular organization

The mitogen-activated protein (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). The MAPKKK activates a MAPKK by phosphorylation on serine and threonine residues within a conserved loop at the N-terminal lobe of the kinase domain. Subsequently, the MAPKK phosphorylates the MAP kinase on a threonine (sometimes serine) and tyrosine residue, which are located adjacent to each other separated by a single aminoacid (Thr/Ser-X-Tyr). This phosphorylation site is located in the activation loop of the catalytic domain; dual phosphorylation on threonine and tyrosine is needed for activation of the MAP kinase. Typically, phosphorylation stimulates transfer of a portion of the activated MAP kinase from the cytosol to the nucleus, where it phosphorylates targets on serine/threonine followed by a proline. Up to date, most of the reported substrates are transcription factors, although MAPK are able to phosphorylate many other substrates including other protein kinases, transcription factors, cytoskeleton-associated proteins and ionic transporters. The MAP kinase remaining in the cytoplasm seems to mediate cytoplasmic events.

In its inactive state, the C-terminal catalytic kinase domain of a MAPKKK is locked by the N-terminal regulatory domain. Activation may occur by phosphorylation or interaction with other proteins, a process that often involves small G-proteins. The activation mechanisms and sensor systems upstream of MAP kinase pathways are diverse and include receptor-tyrosine kinases (in

animal systems), G-protein-coupled receptors, phosphorelay systems, and others.

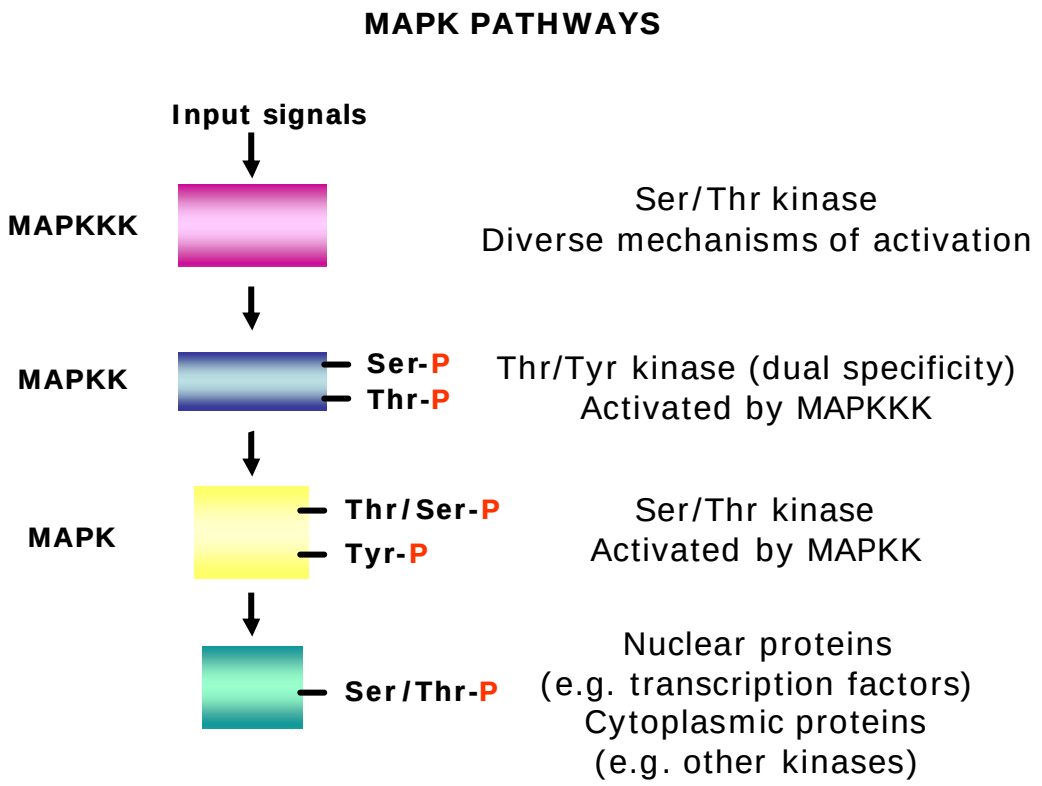

Figure 1. Schematic diagram of a MAPK pathway module. The core module of a MAPK pathway is composed of three kinases; a MAPK kinase kinase, a MAPK kinase and a MAPK, that are sequentially activated by phosphorylation.

2.2. Signalling specificity in MAPK pathways

Different MAPK pathways within the same organism often share protein kinases which are regulated by multiple signalling inputs. Especially in higher eukaryotes but also in *S. cerevisiae*, this situation results in highly complex network systems of signaling pathways. Given this complexity, signalling specificity is achieved by scaffolding proteins. Scaffold proteins bind and sequester selected MAPK pathway components, thereby favoring the rapid signal transmission through the cascade, preventing unwanted crosstalk between MAPK modules, and increasing specificity and selectivity of the signalling by assembly of distinct kinases into distinct MAPK modules. As a result, they ensure the coordinated and efficient activation of MAPK components in response to

specific types of stimuli (Pawson and Scott, 1997). Some scaffold proteins can function as simple adaptors, but in some MAPK pathways the signalling components themselves perform scaffolding functions, such as the yeast MAPKK Pbs2 (see below). Another scaffold and adaptor function could be adscribed to the yeast Ste5 protein, which recruits three protein kinases of the pheromone-signalling pathway into a distinct MAPK module (Printen and Sprague, Jr., 1994).

Besides scaffold proteins, other mechanisms exist to ensure signal specificity, including sensing input systems and MAPK substrate specificity. Substrate selectivity is often conferred by specific MAPK docking sites present on physiological substrates, often at considerable distance from the phosphorylation site in the primary sequence. This allows for a strong interaction with selected MAPK subfamilies and exclusion of others (Kallunki et al., 1996; Yang et al., 1998; Deacon and Blank, 1999; Tanoue et al., 2000). On the other hand, MAPK pathways are negatively controlled by protein phosphatases acting on both the MAPKK and the MAPK (serine-threonine phosphatases) or only on the MAPK (tyrosine phosphatases) (Keyse, 2000).

2.3. Yeast MAPK pathways

S. cerevisiae has five MAP kinases. Based on genetic analyses as well as studies on the transcriptional readout upon physiological, pharmacological and genetic stimulation, the five MAP kinases are allocated to five distinct MAP kinase cascades (Hohmann, 2002) (Figure 2):

(i) The mating pheromone response pathway (MAP kinase Fus3p)
(ii) The pseudohyphal development pathway (Kss1p)
(iii) The HOG pathway (Hog1p)
(iv) The protein kinase C (PKC) or cell integrity pathway (Slt2/Mpk1p)
(v) The spore wall assembly pathway (Smk1p)

These MAP kinase pathways are required for directed cell growth (bud formation, mating projections, and pseudohyphal growth), remodeling of the cell surface associated with growth (cell wall integrity, cell integrity, and HOG pathway), and maintenance of the appropriate turgor pressure (HOG and cell integrity pathways). The Smk1 MAPK regulates sporulation (Krisak et al., 1994). Overall, yeast MAPK pathways have roles in response to developmental and external stimuli, as well as on cell cycle control.

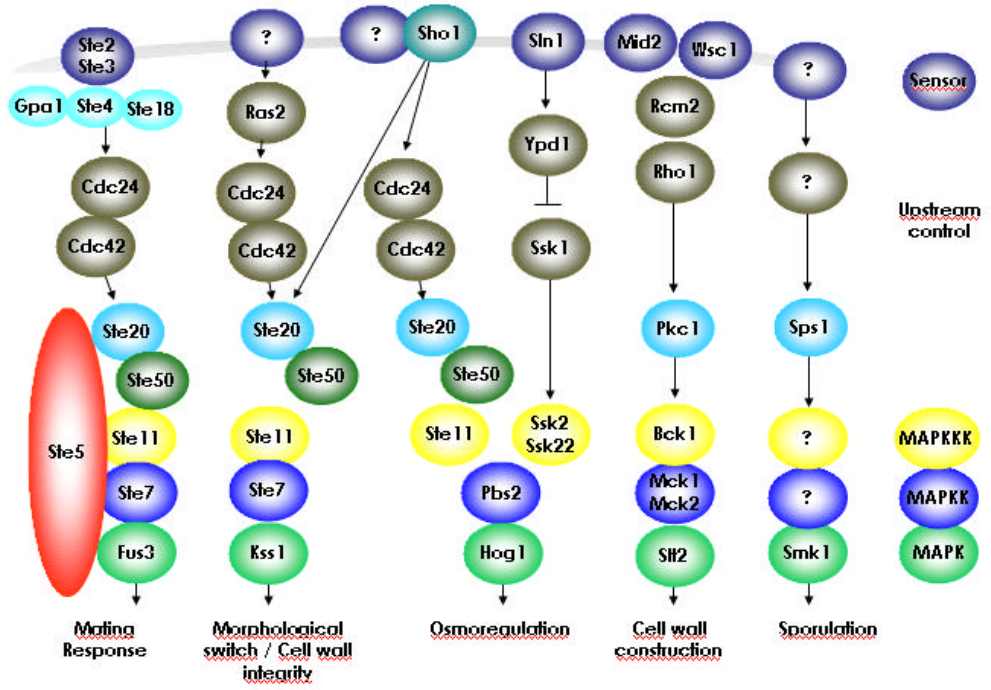

Figure 2. *S.cerevisiae* MAP kinase pathways. *S. cerevisiae* contains five MAPK pathways: mating response, filamentation-invasion, osmoregulation, cell wall integrity and sporulation pathways. Some of the elements are shared between pathways.

3. THE HOG PATHWAY

3.1 Components and organization

The HOG pathway is the best-characterized and understood osmoresponsive system in eukaryotes. Exposure of cells to increased extracellular osmolarity results in rapid activation of the yeast MAPK Hog1, which elicits the program for cell adaptation required for survival under these conditions. Activation of this pathway leads to a set of osmoadaptative responses, including metabolic regulation, cell cycle progression, translation and gene expression regulation (de Nadal et al., 2002; Sheikh-Hamad and Gustin, 2004). The Hog1 MAPK is a prototype of the Stress-Activated Portein Kinases (SAPK) family, equivalent to the mammalian p38 and c-Jun N terminal kinase. The functional conservation between the HOG pathway and the p38 pathway is illustrated by the fact that the corresponding human gene complements the

yeast mutants in the HOG pathway (Galcheva-Gargova et al., 1994; Han et al., 1994; Derijard et al., 1995).

Schematically, the central core of the yeast HOG pathway comprises a layer of MAPKKKs, Ssk2, Ssk22 (Maeda et al., 1995) and Ste11 (Posas and Saito, 1997) that are responsible for the activation of the MAPKK Pbs2. Once activated, Pbs2 phosphorylates and activates the Hog1 MAPK (Figure 3). The pathway is activated predominantly by two independent mechanisms that lead to the activation of either the Ssk2 and Ssk22 or the Ste11 MAPKKKs, respectively. The first mechanism involves a 'two-component' osmosensor, composed of the Sln1-Ypd1-Ssk1 proteins. The second mechanism involves the transmembrane protein Sho1, the MAPKKK Ste11, the Ste11-binding protein Ste50, the Ste20 p21-activated kinase (PAK) and the small GTPase Cdc42 (Posas *et al.*, 1998; Maeda et al, 1995; reviewed in de Nadal et al, 2002) (Figure 3).

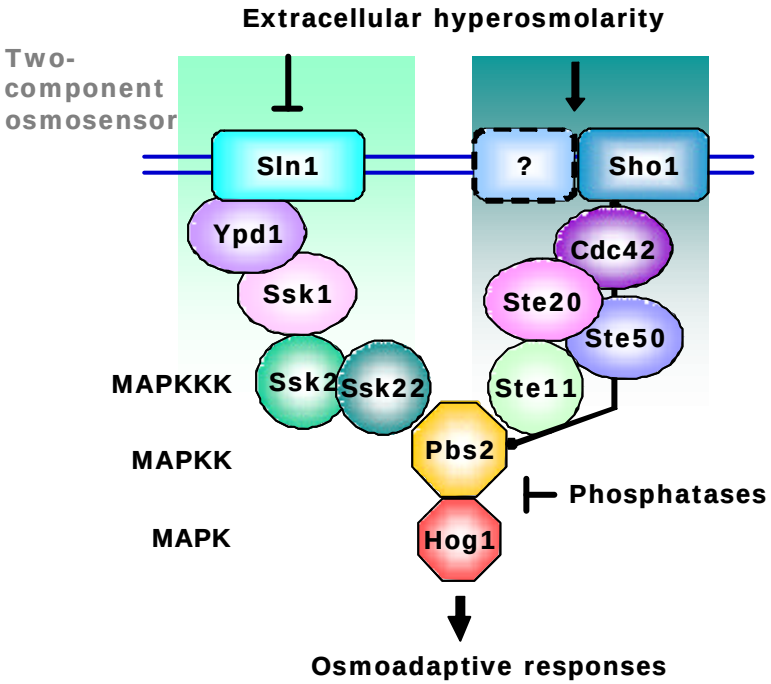

Figure 3. Schematic diagram of the yeast HOG pathway. Two major independent upstream osmosensing mechanisms lead to the activation of specific MAPKKKs and converge on a common MAPKK, Pbs2. Under osmostress, activated Pbs2 activates the MAPK Hog1, which induces a set of osmoadaptive responses.

3.2. Osmostress sensors

3.2.1. The Sln1 branch

The Sln1 branch involves a "two-component" osmosensor: a sensor molecule and a response-regulator molecule. Typically, a sensor protein has an extracellular input domain and a cytoplasmatic histidine kinase domain. On the other hand, a response-regulator is a cytosolic protein containing the receiver domain and a DNA binding domain. Upon activation, the sensor protein phosphorylates a histidine residue within its kinase domain and transfers this phosphate group to an aspartic acid in the receiver domain of the response-regulator molecule, resulting on its function activation. In particular, the Sln1 branch is composed by the primary osmosensor Sln1, the phosphorelay protein Ypd1, and the response regulator protein Ssk1. Sln1 is a two-component system itself, since it contains both a histidine kinase domain and a receiver domain (Ota and Varshavsky, 1993). However, the Ypd-Ssk1 pair functions as a second two-component system. At normal conditions, the osmosensor Sln1 autophosphorylates itself, this phosphate being sequentially transferred to Ssk1. Phosphorylation activates the response regulator Ssk1 which, in turn, represses the activity of two redundant MAPKKs: Ssk2 and Ssk22. At high osmolarity, the Sln1p histidine kinase is inhibited, resulting in an accumulation of unphosphorylated Ssk1p, which then interacts with Ssk2/Ssk22 MAPKKKs to activate the HOG cascade (Posas et al., 1996). Thus, *SLN1* and *YPD1* gene disruption causes lethality, due to the resulting constitutive activation of the HOG pathway (Maeda et al., 1994).

3.1.2. The Sho1 branch

The Sho1 branch is involved in an alternative sensor mechanism that leads to Pbs2 activation independently of the Sln1 branch (Maeda et al., 1995). Sho1 contains four transmembrane domains and a COOH-terminal cytoplasmic region with a Src homology 3 (SH3) domain (Maeda et al., 1995).

Activation of the Sho1 branch involves 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). The complex consists of at least Sho1 and Pbs2. These two proteins interact via a proline-rich region in the N terminus of Pbs2

and the SH3 domain located in the hydrophilic C terminus of Sho1 (Maeda et al., 1995; Posas and Saito, 1997). The complex also contains the PAK Ste20, the Rho-like G protein Cdc42 (Raitt et al., 2000; Reiser et al., 2000) and the MAPKKK Ste11 (Posas and Saito, 1997; O'Rourke and Herskowitz, 1998) as well as Ste50, required for Ste11 function (Jansen et al., 2001) (O'Rourke and Herskowitz, 1998; Posas et al., 1998).

At present, the initial signalling event of this branch is still unknown. Since Sho1 does not seem to function as a sensor itself (Raitt et al., 2000), additional proteins are probably required for this event. In high osmolarity conditions, Sho1 binds Pbs2 and thereby recruits it to the cell surface. Then Cdc42 is recruited and activated. Cdc42 not only binds and activates the PAK-like kinase Ste20, but also binds to the Ste11-Ste50 complex through a conserved C-terminal RAS-association (RA) domain in Ste50, to bring activated Ste20 to its substrate Ste11 (Truckses et al., 2006; Tatebayashi et al., 2006). Activated Ste11 and its HOG pathway-specific substrate, Pbs2, are brought together by Sho1; the Ste11-Ste50 complex binds to the cytoplasmic domain of Sho1 (Zarrinpar et al., 2004), to which Pbs2 also binds. Then, Ste11 activates Pbs2, which in turn, activates Hog1 (Posas and Saito, 1997). Thus, Cdc42, Ste50 and Sho1 act as adaptor proteins that control the flow of the osmostress signal from Ste20 to Ste11 and then to Pbs2.

On the other hand, the MAPKKK Ste11 is used in three functionally distinct MAPK cascades in yeast (Posas and Saito, 1997) (see Figure 2). The ability of Ste11 to function in separate pathways requires stable associations with pathway-specific proteins. For example, Ste11 interacts with the pheromone response pathway-specific scaffold protein Ste5 (Choi et al., 1994; Inouye et al., 1997); (Printen and Sprague, Jr., 1994; Marcus et al., 1994b). In the HOG pathway, the MAPKK Pbs2 serves as a scaffold protein, interacting with Ste11 (Posas and Saito, 1997) and Sho1 (Maeda et al., 1995).

3.2.3. Specific roles of Sln1 and Sho1 branches

Genetic evidences suggest that the upstream branches of the HOG pathway operate independently of each other; blocking one branch of the pathway still allows rapid Hog1 phosphorylation upon an osmotic shock, and such cells are apparently fully resistant to high osmolarity. Although these

observations suggest redundant functions of the two branches, it is unlikely that the cell maintains two different complex pathways to activate Pbs2. It has been proposed that different sensitivities of the two branches may allow the cell to respond over a wide range of osmolarity changes (Maeda et al., 1995). Data suggest that Sln1 is more sensitive than the sensor of the Sho1 branch. It also appears that the Sho1 branch operates in an on-off fashion, while the Sln1 branch shows an approximately linear dose response up about 400 mM NaCl.

Taken together, the observed different sensitivities and responsiveness of the two branches may rather reflect different mechanisms of stimulation, i.e., the two branches may interpret osmotic changes via different physical stimuli, such as membrane stretch, cell wall stress, or impact on the cytoskeleton. If so, it should be possible to find conditions under which only one of the two branches is activated. For instance, components of the Sho1 branch are localized or recruited to places of active cell growth (Raitt et al., 2000; Reiser et al., 2000) indicating that it plays a specific localized role in osmosensing during cell growth and expansion. Meanwhile, the Sln1 branch would be implicated in sensing osmotic changes in the environment (Hohmann, 2002).

Besides the Sln1 and the Sho1 branches, a third putative osmosensing branch working in parallel with the Sho1 branch has been proposed. The observation that in the absence of Sho1 some residual Ste11-dependent signalling still occurs lead to propose the existence of another osmosensor upstream of Ste11. This new branch requires the membrane protein Msb2 (O'Rourke and Herskowitz, 2002). A role for Msb2 has been seen in strains defective in the two known branches that activate Pbs2: an *ssk1*Δ *sho1*Δ *msb2*Δ strain is more osmosensitive than an *ssk1*Δ *sho1*Δ *MSB2* strain. However, the biological importance of Msb2 is not clear yet.

3.3. Signalling through the HOG pathway

Downstream of the sensor systems, any of the MAPKKKs Ssk2p/Ssk22p and Ste11p, is able to activate the MAPKK Pbs2 by phosphorylation on Ser514 and Thr518. Pbs2 is a cytoplasmic protein and appears to be excluded from the cytoplasm, and thus phosphorylation of its substrate, the Hog1 MAPK, occurs in the cytosol. Dual phosphorylation on the conserved Thr174 and Tyr176 activates Hog1 (Brewster et al., 1993; Schuller et al., 1994). Phosphorylation on both

sites is necessary and sufficient to cause a rapid and marked concentration of Hog1 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). The catalytic activity of Hog1, however, is not required for transfer to the nucleus, since a catalytically inactive mutant of Hog1 is transferred to the nucleus very much like the wild-type. Both phosphorylation and nuclear localization of Hog1 are transient effects: under mild osmotic stress (0.4M NaCl) Hog1 phosphorylation peaks within 1 minute and disappears within about 30 minutes. However, the more severe the osmotic shock, the longer it takes until active Hog1p is dephosphorylated and activated (Rep et al., 1999; Van Wuytswinkel et al., 2000).

Nuclear accumulation of Hog1 suggests that an important part of Hog1 functions take place in the nucleus. However, a portion of activated Hog1 remains in the cytosol where it mediates regulatory effects. Among the best documented of such effects is the activation of the protein kinase Rck2 (Bilsland-Marchesan et al., 2000; Teige et al., 2001), which controls translation efficiency (Teige et al., 2001), and the phosphorylation of ion transporters, essential for the rapid reassociation of proteins, previously dissociated from chromatin due to osmotic stress (Proft and Struhl, 2004).

3.4. Modulation and feedback control of the HOG pathway

The HOG pathway is controlled by specific feedback events, as indicated by the transient phosphorylation and activation of the MAP kinase (Maeda et al., 1994; Jacoby et al., 1997; Tamas et al., 2000) and the modulation of the responses depending on the strength of the osmostress input.

One of these feedback mechanisms include two phosphotyrosine phosphatases (Ptp2 and Ptp3), as well as three phosphoserine/threonine phosphatases (Ptc1 to Ptc3). Overexpression of any of these phosphatases suppresses the lethality caused by inappropriate activation of the pathway (reviewed in Hohmann, 2002). 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. Specifically, Ptp2 and Ptp3 interact

directly with phosphorylated Hog1 and dissociate rapidly from the kinase after dephosphorylation. Ptp2 seems to be more important for Hog1 dephosphorylation than Ptp3, possibly because Ptp2 is predominantly nuclear, as is activated Hog1, while Ptp3 is located in both the cytosol and the nucleus (Mattison et al., 1999). Active Hog1 enhances the Ptp2 activity (Wurgler-Murphy et al., 1997) and induces Ptp3 expession (Jacoby et al., 1997), therefore existing a negative feedback loop between these proteins. A catalytically inactive Hog1 mutant, when phosphorylated by active Pbs2, remains tyrosine-phosphorylated much longer than wild-type Hog1, apparently because there is no induction of PTP activities (Wurgler-Murphy et al., 1997).

Since in the *ptp2*Δ *ptp3*Δ double mutant the level of tyrosine-phosphorylated Hog1 is still responsive to osmotic shock, additional dephosphorylation mechanisms must exist (Jacoby et al., 1997; Wurgler-Murphy et al., 1997). Ptc1 inactivates the pathway by dephosphorylating the MAPK phosphothreonine *in vitro* (Warmka et al., 2001) indicating that Ptc1 is perhaps the main phosphatase that acts on the phosphothreonine in Hog1. Moreover, it has been described that Ptc1 is recruited to the Pbs2-Hog1 complex by the Nbp2 adapter (Mapes and Ota, 2004) and this recruitment is essential for Ptc1 to work as a negative regulator of Hog1.

4. PHYSIOLOGICAL ROLES OF HOG1

Once activated, Hog1 elicits the program for cell adaptation to osmotic stress, which includes modulation of several aspects of cell biology essential for cell survival, such as gene expression, cell cycle progression, protein synthesis and metabolic adaptation (Figure 4).

4.1. Metabolic adaptation

As mentioned above, one of the roles of Hog1 upon osmotic stress is metabolic adaptation (Figure 4). Among the targets of the HOG pathway are important transcription factors such as Sko1 and Msn2/4. These transcription factors induce the expression of osmolyte-synthesizing genes, e.g. *GPD1* (encoding glycerophosphate dehydrogenase 1) and *TPS2* (encoding trehalose

phosphate phosphatase) (Ruis and Schuller, 1995). The resulting increase in the levels of the compatible osmolytes glycerol and trehalose leads to the replacement of excessive inorganic ions and the restoration of intracellular electrolyte homeostasis in situations of hyperosmotic stress. Moreover, activation of the HOG pathway upon osmotic stress leads to phosphorylation and activation of the 6-phosphofructo-2-kinase (PF2K) (Dihazi et al., 2004). This activation causes a stimulation of the upper part of glycolisis, a precondition for glycerol accumulation. Yeast cells containing *PF2K* accumulate three times more glycerol than cells lacking *PF2K*, which are not able to grow as efficiently under hypertonic stress (Dihazi et al., 2004).

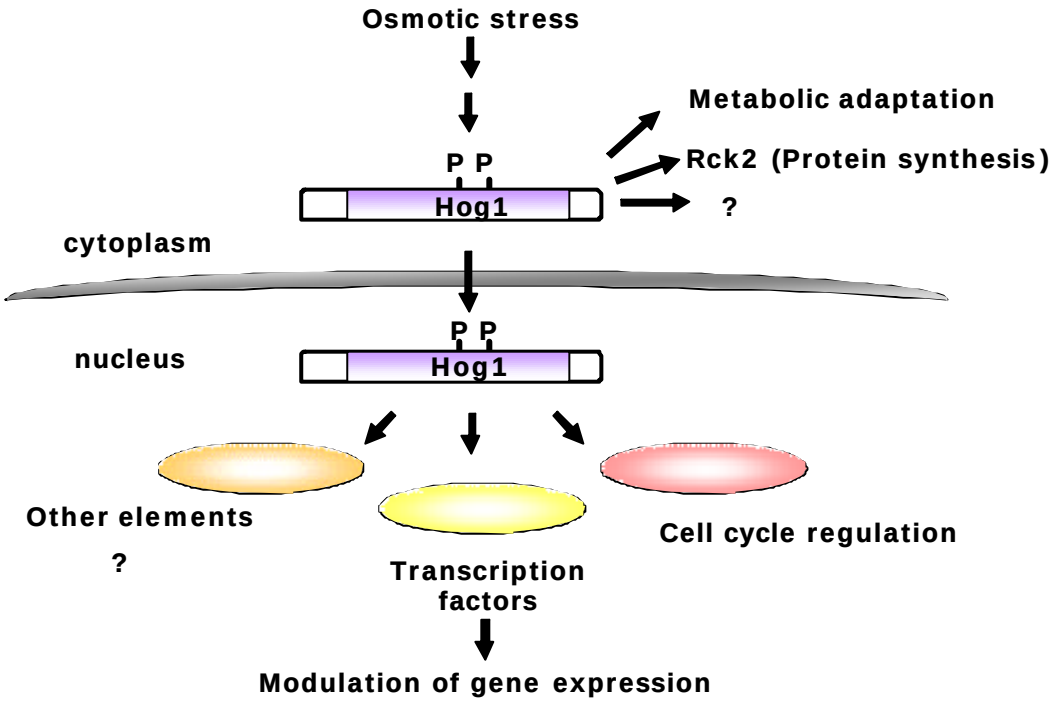

Figure 4. Functions of the MAPK Hog1. Once Hog1 is phosphorylated and activated, it controls several functions, such as cell cycle regulation, modulation of gene expression, metabolic adaptation and regulation of protein synthesis.

4.2. Regulation of protein synthesis

In response to increases in external osmolarity, there is a transient decrease in protein synthesis (Norbeck and Blomberg, 1998; Uesono and Toh, 2002) caused by a decrease in amino-acid uptake, repression of ribosomal protein genes and a decrease in translation efficiency. The HOG pathway

appears not to be involved in the initial inhibition of translation, but rather in the reactivation of translation under stress as an adaptation mechanism (Uesono and Toh, 2002). The yeast Rck2 kinase, which is a member of the calmodulin protein kinase family, is targeted by Hog1 (Bilsland-Marchesan *et al*., 2000; Teige *et al*., 2001). Rck2 affects translation by directly regulating the elongation factor EF-2, as it phosphorylates and thereby inhibits this translation elongation factor (Bilsland-Marchesan et al., 2000; Teige et al., 2001). 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 required for stress adaptation is stimulated and their translation has to be ensured. This suggests that mechanisms must exist that allow the preferential translation of subsets of mRNAs under certain conditions, but little is known about the molecular bases underlying this phenomenon.

4.3. Regulation of cell cycle progression

Progression through the cell cycle is critically dependent on the presence of nutrients and stress stimuli. In response to osmostress, Hog1 mediates a transient cell-cycle arrest to allow cell adaptation.

In *S. cerevisiae*, 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. This regulation is possible due to diverse checkpoints that 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 yeast, Cdc28 is the unique CDK (Cyclin Dependent Kinase) that controls cell cycle progression, and cyclins are the proteins responsible for binding and activating the CDK. Cdc28 cyclins can be G1 cyclins (Cln1, Cln2 and Cln3), that regulate events during the interval between mitosis and DNA replication, and B-type cyclins (Clb1- Clb2, Clb3-Clb4 and finally Clb5-Clb6), that control DNA replication until cytokinesis (Figure 5). Cdc28 activity can be regulated through the synthesis and degradation of cyclins, through association with CDK inhibitors (Sic1 and Far1) (Figure 5), and through phosphorylation and dephosphorylation of Cdc28 by Swe1 and Mih1.

In (Clotet et al., 2006), it is proposed a novel regulatory mechanism of the 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 Hsl1 kinase. Upon osmotic stress, Hog1 phosphorylates Ser1220 of Hsl1, which is the checkpoint kinase (Shulewitz et al., 1999; Cid et al., 2001) promoting delocalization of Hsl7 from the bud neck. This prevents Swe1 from being recruited to the bud neck and from being phosphorylated, which leads to Swe1 accumulation and G2 arrest. 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).

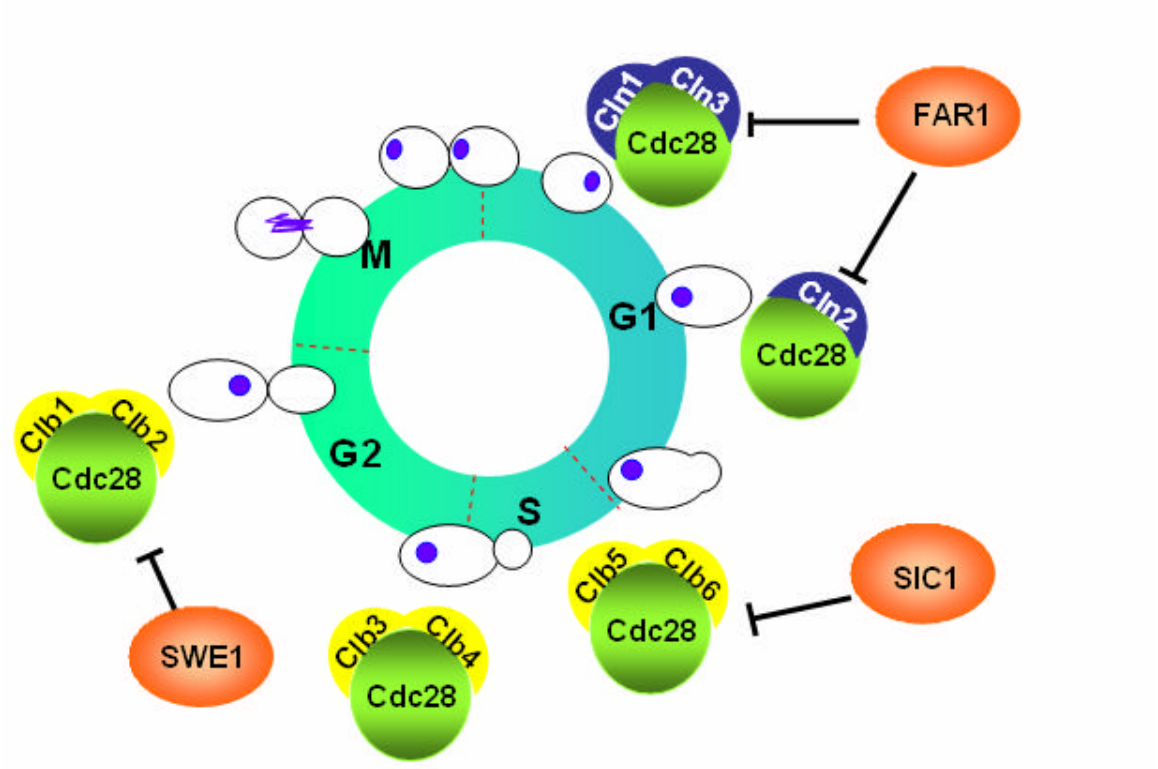

Figure 5. Schematic diagram of *S. cerevisiae* cell cycle. Cdc28 associates to different cyclins at different phases of the cell cycle. Here it is illustrated the presence of bud and position of the nuclei along the cell cycle.

4.4. Regulation of gene expression

In *S. cerevisiae*, genome-wide transcription studies revealed that a large number of genes (~ 5-7%) show significant changes in their expression levels after a mild osmotic shock and that the Hog1 MAPK plays a key role in much of this global gene regulation (Posas et al., 2000; Rep et al., 2000; Gasch et al., 2000; Causton et al., 2001). Osmostress-regulated genes are implicated in carbohydrate metabolism, general stress protection, protein biosynthesis and signal transduction. Another conclusion drawn from these genome-wide studies was that different stress conditions (such as time of exposure to salt and osmolyte concentration) result in a different pattern of expression, i.e, delayed transcriptional response. This PhD thesis intends to give insights into the role of Hog1 in the regulation of gene expression.

5. REGULATION OF TRANSCRIPTION BY Hog1

5.1. The Eukaryotic Transcription cycle

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 exclusively sinthetizes ribosomal RNA genes, RNA polymerase II is used to transcribe most of protein-coding genes, and the RNA polymerase III is responsible for the transcription of tRNA genes, the 5S RNA genes and the snRNAs.

The RNA Polymerase II itself is a large multisubunit enzyme (with a mass of about 0.5 MDa) composed of 12 different proteins encoded by the genes *RPB1* to *RBP12*. All of them are essential, except for *RPB4* and *RPB7* (Woychik and Young, 1989). Subunits Rpb1 and Rpb2 are the largest and most evolutionarily conserved, and form a central core which contains the catalytic site of the enzyme. A unique feature of the RNA polymerase II is the C-terminal domain (CTD) of Rpb1, formed by multiple repeats of the heptapeptide sequence YSPTSPS (Figure 6). The number of these repeats increases with genomic

complexity: 26 in yeast, 32 in *Caenorhabditis elegans*, 45 in *Drosophila*, and 52 in mammals. Studies using functional assays together with specific antibodies revealed the existence of a hypo- and hyperphosphorylated form of RNA Polymerase II. The hypophosphorylated form is recruited to promoters binding to General Transcription Factors (GTFs), whereas transcription-competent RNAP II is heavily phosphorylated on its CTD (Sims et al., 2004). Moreover, the CTD residues that become phosphorylated vary along the transcription process (O'Brien et al., 1994; Komanirtsky et al, 2000). RNAP II phosphorylated predominantly at Ser 5 of the heptapeptide is associated with promoter-proximal regions of transcribed genes, correlating with transcription initiation and early elongation (promoter clearance), and is apparently not detected at the 3'-end region. At the same time, the amount of the enzyme phosphorylated at Ser 2 increases toward the 3'-end of genes. It is now established that the RNAP II CTD plays a central role in the regulation of transcription initiation and elongation, as it recruits protein factors involved in elongation, as well as in mRNA maturation, surveillance, and export (Hirose and Manley, 2000; Orphanides and Reinberg, 2002; Proudfoot et al., 2002). Moreover, these processes are known to occur cotranscriptionally, and many events during the synthesis of a mature mRNA are coregulated (Maniatis and Reed, 2002; Orphanides and Reinberg, 2002).

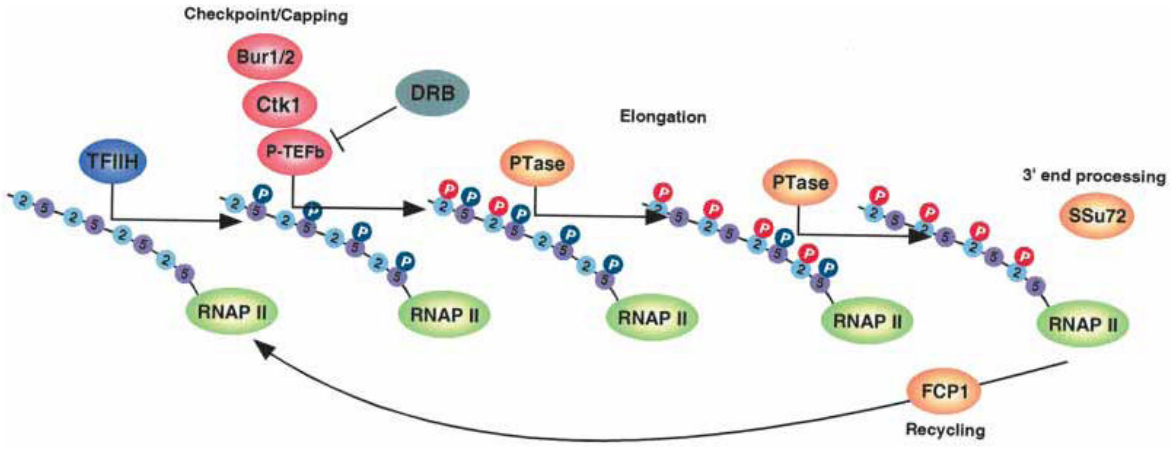

Figure 6. The phosphorylation cycle of the CTD of the large subunit of RNA Polymerase II. Initially, the unphosphorylated RNAP II CTD is targeted on Ser5 by the Kin28 subunit of TFIIH. Early after transcription initiation, the TEC (Transcription Elongation Complex) is arrested at a "checkpoint" to ensure proper mRNA capping. Release from this checkpoint involves the kinase action of P-TEFb (Bur1/2 or Ctk1 in yeast), which targets Ser2 on the CTD. Subsequently during elongation, protein phosphatases (Ssu72 and FCP1) dephosphorylate Ser 5 residues within the CTD and mediate recycling of the RNAP II for subsequent rounds of transcription. (Source: Sims et al., 2004).

5.2. The transcription initiation machinery

Transcription regulation of eukaryotic protein-coding genes is an orchestrated process that requires the concerted functions of multiple proteins, including sequence-specific DNA-binding transcription factors, general/basal transcription factors and coactivators and corepressors.

5.2.1. Transcription factors under the control of Hog1

Transcription factors are elements that bind to proximal promoter elements and/or more distal regulatory sequences (i.e., enhancers and silencers) and modulate the rate of transcription of specific target genes in a tissue and developmental stage specific manner or in response to physiological or environmental stimuli.

SAPKs can modify gene regulation by direct phosphorylation of some transcription factors, both activators and repressors. In yeast, five transcription factors have been proposed to be controlled by the Hog1 MAPK: the stress-generic and redundant zinc finger proteins Msn2 and Msn4 (Schuller et al., 1994), Hot1 (which does not belong to a known family of transcription factors) (Rep et al., 1999b), the bZIP protein Sko1 (Proft et al., 2001; Proft et al., 2005; Alepuz et al., 2001) and the MADS box protein Smp1 (de Nadal et al., 2003). Therefore, depending on the promoter context, Hog1 can function through a variety of structurally unrelated transcription factors. Each factor seems to be controlling a small subset of the osmoresponsive genes and thus, deletion of a particular transcription factor has a very limited effect on general osmostress gene expression. Still remains the possibility that additional transcription factors are required for gene expression upon stress.

Besides phosphorylating transcription factors, Hog1 can associate with chromatin at promoter regions of target genes through interaction with specific transcription factors upon osmotic stress (Alepuz et al., 2001). In fact, Hog1 has been shown to play several roles when associated with promoter regions: as a derepressor (as it alleviates negative regulation by the Sko1 transcription factor), as a delivery vehicle for unbound activators to the promoter, as initiator of transcription factors' transactivation function, as a structural adaptor to the

general transcriptional machinery, and as a kinase to phosphorylate and activate components of the general transcription system (Alepuz et al., 2001).

5.2.2. General Transcription Factors (GTFs)

The general (or basal) transcription machinery is ubiquitous and is composed of Pol II and six GTFs, which include TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. Despite its complexity, Pol II is unable, on its own, to recognize promoters and accurately initiate transcription. Specific transcription initiation by Pol II requires the recognition by GTFs of various core promoter DNA elements (which include the TATA and the initiator (INR)) and the cooperative assembly of GTFs and Pol II into a pre-initiation complex (PIC) at the core promoter (Orphanides et al., 1996; Hampsey, 1998). The first step of PIC formation involves the binding of TFIID to the core promoter. TFIID is a stable complex composed of the TATA-binding protein (TBP) and 14 distinct TBP-associated factors TAFs, which are phylogenetically conserved (reviewed in Burley and Roeder, 1996; Green, 2000; Martinez, 2002). The binding of TFIID to the core promoter is stabilized by TFIIA. TFIIB further stabilizes the TBP-TATA complex and allows the recruitment of TFIIF, Pol II, TFIIE and TFIIH. TFIIE has a role in recruiting TFIIH and in stimulating its activity. Ser5 of the CTD of Rpb1 is then phosphorylated by the kinase activity of TFIIH (Cdk7 in mammalian cells, Kin28 in *S. cerevisiae*) (Feaver et al., 1994; Valay et al., 1995), a step that facilitates promoter clearance by disrupting interactions of the CTD with components of the PIC.

After PIC formation, transcription initiation and promoter clearance, the CTD-phosphorylated Pol II in association with TFIIF elongates downstream of the gene, while TFIIA and TFIID remain bound to the core promoter. Thus, subsequent rounds of transcription initiation do not require *de novo* recruitment of TFIIE, TFIIH or the Pol II holoenzyme (Yudkovsky et al., 2000). Furthermore, admission of the transcription initiation complex requires the modification and remodelling of the nucleosomal structure underlying the respective gene and its promoter (see below). Finally, transcription initiation is also regulated at the level of PIC formation at core promoters, due to many distinct PICs with varying composition and distinct promoter recognition (Muller and Tora, 2004).

In addition to the mentioned role of the MAPK Hog1 in regulating transcription factor activity by direct phosphorylation, several studies demonstrate that the MAPK can associate with chromatin at promoter regions of target genes through interaction with the transcription factors and other components of the transcription initiation machinery (reviewed in de Nadal et al., 2002). In fact, the presence of Hog1 in osmoresponsive promoters is important for inducing gene expression because it facilitates the recruitment of components of the transcription initiation complex and the RNA Polymerase II itself (Proft and Struhl, 2002; Alepuz et al., 2003; Alepuz et al., 2001).

5.2.3. Coactivators and corepressors

Despite the complexity of the basal transcription machinery described above, sequence-specific DNA-binding regulators (activators and repressors) are generally unable to significantly modulate the rate of transcription from their target promoters in systems reconstituted with highly purified GTFs and Pol II, and require the functions of a variety of so-called cofactors (i.e., **coactivators** and **corepressors**). Many of them modulate the activity of the GTFs and Pol II, or manipulate the structure of chromatin. Coactivators are generally recruited to specific promoters via interactions with either regulators and/or components of the basal transcription machinery. Among the most important activators regulated by Hog1 are the Mediator, the SAGA complex and the Rpd3 deacetylase complex.

- **Mediator**

There is considerable evidence that the Mediator complex is a key, and perhaps, the major, target of activator proteins within the basic Pol II machinery (Malik and Roeder, 2000; Myers and Kornberg, 2000). The role of Mediator as a bridge between gene-specific regulatory factors and the general transcriptional machinery allows it to act as a global regulator of transcription and thus, several its subunits are essential for general Pol II transcription in yeast cells. Besides to its role as a scaffold for PIC formation, recent studies suggest that the Mediator could be important in the CTD phosphorylation cycle (Biddick and Young, 2005). Interestingly, cells deficient in mediator subunits are osmosensitive and display

dramatically reduced transcription upon stress, thus mediator is essential to induce gene expression under osmostress conditions (Zapater et al., 2007).

- **The SAGA complex**

SAGA is a large multi-subunit complex containing several enzymatic activities physically linked to activators and nucleosome histones and involved in core promoter selectivity *in vivo*. As seen for Mediator, the SAGA complex is as novel regulator of osmostress-mediated transcription. Interestingly, whereas Mediator was found to be essential for osmostress gene expression, the requirement for SAGA was selective under severe osmotic conditions. This indicates that the requirement for a transcriptional complex to regulate a promoter might be determined in function of the strength of the stimuli perceived by the cell through the regulation of interactions between transcriptional complexes (Zapater et al., 2007).

- **Histone deacetylases: the Rpd3 complex**

The *S. cerevisiae* Rpd3 histone deacetylase regulates the transcription of a wide range of genes by deacetylating mainly histones H3 and H4 (Bernstein et al., 2000). There are two known Rpd3 complexes within *S. cerevisiae* that were identified primarily by their gel filtration elution profiles (Rundlett et al., 1996; Kasten et al., 1997; Lechner et al., 2000): the small Rpd3S complex (0.6 MDa) and the large Rpd3L complex (1.2 MDa). The subunit composition of both complexes has recently been identified. Both complexes contain Rpd3, Sin3 and Ume1. The Rpd3L complex also contains the proteins Pho23, Sap30, Sds3, Cti6, Rxt2, Rxt3, Dep1, Ume6, and Ash1. The Rpd3S complex also contains Rco1 and Eaf3 (Carrozza et al., 2005). Rpd3L and Rpd3S are functionally distinct. The majority of Rpd3-dependent effects on gene expression are due to the Rpd3L complex, which is recruited to promoters. In contrast, the Rpd3S complex is recruited to coding regions through the Eaf3 subunit to repress intragenic transcription through histone deacetylation (Carrozza et al., 2005; Keogh et al., 2005).

Histone deacetylation has been traditionally associated with repression of gene expression (Robyr et al., 2002). However, a genome-wide study of histone

deacetylase function in yeast showed that *RPD3* deletion leads to down-regulation of more than 200 genes (Bernstein et al., 2000). These deletion profiles do not address whether down-regulated genes are direct targets of HDAC-mediated activation or are secondary effects. However, reverse chemical genetic experiments with the HDAC inhibitor TSA indicate that some may be direct targets of Rpd3 activation. In addition, deletion of *RPD3* or *SIN3* results in enhanced gene silencing at HMR, ribosomal loci and telomeres (Sun and Hampsey, 1999; Bernstein et al., 2000). Furthermore, the histone deacetylase Hos2, has been reported to be essential for inducing the expression of the genes *INO1* and *GAL1* (Wang et al., 2002).

Rpd3 can be recruited to gene promoters by at least two mechanisms. One involves direct recruitment to promoters through contacts with DNA binding proteins. On the *INO1* promoter the sequence-specific transcription repressor Ume6 recruits Rpd3 through interaction with the Rpd3 associated protein Sin3 (Kadosh and Struhl, 1997; Rundlett et al., 1998). The second recruitment mechanism involves Rpd3 suppression of genome wide histone acetylation levels that is independent of sequence-specific repressor proteins (Kurdistani and Grunstein, 2003).

Interestingly, one of the mechanisms by which Hog1 regulates gene expression involves the recruitment of the Rpd3 histone deacetylase complex to the promoters of genes regulated by osmostress. Cells lacking the Rpd3-Sin3 histone deacetylase complex are sensitive to high osmolarity and show compromised osmostress gene expression. Moreover, Hog1 physically interacts with Rpd3 *in vivo* to target the deacetylase to specific osmoresponsive genes. Binding of the Rpd3-Sin3 complex to specific promoters leads to local histone deacetylation, entry of RNA polymerase II and induction of gene expression. Thus, targeting of the Rpd3 histone deacetylase to osmoresponsive promoters by the MAPK Hog1 is required to induce gene expression under stress conditions (de Nadal et al., 2004).

5.3. The Transcription Elongation Complex

Transcription elongation is a complex process involving different factors that regulate RNA Polymerase II progression and thus, gene expression (Orphanides and Reinberg, 2002; Price, 2000). The phosphorylation status of

the CTD determines the transition between initiation and elongation. In *S.cerevisiae*, four protein kinases are able to phosphorylate the CTD: Cdk7/Kin28, which is part of the TFIIH (Cismowski et al., 1995; Feaver et al., 1994); Srb10, in the Mediator complex (Liao et al., 1995); Ctk1, catalytic subunit of CTDK1 complex (Lee and Greenleaf, 1991) and Bur1. All of them are cyclin-dependent kinases whose activity occur in different stages of the transcriptional process, and phosphorylate different residues of the CTD either to repress or activate transcription. Kin28 and Bur1 are responsible for phosphorylating Ser 5 in the initiation stages as well as early elongation, whereas Ctk1 phosphorylates Ser 2 at late elongation.

Efficient transcript elongation must overcome several blocks derived from the intrinsic catalytic activity of the RNA P II and its chromatinized template. There are three principle impediments to transcript elongation, which include transcriptional pause, arrest and termination (Uptain et al., 1997; Shilatifard et al., 2003). Many of the identified elongation factors that have been ascribed a mechanistic function serve to counteract or alleviate these blocks. Transcriptional pausing occurs when the RNA polymerase halts the addition of NTPs to the nascent RNA transcript for a time before resuming productive elongation on its own (Sims et al., 2004). Transcriptional arrest can be defined as an irreversible halt to RNA synthesis, whereby the RNA polymerase cannot resume productive elongation without accessory factors. During termination, the RNA polymerase and RNA transcript are released from the DNA, effectively ending the elongation stage of transcription. Transcriptional pause and arrest *in vivo* are most likely caused by a combination of identifiable DNA sequences, protein factors, and the nascent transcript.

5.3.1. Factors that influence transcriptional pausing

Transcriptional pausing by RNAP II occurs in the absence of auxiliary factors and is probably caused by a structuralrearrangement within the enzyme and DNA sequences, which results in the formation of an "unactivated" intermediate (Erie 2002; Neuman et al. 2003). In contrast to transcriptional arrest, pausing is self-reversible, and is thought to be a natural mode of transcriptional regulation. Among the factors that regulate transcriptional pausing there are TFIIF, the ELL family, Elongins, FCP1, CSB, DSIF and NELF

(see below). In *vivo* evidence indicates that perhaps each factor stimulates elongation within specific transcriptional contexts.

The human GTF IIF (**TFIIF**), a heterodimer comprised of RNAP II-associating protein 30 (RAP30) and RAP74, plays an integral role in recruiting RNAP II during PIC formation (Conaway et al., 1991; Flores et al., 1991; Orphanides et al., 1996) and diminishes the time RNAP II is paused and stimulates the rate of RNAP II transcriptional elongation (Bengal et al., 1991; Tan et al., 1994; Lei et al., 1998). Together with TFIIF, the three-subunit **Elongin complex** also stimulates the rate of RNAP II transcription by suppressing transient transcriptional pausing (Bradsher et al. 1993b). However, Elongin-like factors have not been found in yeast and thus, elongins might be higher eukaryotic gene-specific factors. The precise functions of Elongins in RNAP II-mediated elongation are not yet clear. Also absent in yeast, the **ELL family** seems to represent a metazoan adaptation for expression of larger genes (Eissenberg et al. 2002; Shilatifard 2004).

The **P-TEFb** (positive transcription elongation factor b) consists of the Cdk9 kinase that associates either with several cyclins (Peng et al. 1998ab). P-TEFb phosphorylates the Ser 2 on the CTD of RNAP II to facilitate productive elongation upon pausing (Marshall et al. 1996; Ni et al. 2004).

The **DSIF** (DRB-sensitivity-inducing factor) is a heterodimeric complex composed of the *S. cerevisiae* proteins Spt4 and Spt5. (Wada et al. 1998). Spt4 antagonizes the negative effects of RNAP II pausing imposed by the chromatin-remodeling yeast factor Isw1p (Morillon et al. 2003), whereas Spt5 interacts with factors implicated in mRNA maturation and surveillance (Sims et al., 2004). In yeast, mutations in the DSIF components have both positive and negative effects on transcription (Swanson and Winston 1992; Hartzog et al. 1998), suggesting that DSIF might function as an adaptor that connects other modulators to the RNAP II TEC.

The **NELF** (Negative Elongation Factor) complex promotes RNAP II pausing (Yamaguchi et al. 1999). Resumed productive elongation occurs when NELF dissociates from the paused TEC, which arises from P-TEFb-mediated phosphorylation of the RNAP II CTD and the SPT5 subunit of DSIF (Ivanov et al. 2000; Kim and Sharp 2001).

Finally, the **CSB** is a DNA-dependent that directly binds RNAP II and stimulates the rate of elongation, as well affecting the activity of TFIIS (Selby

and Sancar, 1997; Tantin et al., 1997). The yeast homolog of CSB, RAD26, links transcription elongation to transcription-coupled nucleotide repair (Gregory and Sweder, 2001).

5.3.2. Factors that influence transcriptional arrest: TFIIS

Transcriptionally arrested RNA polymerases are usually unable to resume productive elongation without accessory factors, although the enzymes remain catalytically active. Arrest is believed to result from the RNA polymerase "backtracking" relative to the DNA template, causing a misalignment of the catalytic active site and 3'-OH of the nascent RNA transcript. Arrested RNAP II complexes resume productive elongation via an evolutionarily conserved mechanism that requires cleavage of the RNA transcript in a 3'-to-5' direction. The elongation factor TFIIS promotes readthrough of arrested TECs caused by intrinsic DNA sequences, DNA-binding proteins, and drugs that bind DNA (Fish and Kane 2002), by enhancing the preferential removal of the misincorporation at the 3'-end (Jeon and Agarwal 1996). The yeast homolog of TFIIS is termed PPR2; *ppr2* mutants display sensitivity to 6-azauracil (Nakanishi et al., 1995). In yeast, the UTP analog 6-azauracil creates stalled RNAP II complexes effect by lowering the cellular concentration of GTP and UTP (Exinger and Lacroute, 1992). Therefore, mutant yeast strains that display sensitivity to 6-azauracil are considered to have deficiencies in transcript elongation. These approaches have been used to demonstrate that TFIIS functions to alleviate arrested elongating complexes in living cells (Kulish and Struhl, 2001).

5.3.3. Elongation factors that remodel chromatin

The packaging of DNA into chromatin and chromosomal structure plays a central role in many aspects of yeast cell biology, affecting all processes that require DNA access, including replication, transcription and repair. Generally, chromatin is repressive to extraneous access, and this inhibitory effect must be overcome by regulatory factors. The nucleosome is the basic unit of chromatin and consists of ~147bp of DNA wrapped around a single histone octamer. The histone octamer contains two copies each of the histone proteins H2A, H2B, H3

and H4. The core histones are predominantly globular except for their N-terminal “tails”, which are unstructured (Figure 7).

Figure 7. Crystal structure of the nucleosome core particle, consisting of H2A (yellow), H2B (red), H3 (blue) and H4 (green). Each of the four histones share a very similar structural motif consisting of three alpha helices separated by loops. Source: Richard Wheeler (Zephyris) 2006.

Much progress has been made regarding how RNAP II mechanistically elongates in a chromatin environment. The models propose that nucleosomes can undergo compositional alteration, covalent modification and translational repositioning (that is, altering the position of the histone octamer to a new position along the DNA, i.e. nucleosome mobilization and histone depletion), as RNAP II progresses along the chromatin template (Studitsky et al., 2004; Saha et al., 2006). The so-called chromatin remodelling activities are able to either change the location of the nucleosome along a particular DNA sequence or create a remodelled state of the nucleosome that is characterized by altered histone-DNA interactions. Pertinent to all chromatin remodelling complexes is an ATPase subunit which is in fact an ATP-dependent DNA translocase (Saha et al., 2002). There are several known chromatin remodelling complexes: the SWI/SNF family, ISWI family, the RSC complex, the CHD1 ATPase, the INO80 complex, the SWR1 complex, and the related histone chaperones FACT and Spt6.

- **SWI/SNF**

The first ATPase activity found was the Swi/Snf2 ATPase BRG1 (Sullivan et al., 2001), whose function allows for DNA translocation as well as nucleosome transfer from one piece of DNA to another. SWI/SNF is a multiprotein complex,

and certain remodeler subunits contain multiple bromodomains with a preference for particular acetylated residues in the histone tails (Kasten et al., 2004), which might help specify the targeting of the remodeling complex to particular nucleosomes. In yeast, mutations in the SWI/SNF2 gene cause the inability to undergo mating type switching (*swi*$^-$) and sucrose nonfermenting (*snf*$^-$) growth defects due to defects in gene expression of characteristic set of genes (Laurent et al., 1991; Peterson and Herskowitz, 1992).

- **ISWI**

The ISWI family is a highly conserved group of ATP-dependent chromatin remodeling factors that share an identical ATPase subunit, ISWI, but have unique associated proteins that specialize each ISWI-containing complex (NURF, CHRAC and ACF) (Saha et al., 2006). The yeast homolog ISWI complex associates in two different complexes (Vary et al., 2003). On one hand, Isw1a complex consists of Isw1 and Ioc3, and functions to negatively influence initiation and nucleosome positioning (Morillon et al., 2003). On the other hand, the Isw1b complex, composed of Isw1, Ioc2 and Ioc4, regulates and couples elongation to mRNA maturation and termination (Morillon et al., 2003). Different subunits of the Isw1b complex appear to specifically regulate either Ser5 (Ioc2) or Ser2 (Ioc4) phosphorylation (Morillon et al., 2003). Besides, Isw1 is recruited to chromatin dependent on the recognition of H3-K4 di- and trimethylated histone tails, and its activity is necessary for normal RNAP II distribution over the coding region of the selected gene (Santos-Rosa et al., 2003).

- **RSC**

RSC (Remodel the Structure of Chromatin) is an abundant ATP-dependent nucleosome-remodeling complex in yeast cells, and it is the only such complex that is essential for growth (Cairns et al., 1996). This 15-subunit complex was isolated from *S.cerevisiae* on the basis of homology to the SWI/SNF complex, and the two complexes contain some common subunits (Cairns et al., 1998). Sth1, a homolog of Swi2, is the catalytic ATPase subunit of the RSC complex, essential for viability and also sufficient for remodelling *in vitro* (Du et al., 1998; Saha et al., 2002). Biochemical studies suggest the existence of distinct RSC

complexes. Rsc1 and Rsc2 are related proteins that associate with the other RSC subunits in a mutually exclusive manner (Cairns et al., 1999). Unlike other Rsc subunits, loss of either Rsc1 or Rsc2 does not significantly affect cell growth, although the resulting strains show common distinct phenotypes. Loss of both Rsc1 and Rsc2 causes lethality, suggesting that there are Rsc1 and Rsc2 isoforms that have related, though nonidentical functions (Cairns et al., 1999).

Mutations in several RSC subunits show a typical G2/M arrest characterized by large budded cells containing 2N of 4N chromosomes (Cao et al., 1997; Tsuchiya et al., 1998; Angus-Hill et al., 2001). The basis for this G2/M arrest is unknown, but it depends on the spindle pole body checkpoint. Whole-genome analysis of gene expression in *rsc3* and *rsc30* mutants indicates that RSC affects the expression of ribosomal protein and cell wall genes (Angus-Hill et al., 2001). Recent findings suggest that RSC might have a direct role in transcriptional regulation (Ng et al., 2002). Inactivation of the Sth1 and Rsc8, but not hte Sfh1 component of RSC leads to inappropriate expression of the *CHA1* gene under noninducing conditions, indicating that RSC might negatively regulate *CHA1* expression (Moreira and Holmberg, 1999).

Genome-wide location analyses have been used to identify the physiological targets of the RSC complex (Ng et al., 2002). This study reveals that both Rsc1 and Rsc2 associate with the same genes, that RSC is generally targeted to RNAP II promoters (aproximately 700 in the yeast genome), and that it is specifically recruited to Pol II promoters in response to transcriptional activation or repression (Ng et al., 2002). RSC binds to Pol II promoters involved in specific cellular functions such as mitochondrial organization, nitrogen and carbohydrate metabolism, and transcriptional regulation. Other enriched RSC targets are promoters expressing histones, snRNAs, S-phase cyclins and the Chromatin Assembly Complex. In some context, for instance promoters involved in nonfermentative carbohydrate metabolism, RSC is recruited in a manner that correlates with transcriptional activation. In contrast, RSC recruitment to the *HTA/HTB1* promoter requires both the Hir1 and Hir2 corepressors, and this recruitment is correlated with transcriptional inactivity (Ng et al., 2002).

Similar genome-wide localization analysis of the subunit Rsc9 revealed a relationship between genes targeted by Rsc9 and genes regulated by stress; treatment with hydrogen peroxide of rapamycin, which inhibits TOR signalling,

resulted in genome-wide changes in Rsc9 occupancy (Damelin et al., 2002). In this study, Damelin et al used a rsc9-1 mutant termosensitive allele which had a Gln489→Stop mutation supposedly encoding a truncated form of the protein. By means of this allele, they identified many stress-response genes (including genes activated by Msn2 and Msn4) as targets of Rsc9, and conclude that Rsc9 is important for both repression and activation of genes regulated by TOR signall¡ng (Damelin et al., 2002).

- **CHD1**

The ATPase CHD1 (chromo-ATPase/helicase-DNA binding domain) remodels nucleosomes in vitro and appears to function in both elongation and termination (Tran et al., 2000). As Chd1 contains a chromodomain, which can recognize methylated histone tails, it is tempting to speculate that Chd1 specifically recognizes methylated histones, perhaps spatially regulating its localization (Sims et al., 2004).

- **INO80**

The budding yeast INO80 complex is a conserved ATP-dependent nucleosome remodeler containing actin-related proteins Arp5 and Arp8. Its components are recruited to HO endonuclease-induced DNA double strand break (DSB) sites, where a phosphorylated form of H2A accumulates (van Attikum et al., 2004). Reportedly, Ino80 can evict nucleosomes surrounding this HO-induced DSB to facilitate efficient homologus recombination upon DNA damages.

- **SWR1**

Swr1 is the catalytic core (ATPase) of a multisubunit, histone-variant exchanger Swi/Snf2-related complex known as SWR1. It has the remarkable ability to efficiently replace conventional histone H2A from H2A-H2B dimers with histone H2AZ (also Htz1) to regulate a specific subset of yeast genes (Mizuguchi et al., 2004).

- **Histone Chaperones**

The **FACT** (Facilitates Chromatin Transcription) complex is a heterodimeric complex comprised of hSpt16 and SSRP1 (with yeast homologs Spt16/Cdc68 and Pob3). It is highly conserved among eukaryotes and physically interacts with histones to allow RNAP II elongation through the destabilized chromatin structure (Kireeva et al., 2002).

Spt6 (Suppressor of Ty 6) is an elongation factor that promotes nucleosome assembly and also interacts with histones, preferentially histone H3 (Bortvin and Winston, 1996) at actively transcribed regions together with the elongating form of RNAP II (Hartzog et al., 1998; Andrulis et al., 2000; Kaplan et al., 2000).

5.3.4. Histone modification and elongation

A striking feature of histones, and particularly their highly accessible N-terminal histone tail residues, is the large number and type of postranslational, covalent modifications they undergo, which possess information pertinent to transcriptional regulation (Strahl and Allis, 2000; Jenuwein and Allis, 2001). There are at least eight distinct types of modifications found on histones: acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination, and proline isomerization (Kouzarides, 2007). Moreover, there are over 60 different histone residues which can be modified. Extra complexity comes from the fact that methylation can be mono-, di- of trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines. This vast array of modifications gives enormous potential for functional responses, many of them being the regulation of transcription, repair, replication and condensation (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Kouzarides, 2007).

- **Histone Acetylation**

Transcriptionally active genes are typically enriched for histones containing acetylated residues in comparison to genes that are silent. Histone acetylation destabilizes chromatin structure by disrupting internucleosome associations as well as histone tail interactions with linker DNA, thus allowing for

productive transcription (Sims et al., 2004). Acetylation (with Acetyl-CoA as a donor enzyme) is carried out by histone acetyltransferases (HATs), which can be grouped into five families in yeast, based on homology: the Gcn5 family, the MYST familiy (including Sas3 and Esa1), the p300 family, TAF1, TFIIC and Nut1 and the Elongator.

Histone acetylation is a reversible process and, accordingly, histone deacetylases (HDACs) catalyze the deacetylation reaction. There are 10 known HDACs in *S. cerevisiae* classified in three families, based on homology:

- Class I HDACs, include Rpd3, Hos1 and Hos2.
- Class II HDACs, include Hda1 and Hos3.
- Class III HDACs, include Sir2 and four Hst proteins.

- **Histone methylation**

Histone methylation is one of the most studied histone modifications. Silent or repressed genome regions are associated with methylation at the histone lysine residues H3-K9, H3-K27, and H4-K20, and transcriptionally active domains are typically associated with methylation at H3-K4, H3-K36, and H3-K79 (Lachner et al., 2003; Sims et al., 2003). Experiments in yeast suggest that histone methylation and ubiquitination are interconnected, and appear to play a role in elongation. Particularly, H2B monoubiquitination leads to the proteasome recruitment, which degrades monoubiquitinated H2B and allows for H3-K4 and H3-K79 methylation (Ezhkova and Tansey, 2004).

The yeast Set2 protein functions as an H3-K36-specific histone methyltransferase and preferentially associates with the hyperphosphorylated form of RNAP II at the coding region of genes (Krogan et al., 2003c; Shaft et al., 2003; Xiao et al., 2003) in a PAF-dependent manner. Similarly to Set2, Set1 functions as a specific histone H3-K4 methyltransferase in yeast and associates with the RNAP II CTD and the PAF complex (Krogan et al., 2003a; Ng et al., 2003b). However, Set1 preferentially interacts with the Ser5 phosphorylated form of RNAP II, which is associated with early transcription events (Ng et al., 2003b). Set1-mediated histone H3-K4 methylation occurs at promoters and within the coding region of active genes in yeast. Moreover, H3-K4 dimethylation occurs on a genome-wide scale, whereas trimethylation of H3-K4 strictly corresponds to actively transcribed genes (Ng et al., 2003b). The

PAF complex, comprised for five subunits (Paf1, Ctr9, Cdc73 and Leo1) is therefore required for the recruitment of both Set1 and Set2 to coding regions, with resultant histone H3-K36 and H3-K4 methylation respectively (Krogan et al., 2003a). Additionally, experimental work suggests that H2B monoubiquitination promotes Set1-mediated methylation of H3-K4, subsequently followed by H2B deubiquitination by Ubp8 and Set2-mediated H3-K36 methylation (Grant et al., 1997; Henry et al., 2003). Thus, evidence points at PAF being a passive elongation factor, regulating and coordinating the many aspects of transcript elongation and downstream events of mRNA biogenesis.

Until the recent discovery of histone demethylases LSD1 and JmjC (Shi et al., 2004; Tsukada et al., 2006; Tu et al., 2007), methylation was generally considered as a stable modification, well suited as an epigenetic mark to persist through cell generations (Sims et al., 2003). Methylation is clearly a reversible and dynamic modification, thus controlling the recruitment of HATs and HDACs to the chromatin in order to either repress or activate transcription of target genes (reviewed in Mellor, 2006).

5.3.5. Regulation of downstream events during elongation

Transcription is a complex process that integrates mRNA production with its maturation, surveillance, and export (Maniatis and Reed, 2002; Orphanides and Reinberg, 2002). Messenger RNA processing takes place most efficiently cotranscriptionally and involves the addition of a 5'-cap, the excision of intronic sequences by splicing factors, and the addition of a 3'-poly(A) tail. The CTD of RNAP II serves as a platform for many factors required for mRNA maturation (Meinhart and Cramer, 2004). Depending on the CTD conformation and phosphorylation state, it recruits a wide variety of proteins responsible for capping, splicing and 3'end processing (Fong and Bentley, 2001) concomitant to elongation. Thus, the CTD may have a "code" comparable to the histone code (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Buratowski 2003). For instance, the capping enzyme is recruited and stimulated by the Spt5 subunit of the DSIF and the Ser 5 phosphorylated CTD (Sims et al., 2004). Similarly, the spliceosome complex is recruited and enhanced by the hyperphosphorylated

CTD (Fong and Bentley, 2001), and the 3'end cleavage machinery is dependent on the interaction with the phosphorylated CTD-Ser 2 (Barilla et al., 2001; Licatalosi et al., 2002). Still, many of the mechanistic details around the transcript cleavage and polyadenylation of the nascent transcript are unclear. It seems though that RNAP II pausing contributes directly to termination and 3'-end processing (Yonaha and Proudfoot, 1999).

The process of transcript elongation can affect other events involved in mRNA metabolism, such as transcription-dependent recombination and mRNA export. The complex responsible for the transcription of long transcripts, of those with high GC content, is known as THO, and consists of Tho2, Mft1, Thp2 and Hpr1 (Chavez et al., 2001). Together with Yra1 and Sub2, it comprises a larger complex termed TREX (transcription/export) which is necessary for efficient transcript elongation, mRNA export and surveillance (Strasser et al., 2002; Rondon et al., 2003; Libri et al., 2002).

OBJECTIVES

Globally, the research in our group is focused on understanding the mechanisms used by the stress-activated kinase p38 and its yeast homolog Hog1 to regulate adaptive responses. Because one of the most important responses controlled by the Hog1 SAPK is the regulation of gene expression, the aims of this thesis project were to give insight into the mechanisms by which Hog1 regulates gene transcription in response to osmotic stress.

Specifically, the main objectives of this PhD project were as follows:

(1) The study and characterization of a new role of Hog1 in the transcriptional process as a transcription elongation factor selective for osmoresponsive genes.

(2) The identification of RSC as a novel chromatin remodelling complex involved in Hog1-mediated histone eviction at osmoinducible genes, which is necessary for proper osmostress gene expression.

RESULTS AND DISCUSSION

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

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One of the challenges of cells existing in dynamic environmental conditions is the need to rapidly and appropriately respond to extracellular stimuli. As mentioned in the introduction, this is in part achieved by signalling through the mitogen-activated protein kinase (MAPK) cascades (Widmann et al., 1999), which elicit appropriate responses through modulation of gene expression, metabolism, cell death and proliferation. Exposure of yeast cells to high extracellular osmolarity results in rapid activation of the MAPK Hog1, and in fact, genome-wide transcriptional studies have demonstrated that a large number of genes required for cellular adaptation are regulated in a Hog1-dependent manner (Posas et al., 2000; Rep et al., 2000). Thus, control of gene expression is a major adaptive response driven by the MAPK Hog1.

There is not a unique mechanism by which Hog1 regulates gene induction. For example, Hog1 physically associates with the promoters of target genes (Alepuz et al., 2001; Proft and Struhl, 2002) by means of interactions with substrates, including the transcription factors Hot1, Msn2/4 and Sko1. These interactions do not interfere with the kinase catalytic site and instead promote the phosphorylation of both the tethered substrate (such as the transcription factor) and other proteins in the local vicinity, including 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).

Together, these studies indicate that Hog1 can physically associate with the regulatory regions of target genes (promoters) in order to regulate transcription initiation. However, the present publication and other recent studies extend this concept by demonstrating that Hog1, as well as the protein kinases Fus3, Kss1, Ste5, and Tpk1/2/3 can also associate with the entire transcribed region of target genes (Pokholok et al., 2006; Proft et al., 2006).

Thus, SAPKs may have a more general role as chromatin-associated enzymes than previously anticipated (Chow and Davis, 2006).

Hog1 interacts with the Rpb1 subunit of the RNA Polymerase II.

The process of transcription by RNAP Ⅱ coordinates multiple steps in mRNA biogenesis and maturation. As a consequence, several complexes travel together with the polymerase in the so-called Transcription Elongation Complex (TEC) to affect the elongation rate or processivity of Pol II or mRNA maturation (Sims et al., 2004). In fact, elongation factors are defined by their ability to affect the activity of or to associate with the TEC (Sims et al., 2004). One of the aims of this work was to study whether Hog1 was interacting with the elongating Pol II and with general components of the TEC, and thus participating at the level of transcription elongation, besides from its role in initiation.

Although it was known that Hog1 was interacting with the RNAP II *in vivo* (Alepuz et al., 2003), we attempted to identify the specific subunit of the RNAP II that directly interacted with Hog1 by using a photocrosslinking label transfer strategy (Brown et al., 2001). This assay consists on conjugating GST-fused Hog1 with the Sulfo-SBED photocrosslinking agent and incubating it with purified yeast Pol II from a TAP-Rpb9 tagged strain (Rani et al., 2004). By performing Western Blot analysis, Rpb1 was identified by the specific antibody 8WG16 as the target of GST-Hog1. A lower-migrating band also was detected, possibly corresponding to Rpb2, which could as well be an interacting partner of Hog1.

Because the CTD of the Rpb1 subunit of RNAP II is extensively phosphorylated during different stages of transcription and acts as a “docking site” for factors required for productive elongation and mRNA maturation, we studied whether Hog1 associated with phosphorylated Rpb1. For this purpose, we expressed GST-Hog1 in yeast cells and performed GST pull-down experiments with gluthathione-Sepharose beads. The presence of Rpb1 was probed by Western Blot with specific antibodies against total (8WG16), Ser 5 phosphorylated (H14) or Ser 2 phosphorylated (H5) Rpb1. Hog1 coprecipitated more efficiently with Rpb1 upon osmostress induction, when Rpb1 CTD is phosphorylated. These results were confirmed by *in vitro*

coimmunoprecipitation experiments, in which GST-Hog1 expressed and purified from *E.coli* was incubated with yeast purified TAP-Rpb3 complex either treated or untreated with alkalyine phosphatase. Conclusively, the interaction between Hog1 and Rpb1 increased when the Pol II CTD was phosphorylated, since the interaction was partially lost upon alkaline phosphatase treatment.

Genome-wide location analysis reveals binding of stress-activated Hog1 to promoter and 3'regions of osmoresponsive genes

To give insight into the mechanism of recruitment of activated Hog1 to its target genes, we used cells expressing HA-tagged Hog1 to characterize the MAPK occupancy by ChIP-on-chip (combining chromatin immunoprecipitation with DNA microarray technology). Cells were subjected to a 5-minute osmotic shock and processed to obtain input and immunoprecipitated samples, which were amplified, labeled with fluorescent dyes and hibridized with microarrays containing almost all yeast intergenic regions. Due to the fact that Hog1 is bound to chromatin through other partners -it does not have a DNA binding domain-, its relative binding values were generally low. Hovever, data analysis identified 72 genes that were enriched in Hog1 occupancy at least 1.5-fold upon osmostress. If compared with other genome-wide transcriptional profiles (Posas et al., 2000; Rep et al., 2000), most of the identified intergenic regions (87%) corresponded to genes that were in fact >3-fold upregulated under osmotic stress conditions. For instance, among those, there were some very well-characterized osmoresponse genes such as *STL1*, *GRE2*, *CTT1*, *GPD1* and *TSL1*. Strikingly, it was of crucial importance to note that besides promoter regions, many of the detected Hog1-interacting regions corresponded to 3'-end regions or both promoter and 3'-end regions of osmoresponse genes. This evidence, and the fact that Hog1 interacts more efficiently with phosphorylated Rpb1, prompted us to analyze whether Hog1 association was occurring along the coding regions of target genes together with elongating RNAP II. We utilized chromatin immunoprecipitation (ChIP) experiments to analyze the binding of Hog1 and other components of the transcriptional machinery to various regions of six highly induced osmostress-activated genes (*STL1*, *GRE2*, *CTT1*, *FAA1*, *GPD1* and *TSL1*) in the absence or presence of NaCl treatment. Although induction of these genes is Hog1-dependent, the transcription factors

are specific for each gene: Sko1 is found in *GRE2* and *FAA1* (Rep et al., 2001; Proft et al., 2005), Hot1 regulates *GPD1* and *STL1* (Rep et al., 1999; Alepuz et al., 2003), and Msn2/4 control the expression of *CTT1* and *TSL1*.

According to previous results, the analysis showed that the transcriptional machinery (TBP and Pol II) was recruited to all loci only in response to stress, TBP being restricted to TATA box regions and Pol II occupying the entire transcribed region. Furthermore, when we analyzed the occupancy of the activators, Hot1 was detected under inducing conditions and Sko1 had a constitutive binding pattern, both exclusively associated at promoter regions of target genes. Importantly, distribution of Hog1 was not confined to promoters of these genes, but displayed an occupancy profile more similar to that of RNAP II, spanning the open-reading frame (ORF) of osmoresponsive genes under stress conditions. This finding suggested a novel role of the MAPK as transcription elongation factor traveling with the elongating Pol II along the ORFs.

As control experiments, ChIP analysis were performed testing for the presence of RNAP II and Hog1 at the coding regions of constitutively active genes (*ACT1* and *ADH1*). As expected, whereas Pol II was associated to the ORFs of these genes, Hog1 was not, thus indicating that the kinase occupancy at promoters and coding regions was strictly restricted to genes activated by osmotic stress.

We next assessed the kinetics of occupancy of Hog1 and Pol II at the prototypical osmoresponsive gene *STL1*. Chromatin IP samples were taken following a time course every 5 minutes and analysed for the presence of the MAPK and the Rpb1 subunit of Pol II. At 5 minutes, both proteins were found to occupy the coding region of *STL1* in a very similar fashion. Nonetheless, the binding pattern changed as time advanced, because Pol II association was maintained up to 20 minutes after salt addition while 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. Among the possible functions required at the initial rounds of elongation, there could be the modification of the chromatin in the stress-responsive coding regions. In fact, several studies have shown that histone H3 is a phosphorylation target of the MAPK MSK1 and MSK2, which act downstream of the ERK and p38 MAP kinases (Clayton and

Mahadevan, 2003; Soloaga et al., 2003). An alternative function of Hog1 could be related to the coordination of the cotranscriptional recruitment of the mRNA processing complexes required for efficient transcript production.

These findings were recently supported by a publication from Pokholok et al (2006), in which ChIP-on-chip experiments of TAP-tagged protein kinases such as Hog1, Fus3, Kss1 and Tpk1/2/3 revealed that these MAPKs are genomically localized at promoter and coding regions of their corresponding target genes upon inducible treatments. The binding patterns of these kinases were slightly different, indicating the existence of distinct mechanisms involved in their association with genes. Moreover, preferential binding of the kinases to different elongation factors, chromatin regulators, histone modifications, nuclear pore proteins and other components of the TEC could explain the localization of the kinases. The observation that components of different signal transduction pathways physically occupy their target genes upon activation implies a mechanistical regulation of transcription elongation in response to a broad range of environmental cues (Pokholok et al., 2006).

Hog1 interacts with components of the Pol II transcription elongation complex

As pointed out in the introduction, elongation factors are classically defined as molecules that can either affect the catalytic activity of the RNA Polymerase II or are associated with the Transcription Elongation Complex (Sims et al., 2004). Because Hog1 was interacting with elongating RNAP II, we next tested whether Hog1 was in fact part of a larger complex containing other transcription elongation factors required for productive elongation, such as DSIF (Spt4), TFIIS (Dst1), PAF (Paf1) and THO (Thp1). We performed GST-pull down experiments of yeast cell extracts containing GST-Hog1 and TAP-tagged versions of the cited proteins. All the elongation factors tested were interacting *in vivo* specifically with GST-Hog1, and not the GST control. These interactions were also occurring in the presence of DNAse I, and thus were not due to the chromatin templates to which these complexes are bound during elongation. Moreover, a truncated version of Hog1 lacking its C-terminal domain was unable to interact with these factors, confirming that interactions were directly with the kinase. Therefore, our results support the idea that Hog1

is indeed travelling throughout the coding regions of active stress-responsive genes together with the polymerase and the transcription elongation machinery associated with it. However, it remains to be elucidated whether this association occurs in a defined large macromolecular complex and whether Hog1 is able to phosphorylate or activate the activity of a specific factor.

Elongation factors are important for transcription in response to osmotic stress.

The latter results suggested that the physical interaction of Hog1 with several transcription elongation factors could be functionally relevant for the transcriptional output of osmostress-responsive genes, and thus for the adaptive responses to osmostress conditions. To test it, we assayed cell survival under high osmolarity and the transcriptional response of several mutant strains lacking specific transcription elongation factors, such as Spt4, Dst1, Thp1 and Paf1. The corresponding knockout strains did not have compromised cell viability under non-stress conditions, but when subjected to high osmolarity they were osmosensitive, as well as showed significantly impaired osmostress gene expression. Taken together, these results indicated that an intact transcription elongation machinery was necessary for proper transcriptional activation upon osmotic shock as well as to guarantee cell survival under such conditions.

Hog1 association with coding regions is important for mRNA production of osmoresponsive genes upon stress.

To test whether the interaction of Hog1 with the elongation complex was functionally significant, we had to take into account that Hog1 is already essential for the recruitment the transcriptional machinery in order to initiate transcription in response to osmotic stress (Alepuz et al., 2001). Therefore, with the purpose of specifically studying the role of Hog1 in transcription elongation, we had to uncouple 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. The expression of these genes became constitutive, as it was driven

by the LexA-VP16 transcriptional activator. When following mRNA levels of these constitutively expressed artificial constructs in the presence and absence of osmotic stres, the amount of mRNA of the osmotress-responsive genes was 7-fold higher after 10 min of NaCl treatment. Importantly, this induction was dependent on the presence of the MAPK Hog1, but independent on the transcription factor Hot1 in the case of the *STL1* gene –confirming that transcription initiation was independent on both Hog1 and Hot1-. Similar results were observed for the *CTT1* osmostress genes. As control experiments, we fused the LexA promoter with other genes such as *ADH1* and *LacZ*, whose expression is known to be independent on Hog1. As expected, no increase in the corresponding mRNA levels was observed upon osmostress, suggesting that the ability of Hog1 to increase mRNA production was specific for the coding region of its target genes.

To confirm that Hog1 was selectively affecting the coding regions of the osmostress genes fused to the LexA promoter, we performed Chromatin Immunoprecipitation analysis. Our previous results had demonstrated that osmotic stress induced the interaction of Hog1 with both the promoter and coding regions of *STL1*. However, Hog1 association at the artificial *LexA-STL1* system was observed only at the coding region, and not at the LexA promoter, which only contains binding sites for the LexA binding domain. Therefore, although the recruitment of the Hog1 by sequence-specific DNA-binding proteins or transcription factors provides a mechanism of gene targeting to the promoter regions, it seems that it does not account for the recruitment to the entire transcribed region. Accordingly, we showed that osmotic stress causes the recruitment of Hog1 to the promoter, but not the coding region, of a chimeric gene that contains an osmoresponsive promoter and a nonstress responsive ORF, the *STL1::LacZ* reporter gene. Together, these data indicate that the recruitment of Hog1 to promoters is neither sufficient nor necessary for its recruitment to the coding regions of target genes. Moreover, the association of the kinase to coding regions of stress-responsive genes results in proper Hog1-dependent mRNA expression of the target gene upon osmostress treatment. Nevertheless, to directly test the hypothesis that target gene occupancy by Hog1 is functionally significant, and discard that Hog1 occupancy is not a passive consequence of the Hog1-dependent transcription, other

approaches could be used to demonstrate that the selective loss of Hog1 occupancy causes defects in target gene expression.

Due to the fact that Hog1 is a protein kinase, we next addressed the question whether its kinase activity was necessary for binding to the *STL1* coding region and for mRNA induction in the LexA inducible system. Experiments revealed that the catalytically inactive Hog1 mutant was not able to bind to the coding region of the *LexA-STL1* construct upon osmostress. Correspondingly, the catalytic activity was needed for increasing mRNA expression. Therefore, although the relevant substrates for the kinase in the elongation complex are unknown, data suggests that the kinase activity is important for the functional role of Hog1 in elongation.

Stress-activated Hog1 stimulates Pol II density at osmoresponsive coding regions

Once established that Hog1 was important for proper mRNA production under osmostress conditions, an interesting question was to understand whether Hog1 was stimulating the elongation rate or processivity of the RNAP II. The elongation rate is defined as the rate at which elongating RNAP II traverses the gene, and the processivity of the RNAP II is the ability of elongating Pol II to travel the entire length of the gene (Mason and Struhl, 2005). Recent studies demonstrate that the vast majority of the so-called elongation factors do not actually affect the rate at which Pol II travels down the gene, but some of them (Spt4-Spt5, THO, TFIIS, CTDK1) actually contribute to RNAP II processivity under genetic or environmental perturbations (Mason and Struhl, 2005). We thus investigated whether Hog1 affected the processivity of the RNAP II in the coding region on the artificial *LexA-STL1* gene. Indeed, 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. Furthermore, a similar ChIP analysis following binding of the elongation factor Spt4 to the *LexA-STL1* construct revealed that the presence of Spt4 at the coding region of *STL1* required osmostress induction as well as the MAPK. Taken together, these data indicated that Hog1 was important for increased association of the RNAP II and the Spt4 elongation factor at coding regions of

target genes. Nevertheless, this increase in RNAP II density by Hog1 at the *LexA-STL1* locus was still less pronounced than the corresponding 7-fold rise of its messenger RNA upon stress. This incongruity could be due to the differences in experimental background levels between ChIP experiments and mRNA level determinations. However, if that were not the case, this results would rise the interesting possibility that, besides stimulating elongation by recruiting the TEC, Hog1 could be affecting mRNA stability or processing as well. In fact, the occupancy of the kinase at the coding regions together with the TEC anticipates that the protein kinase may contribute to the multiple processes that occur during elongation, including capping, splicing, 3'end cleavage, polyadenylation, and mRNA transport (Howe, 2002; Svejstrup, 2004). Further studies are needed to elucidate possible roles of the tethered kinase in these processes.

Binding of Hog1 to stress-responsive coding regions depends on the 3'UTR

The recruitment of Hog1 at promoter regions does not guarantee the recruitment of the kinase to the transcribed regions of target genes. 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 constructs 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::Lac conferred osmotic-stress induced recruitment of Hog1 to the transcribed region of the heterologous gene. Furthermore, when we replaced the 3' UTR of a noninducible gene such as *ADH1* by the 3'UTR of *STL1*, we found that it conferred the ability of Hog1 to occupy the coding region of *ADH1*. 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. Further studies are needed to elucidate whether this recruitment affects the expression of the chimeric osmoresponsive genes, and how the 3'noncoding region can account

for Hog1 binding to the transcribed regions. It is possible that some factors associated with 3'UTR sequences, 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.

Personal contribution to this work: except for the genome-wide location analysis, which was performed by Dr. Proft, and the binding assays shown in figures 1 and 3, I have been fully involved in the design, execution and discussion of the experiments and results described in this article.

The Hog1 MAPK targets the RSC complex to mediate eviction of H3 modified nucleosomes in osmo-responsive genes

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(Submitted)

The Hog1 MAPK targets the RSC complex to mediate eviction of H3 modified nucleosomes in osmo-responsive genes

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Regulation of gene expression by MAP kinases is essential for proper cell adaptation to extracellular stimuli. The p38 MAPK Hog1 is an important regulator of transcription involved in the modification of transcription factors, recruitment of chromatin modifying factors and in transcription elongation. Here, we show that Hog1 physically interacts with the RSC complex to direct its association with the ORF of osmoresponsive genes. RSC mutants are sensitive to osmostress and display reduced osmostress gene expression. During stress there is a dramatic change on the nucleosome organisation of stress responsive loci that depends on Hog1 and RSC function. A deletion of the *SET1* histone methylase or amino acid substitution of the histone H3 K4 partially bypasses the requirement for RSC indicating that the H3K4 methylation imposes an extra layer of control on stress-responsive genes.

Adaptation to environmental stress requires changes in many aspects of the cell biology. In eukaryotic cells, stress-activated protein kinases (SAPK) play an essential role for proper cell adaptation to extracellular stimuli (Kyriakis and Avruch, 2001). Exposure of cells to high osmolarity results in rapid activation of a conserved family of SAPKs, which include the mammalian p38 and the yeast Hog1 (de Nadal et al., 2002; Sheikh-Hamad and Gustin, 2004). SAPKs are signaling molecules that can modulate gene expression in response to specific enviromental stimuli. Until recently, the prevailing view on how SAPKs, and kinases in general, modulate gene expression has been the direct phosphorylation of transcription factors or co-regulatory proteins. However, the observation that the SAPK Hog1 is recruited to chromatin has lead to a new role for signaling kinases as integral components of transcription complexes, influencing gene expression in an unexpected way (Alepuz et al., 2001; Proft and Struhl, 2002). The recruitment of Hog1 to promoters via its association with transcription factors bound at target promoters has shown to be important to stimulate recruitment of Pol II, the Rpd3 histone deacetylase and SAGA complexes allowing for proper transcription initiation (Alepuz et al., 2003; de Nadal et al., 2004; Zapater et al., 2007). Thus, binding of Hog1 to promoters is critical to induce gene expression upon stress. Recent reports have shown that binding of the Hog1 MAPK to chromatin is not restricted to promoters but it also extents to coding regions of stress-responsive genes (Proft et al., 2006;

Pokholok et al., 2006). Activated Hog1 associates with elongating Pol II and components of the elongation complex and is found selectively recruited to the entire coding region of osmotic stress genes. Hog1 is important for the amount of the Pol II elongation complex and of mRNA produced. Thus, in addition to its various functions during transcriptional initiation, Hog1 behaves as a transcriptional elongation factor that is selective for genes induced upon osmotic stress (Proft et al., 2006). Global *in vivo* binding analyses (ChIP on chip) have revealed that in addition to Hog1, other signalling kinases such as Tpk1 (the catalytic subunit of the PKA) or the Fus3 and Kss1 MAP kinases are also found in coding regions of specific genes (Pokholok et al., 2006). Thus, protein signalling kinases may have a more general role as chromatin-associated enzymes than previously anticipated. At this point, however, the role of these kinases at the coding region of the genes remains unclear, which opens a new dimension on transcription regulation by signalling kinases. Chromatin structure imposes significant obstacles on all aspects of transcription, from initiation to elongation. Modifications at the surface of nucleosomes are critical for transcription since they can disrupt chromatin contacts or affect the recruitment of non-histone proteins to chromatin (Li et al., 2007; Kouzarides, 2007). Many classes of modifications have been characterised to date that target different sites in histones (e.g. methylation, acetylation, phosphorylation, ubiquitination,…). One of the properties of such histone marks is the modulation of the regulation of chromatin dynamics. The dynamics of chromatin structure are tightly regulated through multiple mechanisms including chromatin remodelling, histone variant incorporation, histone eviction and histone modification. Chromatin remodellers are specialized multi-protein complexes that enable access to nucleosomal DNA by altering the structure, composition and positioning of nucleosomes (Saha et al., 2006). The family of SWI/SNF of remodellers is known to participate in many aspects of gene expression, in general to promote transcription. The RSC complex is a member of the SWI/SNF family and is characterized for modifying nucleosome structure through ATP hydrolysis. Interestingly, it has been shown that *in vitro* stimulation of transcription through a chromatin template requires RSC for efficient Pol II elongation via DNA loop formation to make nucleosomal DNA accessible and making possible the process of nucleosome mobilisation and histone eviction (Lorch et al., 2006; Carey et al., 2006; Zhang et al., 2006). Global binding assays showed that Rsc1 and Rsc2 associate to about 700 target promoters that include RNA PolIII and RNA PolII dependent genes. RSC display distinct modes of association with promoters but, it is worth noting that regulated association of RSC is correlated with transcriptional activation of genes involved in carbohydrate metabolism and occurs prior RNA PolII recruitment. In addition, genome-wide localization of Rsc9 indicated a relationship between genes targeted by RSC and genes regulated by stress (i.e., hydrogen peroxide or rapamycin). Although, these studies did not shed light on how RSC was targeted to chromatin and the specific function of RSC on the transcription process, they clearly showed that external stimuli induced changes on RSC genome-wide localization that correlated with induction or repression of specific families of genes (Ng et al., 2002; Damelin et al., 2002). In this work, we show that the Hog1 MAPK interacts and targets the RSC complex to the osmo-responsive genes. RSC targeting is required for massive nucleosome rearrangements in osmostress genes and for proper gene expression in response to high osmolarity. Lastly, requirement for RSC is partially bypassed by deletion of the *SET1* histone methylase or histone H3 K4 mutation that imposes an extra layer of control on gene expression. Thus, the Hog1 SAPK does not only function at the level of transcriptional initiation, but it also serves to recruit the RSC complex to promote nucleosome reorganization and facilitate gene expression from osmo-responsive genes.

Results

Mutants in the RSC complex are sensitive to osmostress and display reduced osmostress gene expression.

The ability of cells to survive at high osmolarity depends on the HOG signaling pathway and the control of gene expression exerted by the Hog1 MAPK. We performed a genome-wide screen to identify activities required for full expression of the gene program required for cell survival upon osmostress. This screen identified several complexes such as the Rpd3 histone deacetylase complex, SAGA, Mediator and components of the TEC that are important for gene expression in response to osmostress (Zapater et al., 2007; Proft et al., 2006). In addition, mutations in *RSC1* and *NPL6* were identified that yielded cells osmosensitive at high osmolarity. Both genes encode non-essential components of the RSC complex. We then analysed whether cells containing mutations on key components of the RSC complex also displayed a similar phenotype. We analysed cell growth in the presence of high osmolarity in strains containing RSC components tagged with a degron mark to stimulate degradation of the proteins. Cells with reduced amount of Rsc8, Rsc3 or Rsc9 are strongly osmosensitive while still grow in media without osmostress (Figure 1A).

We then tested whether mutations in RSC components resulted in decreased osmostress gene expression. Expression of osmoresponsive genes such as *STL1*, *CTT1* and *GRE2* is significantly affected in these mutant strains (Fig. 1B and not shown). These three genes are driven by different transcription factors under the control of the Hog1 MAPK (i.e. Hot1, Msn2/Msn4 and Sko1 respectively) and thus indicate a general defect on stress-responsive genes rather than a defect associated to a given transcription factor. Correspondingly, the analysis of gene expression in a *rsc9*^{ts} strain under non-permissive temperature showed that a *ts* mutation in *RSC9* also strongly reduces expression of osmo-responsive genes upon stress (Figure 6B). Under the same non-permissive conditions, induction of *GAL1* in the presence of galactose was not affected (Figure S1). Therefore, as it is the case for promoter-associated factors and elongation factors, the RSC complex is important for gene expression in response to osmotic stress and for the ability of the cells to grow at high osmolarity.

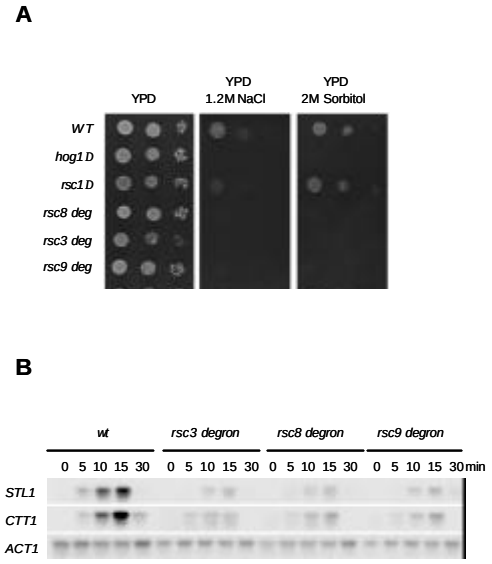


Figure 1. Mutations in components of the RSC complex affect cell survival at high osmolarity and display impaired osmostress gene expression.
(A) Wild-type (BY4741) and the indicated mutant strains were spotted on YPD plates without and with 1.2M NaCl or 2.4 M Sorbitol. Growth was scored after 4 days.
(B) RNA levels in wild-type and mutant strains (*rsc3*, *rsc8* and *rsc9* degron strains) were grown in YPGal medium up to mid-log phase, subjected to 2h at 37ºC and then subjected to osmotic shock (0.4 M NaCl) for the indicated time.

Hog1 interacts with the RSC complex.

We previously showed that Hog1 interacted with several complexes (e.g., SAGA and Rpd3) to recruit them at the osmo-responsive promoters. We therefore tested whether Hog1 is able to interact with the RSC chromatin remodeling complex by performing GST pulldown experiments in extracts from osmotically stressed cells expressing GST-Hog1 and TAP-tagged versions of Rsc3, Rsc8 and Rsc9 (all *bona fide* components of the RSC complex). In all cases, GST-Hog1, but not the GST control, co-precipitate the TAP-tagged RSC

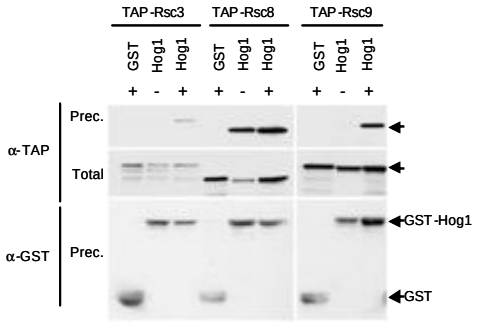


Figure 2. Hog1 physically associates with the RSC complex.
TAP-tagged-Rsc1, Rsc8 and Rsc9 strains that express GST or GST-Hog1 were subjected to a brief osmotic shock (10 min, 0.4 M NaCl). GST proteins were pulled down by gluthathione Sepharose 4B beads, and the presence of TAP proteins was probed by immunoblotting using anti-TAP proteins (PAP, Sigma) (top). Total extracts represents <20% of total input protein (middle). The amount of precipitated GST proteins was detected using anti-GST (bottom).

components (Fig. 2). These observations indicate that Hog1 physically associates with the RSC complex. In addition, they strongly support the idea that Hog1 could be targeting RSC to the stress responsive genes.

RSC associates with osmo-responsive genes upon stress.

Hog1 associates to osmo-responsive gene promoters and throughout the entire transcribed region of target genes in response to stress (Alepuz et al., 2001; Pokholok et al., 2006; Proft et al., 2006). Considering that Hog1 associates with the RSC complex, we asked whether RSC was recruited to osmo-responsive genes in response to stress by Hog1. We utilized chromatin immunoprecipitation to follow the binding of Rsc1 and Rsc9 components of the RSC complex to various regions of osmostress-activated genes (*STL1* and *CTT1*) before and after the addition of NaCl. Chromatin from wild type and *hog1D* cells expressing functional epitope-tagged Rsc1 and Rsc9 from their natural locus were immunopreciptated and analyzed by

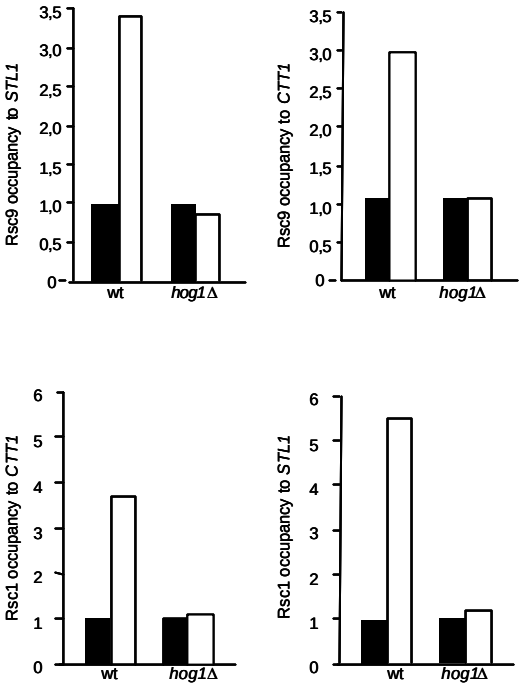


Figure 3. Hog1 mediates the recruitment of the RSC complex to stress-responsive genes in response to osmotic shock. Rsc9 and Rsc1 association *in vivo* by ChIP with the ORF regions of *STL1* and *CTT1* loci in wild type or *hog1D* strains (both contaning HA tagged-Rsc9 or HA-Rsc1) that were (open bars) or were not (filled bars) subjected to osmostress (0.4 M NaCl, 10 min). Results are shown as the fold-induction of treated against the untreated cultures normalized to a telomere internal control.

PCR. As shown in figure 3, the RSC complex associates specifically with osmoresponsive genes only in response to osmostress and, its recruitment is completely dependent on *HOG1*. It is worth noting, that we could only detect binding of RSC to the coding regions and not to the promoters of stress-responsive genes (see discussion). Therefore, our data suggests a possible role for RSC in the process of Hog1-mediated transcription at osmo-stress responsive genes.

Hog1 mediates a dramatic change on nucleosome organization at stress-responsive loci.

It has been shown that RSC can promote nucleosome remodeling when associates to chromatin (Saha et al., 2006).

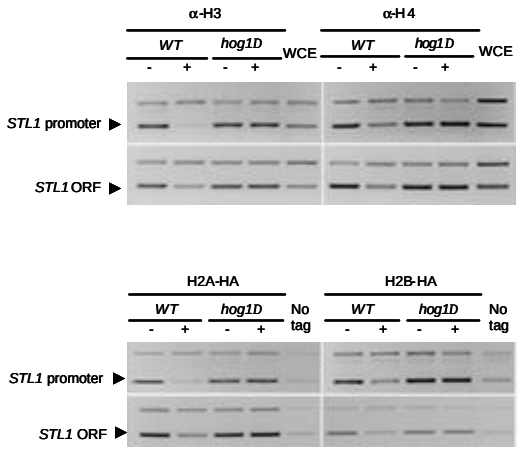


Figure 4. Hog1 is required for nucleosome reorganisation in response to osmostress. Association of histones H3, H4, HA-H2A and HA-H2B to the *STL1* promoter and *STL1* ORF in wild-type and *hog1D* strains that were (+) or were not (-) exposed to hyperosmotic stress. Histones were immunoprecipitated with antibodies against H3 (Abcam), H4 (Abcam) and anti-HA. Binding to *STL1* promoter and open-reading frame (ORF) was determined by ChIP. As internal loading control, PCR samples were amplified with a telomere region (upper band). Control lanes show DNA amplified from extracts prior to immunoprecipitation (Whole-Cell Extract, WCE) or without tagged protein (No tag).

The targeting of RSC by Hog1 to osmostress genes prompted us to analyse whether Hog1 was promoting nucleosome rearrangements at osmosresponsive genes upon stress. Chromatin from HA-tagged H2A or H2B in wild type and *hog1D* cells subjected to osmostress was immunoprecipitated using specific antibodies against anti-HA epitope or anti-H3 and H4 and analyzed by PCR. As shown in figure 4, a dramatic reduction of all histones is observed at both promoter and coding regions of *STL1* gene only in response to osmostress. Interestingly, no reduction in binding of any of the histones is observed in *hog1D* cells. Similar results were observed at *GRE2* and *CTT1* genes (Figures S2A). In addition, no changes on histone composition were observed in a non stress-responsive gene such as *ACT1* (Figure S2B). Kinetic analyses of the *in vivo* binding of histone H3 and H4 at *STL1* in response to osmostress showed that histone eviction is transient and correlates with Hog1 activation (Figure S3A and S3B). Thus, Hog1 is promoting massive histone eviction at osmo-responsive genes in response to osmostress.

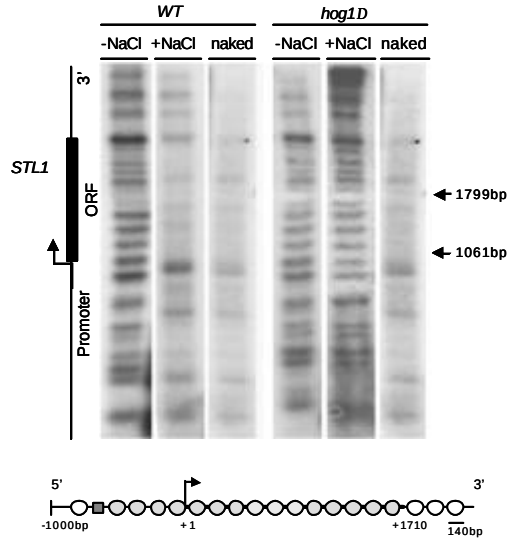


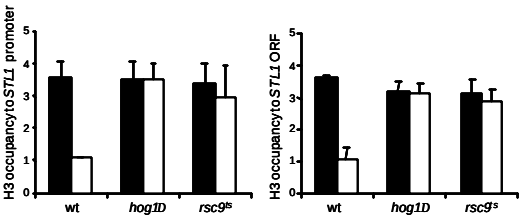
Figure 5. Hog1 mediates major changes in nucleosome content in response to high osmolarity. Chromatin and naked DNA from wild-type and *hog1D* cells treated (+ NaCl) or not (- NaCl) was digested with Micrococcal nuclease (MN), digested with EcoRI restriction enzyme, resolved in agarose gels, and hybridized with a DNA probe to map nucleosomes at chromosomal *STL1*. Arrows indicate positions of DNA ladder. The lower scheme shows the location of nucleosomes (ovals) at the *STL1* gene in the wild type strain without stress.

To support the data from ChIP analyses and shed some light on chromatin organisation we analysed the nucleosome pattern in the *STL1* gene by performing Micrococcal Nuclease (MN) digestion of chromatin before and after stress. Wild type and *hog1* strains were subjected or not to osmostress (same conditions used for ChIP analyses) and cells were fixed before chromatin and nuclease digestion to prevent Hog1 activation during spheroplast preparation (see experimental procedures). We found that the digestion pattern of *STL1* is consistent with the fact that without stress, *STL1* exhibits strongly positioned nucleosomes at the promoter, coding and 3' regions. Interestingly, in response to osmostress the patterning for nuclesome positioning dramatically changes and chromatin displays a similar digestion pattern than naked DNA (Figure 5). Correspondingly with the ChIP analyses, MNase digestion showed that exposure to osmostress of *hog1D* cells did not affect nucleosome organization when compared to non-stressed cells (Figure 5). Nucleosome positioning did not change in the non-stress responsive genes such as *ACT1* or *GAL1* upon stress as it was observed by ChIP (data not shown). Therefore, our ChIP and MNase digestion data supports the idea that Hog1 is inducing strong nucleosome positioning at osmoresponsive genes upon stress.

RSC function is required for nucleosome re-organisation upon osmostress.

The association of Hog1 with the RSC complex suggested the possibility that the RSC complex might be mediating the remodeling of chromatin that occurs in response to osmostress. Thus, to understand the mechanism by which Hog1 stimulates nucleosome reorganization upon stress we assessed whether the RSC complex is important for histone eviction in stress-responsive genes. Chromatin from wild type and *rsc9*^{ts} cells subjected to osmostress was immunoprecipitated using specific antibodies against H3 and analyzed by PCR. As shown in figure 6A, a decrease in histone H3 binding was observed upon osmostress at the *STL1* loci in wild type cells, which was neither observed in *rsc9*^{ts} cells nor *hog1* deficient cells. Similar results were obtained when histone H3 eviction was analysed at the *CTT1* loci (not shown). Correspondingly, gene expression in response to osmostress was reduced in the *rsc9*^{ts} strain (figure 6B). Therefore, RSC function is required for chromatin remodeling at osmo-responsive genes upon stress.

A



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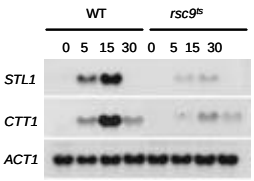

Figure 6. Chromatin remodeling and gene expression upon osmostress is dependent on Rsc9.

(A) Rsc9 is required for histone eviction at *STL1* loci in response to high osmolarity. Association of histone H3 in wild-type, *hog1D* and *rsc9*^{ts} strains termosensitive allele which had a Gln489→Stop mutation supposedly encoding a truncated form of the protein (Rsc9ts). Strains were grown at 25ºC until OD_{660} 0.5, shifted to 37ºC for 1h 30 min and samples were taken for either untreated (filled bars) and treated (open bars) with 0.4 M NaCl for 10 minutes. Histone H3 was immunoprecipitated with an anti-H3 antibody (Abcam). Occupancy to *STL1* promoter and ORF regions was normalized to *TEL* as internal control. Data represent the mean and standard deviation of three independent experiments.

(**B**) Mutation of *RSC9* causes impaired transcription of osmostress genes. Wild type and Rsc9ts strains were grown as in (A) and samples were subjected to osmotic shock (0.4 M NaCl) for the indicated times. Total RNA was assayed by northern blot for *STL1*, *CTT1* and *ACT1* (as a loading control).

Deletion of the *SET1* methylase or mutation of lysine 4 of histone H3 bypasses the requirement for RSC in osmostress gene expression.

A genetic screen searching for suppressors of *rsc9*^{ts} sensitivity to heat stress yielded a mutant version of the *SET1* methylase. This result pointed out a genetic relationship between the RSC complex and methylation (R.D and G.A unpublished observation). We tested then whether deletion of the *SET1* methylase was able to suppress the transcriptional defect observed in the *rsc9*^{ts} strain in response to high osmolarity. Indeed, deletion of *SET1* resulted in a partial suppression of the defects on gene expression observed for *CTT1* and *STL1* caused by RSC inactivation (Figure 7A). Deletion of another histone methylase, *SET2*, did not affect gene expression upon stress. Similar results were obtained when the *rsc9* degron strain was used instead of the *rsc9*^{ts} strain (figure 7B).

RSC inactivation impairs histone eviction and proper gene expression in response to osmostress. We then tested whether mutation of *SET1* also suppresses the impairment for histone eviction observed in a *rsc9*^{ts} strain. As shown in figure 7C, eviction of histone H3 was restored in *STL1* and *CTT1* loci in cells containing *set1* deletion. Suppression of the RSC defect by *set1*Δ is not restricted to *rsc9* mutants because similar results in gene expression and histone eviction were observed in *rsc8 set1*Δ cells (not shown). Therefore, deletion of *SET1* histone methylase bypasses the requirement for RSC to remodel chromatin in response to stress.

Set1 tri-methylates lysine 4 of histone H3. To analyse whether the suppression of RSC by *set1D* was due to the effect of Set1 methylase over histone H3 lysine 4, we analysed gene expression and histone eviction in a *rsc9*^{ts} strain containing a mutation on histone H3 lysine 4 to alanine

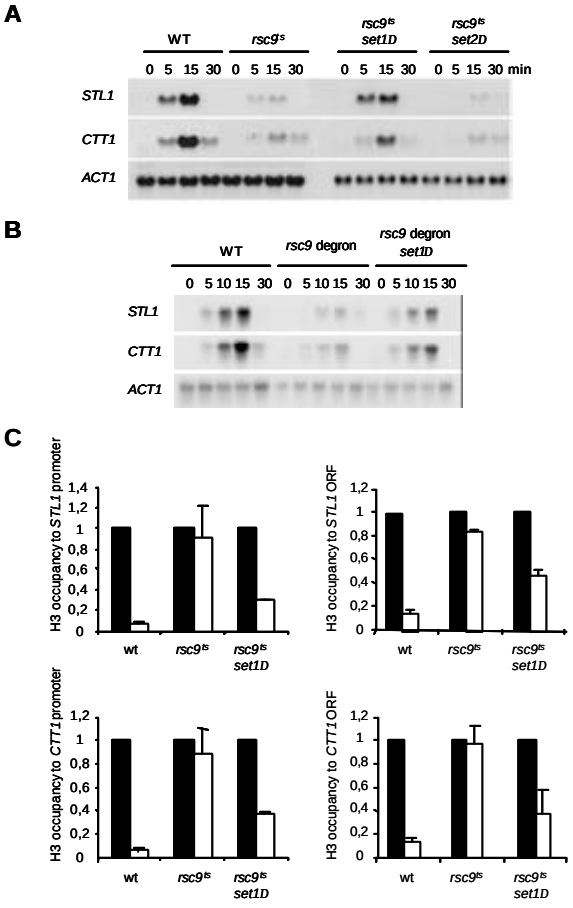

Figure 7. Deletion of *SET1* partially suppresses the deffect of *rsc9* mutations in osmostress gene expression and chromatin remodelling.
(**A)** Deletion of *SET1* restores gene expression in response to high osmolarity in a *rsc9* deficient strain. Indicated strains were subjected to osmotic stress (0.4 M NaCl) for the indicated times. Total RNA was assayed by northern blot for *STL1*, *CTT1* and *ACT1* (as a loading control).
(**B)** Deletion of *SET1* restores gene expression in response to high osmolarity in a *rsc9* deficient strain. Wild-type and *rsc9* degron and *rsc9* degron *set1D* mutant strains were grown in YPGal at 25ºC up to OD_{660} 0.5 , shifted to 37ºC for 2 h and subjected to osmotic shock (0.4 M NaCl) for the indicated times. RNA levels were probed for *STL1*, *CTT1* and *ACT1* (as a loading control).
(**C)** Deletion of *SET1* restores nucleosome eviction in a *rsc9* deficient strain. Association of histone H3 in wild-type, *rsc9*^{ts} and *rsc9*^{ts} *set1D* mutant strains analysed by ChIP before (filled bars) or after hyperosmotic stress (open bars). Real-time PCR was performed to determine relative occupancy of histone H3 to *STL1* and *CTT1* promoter and coding regions (ORF) normalized to the telomere control. Data represent the mean and standard deviation of three independent experiments.

(H3K4A). As shown in figure 8A, histone eviction in a *rsc9*^{ts} *H3K4A* was similar to the *rsc9*^{ts} *set1D* strain in contrast to the lack of histone eviction observed in *rsc9*^{ts} cells. Correspondingly, gene expression also increased in the *rsc9*^{ts} *H3K4A* cells when compared to the *rsc9*^{ts} cells (figure 8B). Thus, these data indicate that methylation of histone H3 K4 prevents histone eviction in absence of RSC which suggests that this mark imposes an extra layer of control on stress-responsive genes. Thus, it would be expected that histone H3 K4 should be already methylated in absence of stress. We performed ChIP analyses monitoring H3K4 tri-methylation and found that whereas no tri-methylation was observed in a histone H3 K4A mutant or in a *set1D* mutant, wild type or *rsc9*^{ts} strains showed methylated H3K4 at the *STL1* or *CTT1* genes already in absence of stress (Figure S4).

Discussion

Stress-activated protein kinases regulate the expression of specific classes of genes that permit the adaptation to environmental stress. Often, SAPKs trigger the necessary response by directly regulating transcriptional initiation through phosphorylation of transcriptional regulatory proteins. In yeast , the Hog1 SAPKs engages the transcription program in response to osmostress. Hog1 associates with the osmostress responsive genes and in addition to control gene expression through the phosphorylation of DNA-binding regulatory proteins also forms an integral component of transcription complexes involved in the recruitment of the Pol II, SAGA and chromatin modifying activities at stress-responsive genes (Alepuz et al., 2003; Zapater et al., 2007; de Nadal et al., 2004).

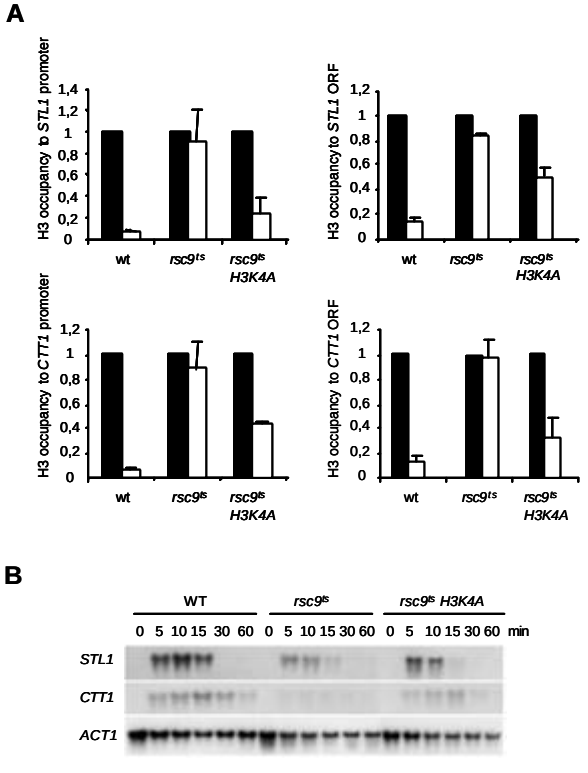

Figure 8. Mutation of K4 in histone H3 partially suppresses the deffect of *rsc9* mutation in osmostress gene expression and chromatin remodelling.
(**A)** Mutation of K4 in histone H3 (H3K4) restores nucleosome eviction in a *rsc9* deficient strain. Association of histone H3 in wild-type, *rsc9*^{ts} and *rsc9*^{ts} *H3K4A* mutant strains analysed by ChIP before (filled bars) or after hyperosmotic stress (open bars). Real-time PCR was performed to determine relative occupancy of histone H3 to *STL1* and *CTT1* promoter and coding regions (ORF) normalized to the telomere control. Data represent the mean and standard deviation of three independent experiments.
(**B)** Mutation of K4 in histone H3 (H3K4A) restores gene expression in response to high osmolarity in a *rsc9* deficient strain. Indicated strains as in (A) were subjected to osmotic stress (0.4 M NaCl) for the indicated times. Total RNA was assayed by northern blot for *STL1*, *CTT1* and *ACT1* (as a loading control).

Recent reports have shown that 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 interacts with elongating Pol II and components of the TEC to stimulate mRNA production, which defines Hog1 as a *bona fide* elongation factor, with the novel feature that its role in elongation is restricted to osmoresponsive genes (Proft et al., 2006; Pokholok et al., 2006)). Actually, in addition to Hog1, other signaling kinases are also present at the coding regions of target genes (Pokholok et al., 2006). Although, the mechanism by which these signaling kinases stimulate transcription is still unknown it opens a novel role for this group of kinases in chromatin (Edmunds and Mahadevan, 2006; Chow and Davis, 2006).

The Hog1 SAPK is part of the transcription elongation complex during the initial phase of osmostress adaptation. Recruitment of Hog1 to ORFs of stress genes is initially identical to that of the Pol II. However, the presence of the SAPK is restricted at the initial phase of the elongation, suggesting a role for the SAPK in elongation at the initial stages of elongation. One candidate for such a function is the modification of the nucleosomes occupying the stress responsive genes (Proft et al., 2006). Here, we show that in response to Hog1 activation there is a transient but dramatic change on nucleosome positioning at osmosresponsive loci upon stress. Hog1 mediates nucleosome rearrangements at stress genes by physically associating and targeting the chromatin remodeling RSC complex to the coding regions of stress-responsive genes.

subset of genes has not been described. Genome wide binding analyses showed that strong changes on localization of RSC components occurs upon different type of stresses indicating that external stimuli promotes changes on RSC activity that can be mediated by signaling molecules (Ng et al., 2002; Damelin et al., 2002). Interestingly, the specificity for the RSC to stress genes might be determined by the direct targeting of the MAPK to stress-responsive genes. Therefore, a function of Hog1 at the stress loci is the recruitment of RSC to mediate chromatin remodeling .

Recruitment of Hog1 to osmoresponsive genes occurs at the promoters via binding to specific transcription factors and independently at the coding regions of the genes through the 3' UTRs (Proft et al., 2006). We have observed that RSC is important to mediate nucleosome reorganization at both promoter and coding region of osmo-responsive genes, however, we only detect recruitment of RSC at the coding regions of stress genes. Although we cannot exclude that binding of RSC also occurs at the promoters with less affinity than in the coding regions it might be that Hog1 mediates recruitment of RSC once the TEC is formed after an initial round of transcription. Actually, maximal histone displacement is observed ten minutes after stress when Pol II is already maximally recruited at the promoters five minutes before (Proft et al., 2006).

Defective histone eviction in response to stress caused by mutations in RSC is suppressed by the deletion of the histone methylase *SET1* or mutation on the lysine 4 of histone H3. Correspondingly, defective gene expression due to RSC deficiency is also suppressed by the lack of methylation on histone H3 K4. These data suggest that at osmo-responsive genes, H3 K4 methylation might be playing a negative role to prevent gene expression until RSC is targeted by Hog1 to remove this mark. Actually, under non-stress conditions, the H3K4 is methylated at stress-loci and recent reports have shown that certain PHD domains can target repressing activities through interaction with trimethylated lysine 4 in Histone H3 (Shi et al., 2006). Alternatively, histone H3 K4 methylation could be a licensing mark for RSC to be the only remodeling complex able to act on osmostress-responsive genes. The absence of tri-methylation could lead to other remodeling factors to act at the osmo-responsive genes. In any case, there is a close relationship between the methylation by Set1 and the RSC complex to control the

Mutations that affect RSC a

remodeling of chromatin in response to Hog1 activation at the stress-responsive loci.

Experimental procedures

Yeast strains and plasmid DNAs

Yeast strains used in this study are the wild type strain BY4741 (*MAT***a** *his3*−Δ1 *leu2*−Δ0 *met15*−Δ0 *ura3*−Δ0) and its derivatives containing chromosomally integrated *RSC3-TAP*, *RSC8-TAP* and *RSC9-TAP* and the derivative deletion strains YGM61 (*MAT***a** *hog1*::*kanMx4*) and *rsc1::kanMx4* (a gift from Dr. M. Peter, ETH Zürich). Derived from BY4741 were strains in lower panels of figure 4: YGM164 (*MAT***a** *HTA1-HA6-HIS3*) and YGM177 (*MAT***a** *HTA1-HA6-HIS3 hog1*::*kanMx4*) were genomically tagged at the C-terminus of the *HTA1* locus with a cassette encoding 6-HA epitope tag and *HIS3* as selectable marker; YGM165 (*MAT***a** *HTB1-HA6-HIS3*) and YGM181 (*MAT***a** *HTB1-HA6-HIS3 hog1*::*kanMx4*) were genomically tagged at the C-terminus of the *HTB1* locus by means of the described *HA6-HIS3* cassette.

Derivatives from W303-1A (*leu2*-3,112 *ura3*-1 *his*-11 *trp1*-1 *can1*-100) were the degron strains *rsc3* degron-URA3 (*MAT***a** *ADE2*; *his3*-11,15; *leu2*-3,112; *trp1*-1; *ura3*-1; *Dlys2::rKWD50N*, $P_{Gal1-10}$*-myc::UBR1::HIS3*; P_{CUP1}*-degron::rsc3::URA3*), *rsc8* degron-URA3 (*MAT***a** *his3*-11,15; *leu2*-3,112; *trp1*-11; *ura3*-1; *Dlys2::rKWD50N*, $P_{Gal1-10}$*-myc::UBR1::HIS3*; P_{CUP1}*-degron::rsc8::URA3*), *rsc9* degron-URA3 (*MAT***a** *his3*-11,15; *leu2*-3,112; *trp1*-11; *ura3*-1; *Dlys2*::rKWD50N, $P_{Gal1-10}$*-myc::UBR1::HIS3*; P_{CUP1}*-degron::rsc9::URA3*), and the deletion strain YGM185 (*rsc9* degron *set1::kanMx4*). Also derived from W303 was the $rsc9^{ts}$ strain (*MAT***a** *fae1-2::TRP1*), (Dr. G. Ammerer, Wien) and its derivatives YGM97 ($rsc9^{ts}$ *set1::kanMx4*), YGM207 ($rsc9^{ts}$ *H3K4A*), and YGM99 ($rsc9^{ts}$ *set2::kanMx4*). Strains in figure 3 were also W303 derivatives where the genomic locus of *RSC9* and *RSC1* were tagged at the carboxyl terminus with a sequence encoding 6-HA epitope tag followed by the *S. pombe HIS3* as selectable marker (YGM17 and YGM23 with a *hog1::kanMx4* deletion for *RSC9-HA6-HIS3*; YGM68 and YGM79 with a *hog1::kanMx4* deletion for *RSC1-HA6-HIS3*).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described previously (Kuras and Struhl, 1999; Alepuz et al., 2001). Yeast cultures were grown to early log phase (OD_{600} = 0.6-1.0) before aliquots of the culture were exposed to osmotic stress treatment (0.4 M NaCl) for the time specified in the figure legends. For cross-linking, yeast cells were treated with 1% formaldehyde for 20 min at room temperature. Antibodies used in this study were rabbit polyclonal to histone H3, H4 and tri-methyl H3K4 (Abcam). Monoclonal anti-HA antibodies were also used to immunoprecipitate HA tagged proteins in fig 3 and 4. Primer mixes were adjusted for balanced signals. Conventional and real-time PCR analysis of stress genes and constitutively expressed genes utilized the following primers (sequences are available upon request), with locations indicated by the distance from the respect ATG initiation codon: *STL1* (-372/-112 for promoter; +402/+630 for coding region), *CTT1* (-452/-160 for promoter; +422/+669 for coding region), *GRE2* (-300/+30 for promoter; +340/+620 for coding region), *ACT1* (-389/+30), *TEL1* (region 490 bp right arm of chromosome VI) used as a negative control. Experiments were performed on three independent chromatin preparations, and quantitative PCR analysis was performed in real time using an Applied Biosystems 7700 sequence detector. Immunoprecipitation efficiencies were calculated in triplicate by dividing the amount of PCR product in the immunoprecipitated sample by the amount of PCR product in the input sample. Data in Figs. 3 and 6A are presented as fold immunoprecipitation over the *TEL1* sequence control.

GST pull-down experiments

To analyze the association of Hog1 with components of the RSC complex, one milligram of yeast extract, from cells expressing specific TAP-tagged proteins, in buffer A (50 mM Tris-HCl pH 7.5, 15 mM EDTA, 15 mM EGTA, 0.1% Triton X-100, 150 mM NaCl, 2 mM DTT plus antiproteases and phosphatase inhibitors) was incubated with 50 µl of glutathione Sepharose 4B beads overnight at 4°C. The beads were washed extensively with buffer A, resuspended in loading buffer and resolved by SDS-PAGE. The antibody used to detect the TAP-tagged proteins was the PAP antibody from Sigma.

Northern blot analysis

Yeast strains were grown to mid-log phase in rich medium and then subjected to osmotic shock (0.4M NaCl) for the indicated times. Total RNA and expression of specific genes was probed using labeled PCR fragments containing

the entire open reading frame of *STL1* (1.7kbp), *CTT1* (1.7kbp), *GAL1* (kpb) and *ACT1* (1.4kbp).

Mnase nucleosome mapping

Yeast spheroplasts and micrococcal nuclease digestions were performed as described previously with modifications (Chavez et al., 1995). Spheroplasts were prepared from mid-log phase cultures grown in SC-Ura with 2% glucose and either untreated or treated with salt (0.4 M NaCl, 10 min), following 1% formaldehyde cross-linking for 20 min, stop with 125mM glycine for 15 min and 4 times TBS washes. Cells were then lysed and immediately digested with 7.5 to 125 mU of micrococcal nuclease. For naked DNA controls, genomic DNA was extracted as previously described and digested with 0.003 to 0.2 mU of micrococcal nuclease under the same conditions. Micrococcal nuclease-cleaved genomic DNA was digested with EcoRI (for *STL1*) and resolved in 1.5% agarose gel without ethidium bromide. For the analysis of *STL1*, the probe used was the 200-bp PCR fragment immediately upstream of the EcoRI site present in *STL1* (at -1052 from the transcription start site).

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Supplemental Figures

S1

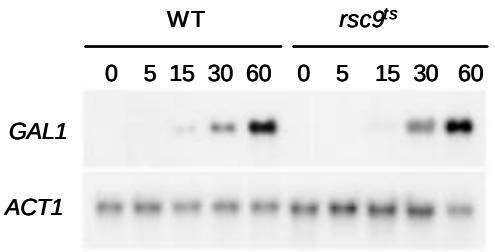


Figure S1. Induction of the *GAL1* gene is not affected by mutation in *rsc9*. Wild type and *rsc9*^{ts} strains were grown at 25ºC in YPRaf, shifted to 37ºC for 1 h 30 min until OD_{660} 0,7-0,9 and induced with Galactose 2% for the indicated times. Total RNA was assayed by northern blot for *GAL1* expression and *ACT1* as a loading control.

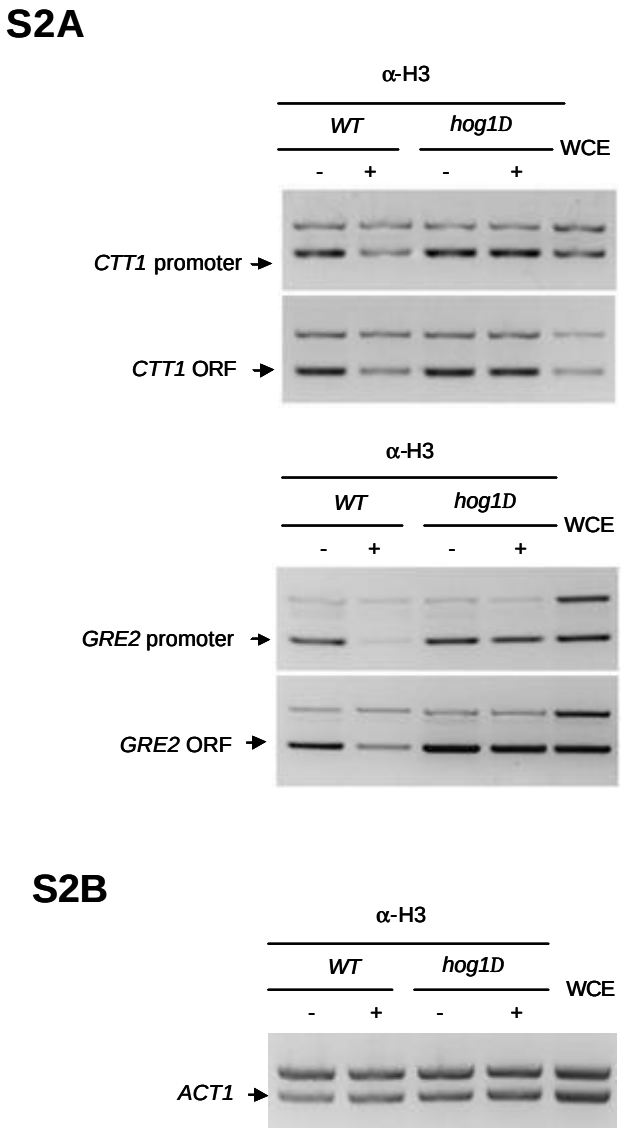
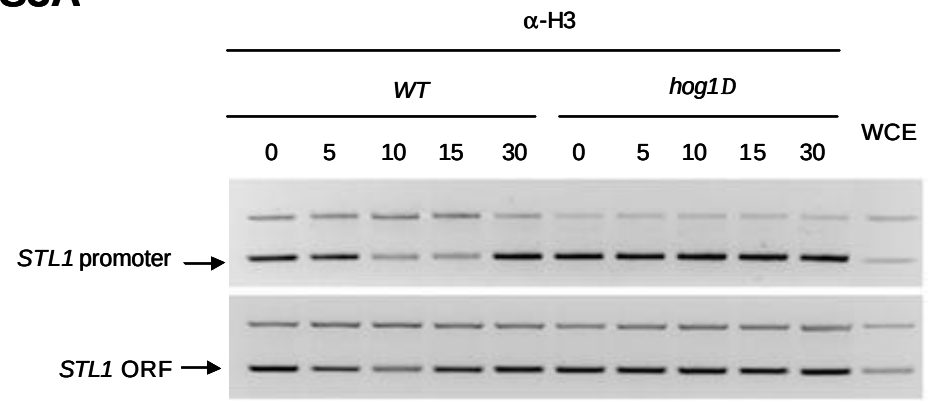


Figure S2. Hog1-dependent histone H3 eviction occurs at osmoresponsive genes but not in *ACT1* under stress conditions.

(**A)** Wild-type and *hog1D* strains carrying endogenous H3 were grown to mid-log phase and induced with 0.4 M NaCl for 10 minutes. ChIP was performed to determine binding of H3 (Abcam antibody) to promoter and coding regions of the osmotress-response genes *CTT1* and *GRE2*. As internal loading control, PCR samples were amplified with a telomere region (upper band). Control lanes show DNA amplified from extracts prior to immunoprecipitation (Whole-Cell Extract, WCE).

(**B)** Chromatin as in (A) was also used to determine histone H3 occupancy at the nonstress responsive gene *ACT1*. Internal and input controls are described in (A).

S3A



S3B

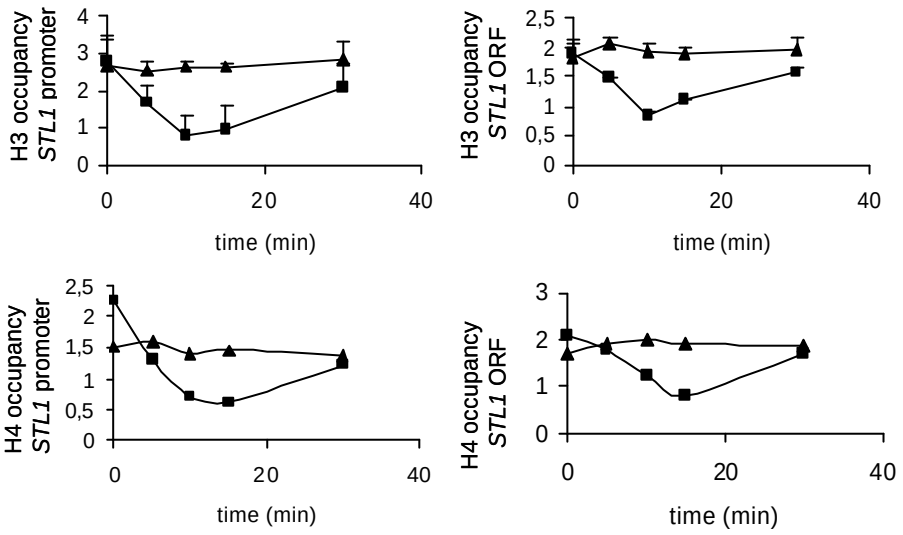


Figure S3. Osmostress induces a transient nucleosome eviction.
(**A)** Histone H3 is transiently evicted from the promoter and ORF of *STL1* in a Hog1-dependent manner. Wild type and *hog1D* strains were grown to mid-log phase and treated with 0.4 M NaCl for the indicated times. Immunoprecipitation against H3 was performed with anti-H3 antibody (Abcam) and binding to *STL1* promoter and open-reading frame (ORF) was determined by PCR. As internal loading control, PCR samples were amplified with a telomere region (upper band). Control lanes show DNA amplified from extracts prior to immunoprecipitation (WCE).
(**B)** Kinetics of histone H3 and H4 binding under osmostress conditions. *In vivo* binding of histone H3 and H4 to *STL1* promoter and coding region analysed in wild type (squares) and *hog1D* (triangles) strains after osmostress. Quantitative data was obtained by real-time PCR and TEL was used as a reference control. Data represents the mean and standard deviation of three independent experiments.

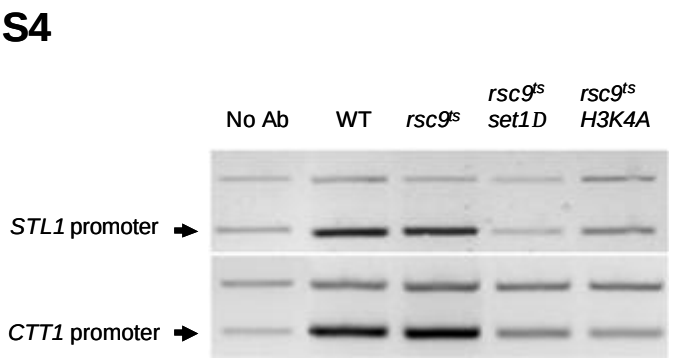


Figure S4. The levels of trimethyl H3K4 are high under non-inducible conditions at osmoresponsive genes. Wild type, *rsc9*^{ts}, *rsc9*^{ts} *set1D* and *rsc9*^{ts} *H3K4* strains were grown at 25ºC, shifted to 37ºC for 1h 30 min until mid-log phase and ChIP samples were then taken to analyze basal levels of trimethyl H3 K4 (Abcam antibody) at the promoter regions of *STL1* and *CTT1*. As a control, chromatin from wild type cells was immunoprecipitated without antibody (No Ab). PCR samples were amplified with TEL as a loading control (upper band).

The Hog1 MAPK targets the RSC complex to mediate eviction of H3 modified nucleosomes in osmoresponsive genes

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(Submitted)

Previous results showed that in response to osmotic stress Hog1 is intimately recruited to chromatin. Once bound to chromatin, Hog1 is able to regulate transcription initiation by the recruitment of the Rpd3 histone deacetylase complex, which promotes the modification of the chromatin at promoters (de Nadal et al., 2004) and the stimulation of the recruitment of the RNA Pol II (Alepuz et al., 2003). Moreover, the role of Hog1 is not restricted to promoters but it also associates with elongating Pol II and components of the elongation complex throughout the coding region of stress-response genes. Hog1 acts as a selective elongation factor, essential for proper mRNA production and for the amount of elongating Pol II at osmoresponsive genes. Global ChIP on chip analyses mapping other yeast signalling kinases have revealed that other kinases such as Tpk1, Fus3 and Kss1 are also found in coding regions of target genes (Pokholok et al., 2006). Thus, protein signalling kinases may have a more general role as chromatin associated enzymes that previously anticipated. Importantly, chromatin structure itself imposes significant obstacles to the process of transcription. By means of several histone modifications and nucleosome remodeling, chromatin is subjected structural changes, which enable access to the transcriptional machinery. Among the chromatin remodelers specialized in altering the structure, composition and positioning of the nucleosomes, the RSC complex has been shown to target several stress-dependent genes (Ng et al., 2002; Damelin et al., 2002).

In the presented work it is shown that the chromatin remodeling RSC complex is required for the Hog1-dependent transcription in response to osmotic stress. Hog1 interacts and targets RSC to mediate nucleosome eviction required for proper gene expression of osmostress genes in response to high osmolarity. Interestingly, requirement for RSC is partially bypassed mutation of the *SET1*

histone methyltransferase or its target histone H3 residue (Lys4). Thus, trimethyl-H3K4 is imposing a negative control to osmostress gene expression.

Mutants in the RSC complex are sensitive to osmostress and display reduced osmostress gene expression.

To identify novel activities involved in Hog1-mediated gene expression, we performed a high-throughput screening looking for mutations that rendered cells osmosensitive. We analyzed the osmosensitive phenotype of the complete yeast knock-out collection by automatically pinning an ordered array of ~ 4700 haploid deletion mutants onto rich medium plates or rich medium plus 2.4 M sorbitol. Since similar screenings had already been reported at mild osmostress conditions, the screen was performed at high sorbitol concentrations. The screen was performed in duplicate and a total of 179 mutants were scored as osmosensitive. As expected, several complexes related to regulation of gene expression such as the Rpd3 histone deacetylase complex, SAGA, Mediator and components of the Transcription Elongation Complex (TEC) were identified as essential for cell survival upon high osmolarity conditions (Zapater et al., 2007). Additionally, mutation of genes encoding non-essential components of the RSC complex (*RSC1* and *NPL6*) rendered osmosensitive cells. To characterize in more detail the importance of RSC in survival at high osmolarity, we analyzed the osmosensitive phenotype of other RSC mutants at high salt and sorbitol concentrations. Due to the fact that many RSC components are essential for viability, we used strains containing RSC factors tagged with a degron mark to reduce the total amount of these proteins. The results showed that reduced amount of subunits such as Rsc3, Rsc8 and Rsc9 strongly affected cell growth at high osmolarity. These results suggested a possible role for RSC in osmostress-induced gene expression. To test it, we analyzed osmostress gene expression by northern blot at mild (0.4M NaCl) osmostress conditions in a wild-type strain and in several RSC degron mutants. Expression of osmoresponsive genes such as *STL1*, *CTT1* and *GRE2* was significantly impaired in these mutant strains. Similar osmoresponsive-gene expression defects were observed when performing northern blot analysis in a *rsc9*^{ts} strain under non-permissive temperature at osmotic stress conditions. Importantly, transcription of the

galactose-induced gene *GAL1* was not affected under such non-permissive conditions.

Globally, these results indicated that an intact RSC complex was necessary for proper transcriptional activation in response to osmotic shock, as well as to guarantee cell viability under such conditions.

Hog1 interacts with the RSC complex

As pointed out in the introduction, Hog1 is able to interact with several transcription-related complexes such as transcription factors, coactivators (i.e. Rpd3, SAGA and Mediator complexes) and TEC components (de Nadal et al., 2004; Zapater et al., 2007; Proft et al., 2006). In some cases, Hog1 interaction with these complexes is necessary to mediate their recruitment at stress-responsive genes to activate gene expression. Because RSC was necessary for osmostress-gene expression, we addressed the question whether Hog1 was physically interacting with this chromatin-remodeling complex. We performed GST-pull down experiments of osmotically stressed yeast cell extracts expressing GST-Hog1 and TAP-tagged versions of Rsc3, Rsc8 and Rsc9 proteins. All the RSC factors tested were interacting *in vivo* specifically with GST-Hog1, and not the GST control. These results supported the idea that Hog1 was strongly associated to the RSC complex, and suggested the possibility that this association occurred to target or activate RSC function at osmoresponsive genes.

RSC associates with osmo-responsive genes upon stress.

Once shown the relevance of RSC in osmostress gene expression and its association with Hog1, we analyzed the presence of this complex at osmoresponsive promoters and the role of Hog1 in its recruitment. For this purpose, we performed ChIP experiments and followed the binding of the epitope-tagged RSC components Rsc1 and Rsc9 before and after stress in a wild type strain and in a *hog1*Δ strain. The results showed that RSC was bound to osmotic stress genes such as *STL1* or *CTT1* only after osmotic stress and in a Hog1-dependent manner. Importantly, recruitment of these RSC subunits could only be detected at coding regions of osmo-responsive genes, and not at the

corresponding promoter regions. Although we cannot exclude the possibility that our ChIP method was unable to detect weak but effective binding of RSC to promoter regions, a temptative explanation would be that Hog1-mediated RSC recruitment took place once TEC was formed after the first round of transcription. In fact, maximal histone eviction is observed at 10 minutes, whereas the RNA Polymerase peaks already at 5 minutes after an osmotic shock.

Taken together, our results suggest that Hog1 is required for targeting RSC to osmotic stress genes upon inducible conditions.

Hog1 mediates a dramatic change on nucleosome organization in stress-responsive loci.

The Hog1-dependent targeting of the RSC complex to osmostress-responsive genes prompted us to analyse whether RSC was playing a role on chromatin remodeling of such genes. The RSC complex, such as its homolog SWI/SNF complex, has a known ATPase dependent chromatin remodeling activity. RSC has been shown to remodel nucleosomes at DNA double-strand breaks upon DNA damage (Shim et al., 2007), and to remodel nucleosomes at chromatin templates *in vitro*, thus facilitating RNA Polymerase II elongation (Carey et al., 2006). Moreover, recent reports show that RSC interacts with the RNA Polymerase II and this interaction is critical for linking chromatin remodelling with transcription *in vivo* (Soutourina et al., 2006).

To test whether osmotically induced genes were subjected to nucleosomal rearrangements, we performed ChIP from H2A-HA or H2B-HA tagged wild-type and *hog1D* cells subjected to a brief osmotic shock. Histones were immunoprecipitated using antibodies against H3, H4 or the HA epitope. In response to osmotic stress, a dramatic reduction of the four core histones was observed at both promoter and coding region of the *STL1* gene. Importantly, such histone eviction was not occurring in a *hog1D* strain. Other osmoresponsive genes such as *CTT1* and *GRE2* had a similar Hog1-dependent massive nucleosome eviction under osmotic shock. Additionally, no changes in histone loading were observed in a non-stress responsive gene such as *ACT1*.

We next analysed the kinetics of the *in vivo* binding of histones H3 and H4 at the *STL1* gene, in response to osmostress. ChIP experiments demonstrated

that the histone loss at both promoter and coding regions of *STL1* was transient and dependent on Hog1. Moreover, the kinetics of histone eviction correlated with that of the kinase activation. Overall, these results suggested that Hog1 was mediating histone eviction upon induction of osmoresponsive genes. Further evidence confirming this hypothesis was obtained by analysis of the nucleosome positioning at the *STL1* gene by Micrococcal Nuclease digestion assays. Wild-type and *hog1Δ* strains were subjected to an osmotic shock following a formaldehyde 1% crosslinking to fix cells before obtaining spherophasts. Formaldehyde treatment was used to prevent activation of Hog1 due to sorbitol addition during spheroplast preparation. Chromatin was then digested with micrococcal nuclease and blotted to hybridize against the *STL1* gene. The results showed a strong nucleosome positioning at promoter and coding region of *STL1* without stress, and a chromatin pattern similar to naked DNA in response to osmostress. Furthermore, *hog1Δ* cells did not exhibit significant changes on nucleosome positioning when subjected to an osmotic stress compared to non-stressed conditions. Thus, Hog1-dependent massive nucleosome eviction is occurring upon osmotic shock, which is consistent with the previous ChIP results. Importantly, nucleosome positioning analysis of non-stress responsive genes such as *ACT1* or *GAL1* showed no changes in chromatin digestion patterns under osmostress conditions. Taken together, our ChIP and MNase digestion data demonstrate that Hog1 mediates strong nucleosome eviction in response to osmostress.

RSC function is required for nucleosome re-organisation upon osmostress

The Hog1-dependent recruitment of RSC to osmoresponsive genes under inducible conditions prompted us to study whether RSC could be involved in the histone eviction that occurs in response to osmostress. Thus, ChIP analysis was performed to determine the relative amount of histone H3 present before and after a brief osmotic shock at the *STL1* gene in wild-type, *hog1Δ* and *rsc9^{ts}* cells. Chromatin was immunoprecipitated using anti-H3 antibodies, and the PCR products confirmed a decrease in histone H3 binding upon osmostress at the *STL1* promoter and coding region in a wild-type strain. Interestingly, this histone eviction was observed neither in *hog1Δ* nor in *rsc9^{ts}* cells. Other osmoresponsive

genes such as *CTT1* and *GRE2* were tested with similar results. In that order, northern blot analysis of *rsc9*^{ts} cells showed a decrease in osmostress gene expression presumably due to the corresponding lack of nucleosome remodelling. Altogether, these experiments established a role of RSC in transcription-dependent histone eviction of osmoresponsive genes upon stress.

Deletion of the *SET1* methylase of mutation of lysine 4 of histone H3 bypasses the requirement for RSC in osmostress gene expression.

A genetic relationship between rsc9 and the set1 histone methyltransferase was established by a genetic screen in search of suppressors of *rsc9*^{ts} thermosensitivity. This result suggested that histone methylation could be imposing a negative control to RSC function on stress genes (R. Dechant and G. Ammerer, unpublished results). Indeed, the transcriptional defect observed for the mutant defective in RSC (*rsc9*^{ts}) in response to high osmolarity was partially suppressed when we deleted the *SET1* methyltransferase. Specifically, mRNA expression of *STL1* and *CTT1* upon osmotic shock was higher in an *rsc9*^{ts} *SET1D* strain compared to the *rsc9*^{ts} strain. Consistently, similar results were obtained when using *rsc9* degron strain instead of the *rsc9*^{ts}. It is worth noting that the suppression defect was specific for the Set1 methyltransferase deletion, as it was not observed when we mutated the Set2 methylase in *rsc9*^{ts} background cells.

We next performed ChIP experiments to analyze histone H3 eviction in both *rsc9*^{ts} and *rsc9*^{ts} *SET1D* strains. Corresponding to the previous gene expression results, eviction of histone H3 was significantly restored in osmostress-responsive genes in a *rsc9*^{ts} strain lacking *SET1*. Besides, suppression of the RSC defect by *SET1* deletion is observed both in *rsc9* degron and *rsc8* degron strains. These observations indicated that re-establishment of nucleosome remodelling caused by *SET1* deletion in cells defective for RSC resulted in increased gene expression profiles of osmostress genes. Thus, *rsc9*^{ts} *SET1D* cells are able to bypass the requirement for RSC to remodel chromatin in response to osmostress.

The Set1 histone methyltransferase has been shown to specifically trimethylate H3K4 residues at promoter and coding regions of transcriptionally active genes (Ng et al., 2003b). This observation prompted us to analyse

whether the remodelling effect derived from the mutation of *SET1* in *rsc9*^{ts} cells was indeed due to the lack of trimethylated-H3K4. ChIP and northern blot analysis where then performed to compare histone eviction and osmostress-gene expression of both *rsc9*^{ts} *SET1D* and *rsc9*^{ts} *H3K4A* mutant cells with *rsc9*^{ts}cells. Interestingly, similar patterns were obtained for *rsc9*^{ts} *SET1D* and *rsc9*^{ts} *H3K4A* mutant strains, confirming that trimethylation of H3K4 by Set1 was preventing histone eviction in the absence of the RSC complex. Hence, trimethyl-H3K4 was unexpectedly imposing a negative regulation at stress-responsive genes, since it was preventing histone eviction and proper gene expression. To confirm this hypothesis, we monitored the level of trimethylated-H3K4 residues by ChIP at osmoresponsive genes in the repressive state. Importantly, wild-type and *rsc9*^{ts} strains showed high levels of trimethylated H3K4 in the absence of stress at *STL1* and *CTT1* genes, whereas no significant trimethylation of H3K4 was observed in *rsc9*^{ts} *SET1D* nor in *rsc9*^{ts} *H3K4A* strains.

I personally contributed to all aspects of the design, execution and discussion of the experiments and results described in this manuscript, with exception of the genetic screen described in figure 1, which was performed by Dra. de Nadal.

GLOBAL DISCUSSION

Elucidating the mechanisms by which the Hog1 MAPK is regulating gene expression under osmostress conditions has been the main interest of this PhD Thesis. The importance of the role of Hog1 in transcription regulation is illustrated by several genome-wide transcriptional studies which revealed that a large number of genes (5-7% of the yeast genome approximately) undergo significant changes in their expression patterns in response to osmotic shock in a Hog1-dependent manner (Posas et al., 2000; Rep et al., 2000; Gasch et al., 2000; Causton et al., 2001; O'Rourke and Herskowitz, 2004). Our research group and other studies have clearly demonstrated the key role of the MAPK Hog1 in regulating transcription initiation upon osmostress (Alepuz et al., 2001; Alepuz et al., 2003; de Nadal et al., 2003; Proft and Struhl, 2002). Among the best known regulatory mechanisms, there is the direct phosphorylation and activation of specific transcription factors by the kinase Hog1 (reviewed in de Nadal et al., 2002), which lead to gene expression induction. Further investigation demonstated that Hog1 itself cross-linked with chromatin at several promoters in response to osmotic stress, this binding being important for eliciting gene transcription through recruitment of the RNA polymerase II holoenzyme (Alepuz et al., 2003). Additionally, the presence of Hog1 at promoters is also important to recruit the Rpd3 histone deacetylase complex, and thus, promote the modification of the chromatin to properly initiate transcription (de Nadal et al., 2004). In sum, the specific chromatin association of Hog1 to stress-responsive promoters opened a new dimension to the regulation of transcription initiation by signalling kinases. However, transcription is a highly regulated process involving many steps (preinitiation, initiation, promoter clearance, elongation and termination) which are coordinated simultaneously. Traditionally, the recruitment of protein factors involved in transcription elongation, as well as mRNA maturation, surveillance and export is coordinated by the phosphorylated CTD of the largest subunit of the RNA Pol II holoenzyme, Rpb1 (reviewed in Sims et al., 2004). The first article presented in this Thesis (Proft et al., 2006), provided evidence that the MAPK Hog1 was physically interacting with the Rpd1 subunit of RNA Pol II, and this interaction increased when the CTD in a phosphorylated state. This result suggested the possibility that Hog1 was selectively associated with the RNA Pol II during the transcription elongation process. Indeed, the interaction was involving other components of the Transcription Elongation Complex (TEC) such as Spt4, TFIIS,

Paf1 and Thp1. Notably, *in vivo* ChIP experiments revealed that Hog1 was travelling with elongating Pol II through the entire coding region of stress-responsive genes. Selective association with osmoresponsive genes was dependent on the 3'UTRs of these target genes. Furthermore, association of Hog1 with the TEC was necessary to increase mRNA expression of osmoresponsive coding regions in response to osmostress. This function of Hog1 required its kinase activity, although the relevant substrates were not known. The discovery that Hog1 can interact with the entire transcribed region of its target genes has been confirmed by Pokholok et al. (2006). These authors also extend this concept by demonstrating that other protein kinases such as Tpk1, Fuss1 and Kss1 are selectively targeted to coding regions of stress-induced genes. Thus, protein kinases might be playing a more general role as chromatin-associated enzymes than previously anticipated. For instance, the tethered protein kinases may regulate phosphorylation of transcription elongation factors, histones, chromatin remodelling enzymes, mRNA processing and export machinery, and other complexes involved in the transcription process. Although in Proft et al. (2007) it is demonstrated that Hog1 occupancy of the *STL1* coding region leads to increased mRNA production and Pol II processivity, further study should be made to address the physiological significance of protein kinase interactions with the entire transcribed region of target genes. Other processes such as mRNA stability, processing and transport could be modified by these kinases in order to properly induce gene expression in response to osmostress. Besides, specific chromatin sequences and structures of actively transcribed genes may enable interactions of protein kinases and other transcriptional regulators. In fact, it is worth noting that the presence of the MAPK was temporally restricted at the initial phase of elongation, suggesting a rapid and transient role at early stages of transcriptional elongation. A possible candidate for such a function is the modification of the chromatin structure of the stress responsive genes to allow accessibility of the transcriptional machinery. The aim of the second article presented in this PhD Thesis (Mas et al., submitted) was to identify chromatin remodelling activities required for the Hog1-mediated transcription in response to osmostress. In this paper, we show that upon osmostress there is a transient but massive histone eviction at promoter and coding regions of osmotically induced genes. Hog1 targets the chromatin remodelling complex RSC to stress loci to mediate nucleosome eviction in

response to osmotic shock. In the absence of the RSC complex, nucleosome remodelling is almost abolished. As a consequence, cells display a transcriptional defect and are not able to properly grow in high osmolarity media.

Taken together, these results provide evidence of *in vivo* nucleosome rearrangements at stress-responsive genes mediated by the RSC complex. Interestingly, genome-wide ChIP on chip analysis of RSC components showed that chromatin binding pattern of this complex changes upon several stress conditions, suggesting that signaling molecules could mediate such binding changes (Ng et al., 2002; Damelin et al., 2002). Thus, in the case of osmostress-inducible genes, Hog1 kinase is targeting RSC to mediate chromatin remodelling.

In point of fact, our laboratory has shown that several chromatin modifying activities are recruited upon osmotic shock to properly induce transcription. The SAGA and the Rpd3 complex are targeted to osmotically induced genes in a Hog1-dependent manner, this targeting being important for proper transcriptional outcome (Zapater et al., 2007; de Nadal et al., 2004). Both complexes contribute to the acetylation and deacetylation balance at stress-responsive genes. In Mas et al., we demonstrate that the methylation of histone residues at osmostress inducible genes is also regulating gene expression. Specifically, mutation of the Set1 methyltransferase or its target histone residue H3K4 in the absence of the RSC complex, resulted in suppression of the gene expression defect due to lack of RSC. Correspondingly, histone eviction in response to stress was enhanced in *rsc9^{ts} SET1D* and *rsc9^{ts} H3K4A* strains compared to *rsc9^{ts}*. Therefore, trimethyl-H3K4 was imposing a negative regulation as it prevented histone eviction and gene expression in the absence of RSC. The fact that under non-stress conditions high trimethyl-H3K4 levels were found at osmoresponsive genes also supports this hypothesis. Although trimethylation of H3K4 by Set1 has been traditionally associated with transcriptionally active genes (reviewed in Sims et al., 2004), it seems that stress regulated genes are regulated in a different way. Other reports suggest similar phenomena for heat shock inducible genes (Zhang et al., 2005). Very recently, evidence that certain repressing activities are targeted through interaction with trimethylated-H3K4 has also give insight into the negative role of this histone mark (Shi et al., 2006). Interestingly, in the absence of tri-methyl lysine 4 at histone H3 in cells defective for RSC, histones are efficiently

evicted in response to osmostress. Thus, either the altered chromatin structure or the presence of other chromatin remodelling complexes could lead to nucleosome eviction upon osmostress. Further experiments should be performed to support this hypothesis. Moreover, it would be interesting to establish the relationship between functions of RSC, the SET1 methyltransferase, SAGA and the histone deacetylase Rpd3 complex regarding the activation of transcription upon osmotic stress. It is not clear whether the recruitment of these complexes is independent or not, and whether methylation and acetylation of histones H3 and H4 respectively provide an altered chromatin structure or specific binding surfaces for the recruitment of regulatory factors.

In conclusion, the results obtained in this PhD Thesis provide useful guidelines for studying the mechanisms by which the Hog1 kinase regulates gene expression and chromatin structure.

CONCLUSIONS

The following conclusions can be reached from the results of the scientific articles presented in this PhD thesis:

- The Hog1 MAPK interacts with elongating RNA polymerase II and with general components of the Transcription Elongation Complex (TEC).
- Hog1 is able to selectively travel through promoter and coding regions of osmostress-responsive genes.
- The selective association of Hog1 to coding regions is mediated by the 3' UTRs of osmoresponsive genes.
- Interaction of Hog1 with the TEC is necessary for the full transcriptional output of osmoresponsive coding regions in response to stress.
- Thus, Hog1 acts as a selective elongation factor for genes induced by osmotic stress.

- The RSC complex has been identified as a novel requirement to induce osmostress-mediated transcription.
- Cells lacking the RSC chromatin remodeling complex are sensitive to high osmolarity and show compromised expression of osmostress genes.
- Hog1 physically interacts with RSC components *in vivo* and targets the chromatin remodelling complex to specific osmoresponsive genes.
- Upon transcriptional induction, core histones are evicted from the entire transcribed region of osmoresponsive genes in a transient and Hog1-dependent manner.
- The RSC complex is necessary for nucleosome eviction at both promoters and coding regions of osmostress genes, which allows for proper induction of gene expression.
- In cells deficient on the RSC complex, both deletion of the *SET1* methyltransferase or mutation on its target residue H3K4 leads to histone eviction and transcriptional induction of osmoresponse genes. Thus, trimethyl H3K4 imposes a negative mark for osmostress gene expression.

Taken together, a better understanding of the mechanisms of transcriptional regulation and chromatin modification driven by the stress-activated kinase Hog1 has been achieved.

SUPPLEMENTARY ARTICLES

During the working period spent in Dr. Posas' laboratory, we were aimed at studying the role of Hog1 in the control of gene expression. Besides the presented results concering Hog1 involvement in transcription elongation and chromatin remodelling, I have also contributed to elucidating other mechanisms of transcriptional regulation driven by the MAPK. Since my personal contribution to these articles was mainly of experimental support, these articles have not been included in the discussion. However, a summary is included (see below).

- The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes.

Eulàlia de Nadal, Meritxell Zapater, Paula M. Alepuz, Lauro Sumoy, Glòria Mas & Francesc Posas.

Nature, Vol 427 pp.370-374, 2004.

- Expression of the HXT1 low affinity glucose transporter requires the coordinated activities of the HOG and Glucose signalling pathways.

Lidia Tomás-Cobos, Laura Casadomé, Glòria Mas, Pascual Sanz and Francesc Posas.

The Journal of Biological Chemistry, Vol 279 pp.22010-22019, 2004.

SUMMARY

In response to hyperosmolarity, the MAPK Hog1 coordinates the transcription programme required for cell adaptation. Hog1 tightly binds chromatin in response to osmostress and elicits gene expression by mechanisms which are not completely understood. To provide new insights into the mechanisms used by Hog1 to activate gene expression, other than simple modification of activators, in de Nadal *et al.* (2004) a genetic screening for chromatin-modifying osmosensitive mutants was performed. Mutant strains lacking specifically the Rpd3 histone deacetylase and its interacting protein Sin3 were sensitive to high osmolarity. Moreover, Hog1-mediated gene expression was impaired by deleting *RPD3*. Microarray analysis also showed that Rpd3 was important for eliciting osmostress gene expression. Correspondingly, mutation of

lysine residues in histone H4 resembling a deacetylated state or a constitutively acetylated state lead to transcriptional hyperactivation or repression, respectively. By means of coimmunoprecipitation experiments, de Nadal *et al*. (2004) also demonstrated that Hog1 physically interacts with Rpd3 *in vivo* and *in vitro* and, upon osmostress, targets the deacetylase to specific osmostress-responsive genes. Binding of the Rpd3-Sin3 complex to specific promoters leads to local histone H4 lysine deacetylation. Furthermore, recruitment of the RNA Polymerase II was compromised in an *rpd3D* strain, confirming that local histone deacetylation of osmoresponse genes by Rpd3 allows for RNA Polymerase entry at promoter regions and induction of gene expression. Together, these data indicated that Rpd3 deacetylase complex had a positive role in gene expression, thus contradicting traditional association of histone deacetylation with transcriptional repression. In fact, heat stress also induced recruitment of the Rpd3 complex to heat-shock responsive promoters, in a Hog1 independent-manner. Therefore, Rpd3 would function as an activator of transcription in stress gene expression, Hog1 being the chief targeting molecule of Rpd3 in response to osmostress.

My personal contribution to this work (de Nadal et al., 2004) was focused on the chromatin immunoprecipitation experiments which lead to the last figure of this article. However, I followed closely the whole work.

As mentioned above, activation of the Hog1 MAPK induces diverse osmo-adaptive responses such as the regulation of gene expression. Genome-wide transcriptional studies showed that a large number of genes are regulated by osmotic stress in a Hog1-dependent manner. Among these, there are genes that encode proteins involved in carbohydrate metabolism such as *HXT1*. Expression of the *HXT1* gene, which encodes a low affinity glucose transporter in *S.cerevisiae*, has been used as a model to study the process of transcriptional activation by glucose. The expression of *HXT1* is regulated positively in response to glucose by the general glucose induction pathway, involving Rtg1 as its transcription factor. In Tomás-Cobos et al. (2004) it is shown that, in addition to the glucose signalling pathway, regulation of *HXT1* expression also requires the HOG pathway. Actually, the Hog1 MAPK is necessary for induction of *HXT1* expression by glucose and osmostress. Moreover, in cells lacking the transcriptional repressors Rtg1 or Sko1-Tup1-Ssn6, from the glucose induction

pathway and HOG pathway respectively, the expression of *HXT1* was overinduced in response to osmostress (even in the absence of glucose). Both Rtg1 and Sko1 were bound to the *HXT1* promoter under low glucose conditions to co-repress gene transcription, and were released from the promoter upon addition of glucose and osmostress. Taken together, these results suggest that regulation of *HXT1* expression is achieved by two independent transcription factors, Rtg1 and Sko1, controlled by the glucose induction and HOG signaling pathways, respectively.

My personal contribution to this article (Tomás-Cobos et al., 2004) was mainly of technical support performing the chromatin immunoprecipitation experiments shown in figure 6. Nonetheless, I was involved in the discussion and progression of the project.

The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes.

Eulàlia de Nadal, Meritxell Zapater, Paula M. Alepuz, Lauro Sumoy, Glòria Mas and Francesc Posas

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Expression of the *HXT1* low affinity glucose transporter requires the coordinated activities of the HOG and Glucose signalling pathways.

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