Meritxell Zapater i Enrique

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CONTROL OF TRANSCRIPTION INITIATION BY THE STRESS ACTIVATED Hog1 KINASE

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Treball dirigit pel Dr. Francresc Posas Garriga

i la Dra. Eulàlia de Nadal Clanchet

Unitat de Senyalització Cel.lular

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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SUMMARY

In *Saccharomyces cerevisiae* changes in the extracellular osmotic conditions are sensed by the HOG MAPK pathway, the functional homolog of the mammalian stress activated MAP Kinases JNK and p38. The Hog1 SAPK elicits the program for cell adaptation that includes modulation of several aspects of cell biology, such as gene expression, translation and cell-cycle progression. At the beginning of this PhD Project, the mechanisms by which Hog1 was controlling gene transcription were not completely understood. Our main objective was then to characterize the molecular mechanisms by which the Hog1 MAPK modulates transcription upon osmostress. Previous results showed that phosphorylation of activators was a mechanism to regulate transcription. However, this could not be the only means to mediate gene expression modulation. Here we have shown that assembly of the general transcription machinery at osmoresponsive promoters depends on the presence of active Hog1. Hog1 interacts with the RNA polymerase II (Pol II) and with general components of the transcription machinery. Thus, anchoring of active Hog1 to osmoresponsive promoters by the transcription factor is essential for recruitment and activation of RNA Pol II, a mechanism that might be conserved among eukaryotic cells.

In addition, we aimed to identify novel chromatin modifying and remodelling activities involved in the Hog1-mediated osmostress gene expression. For this purpose, we performed a genome-wide genetic screening searching for mutations that render cells osmosensitive at high osmolarity and displayed reduced expression of osmoresponsive genes. By this way, Rpd3 histone deacetylase, SAGA and Mediator complexes were identified as novel regulators of osmostress-mediated transcription. Cells lacking Rpd3 show compromised expression of osmostress genes. Moreover, Hog1 interacts physically with Rpd3 and targets the deacetylase to specific promoters, where Rpd3 deacetylates histones leading to entry of RNA pol II and induction of gene expression. Mediator is essential for osmostress gene expression, however, the requirement for SAGA is different depending on the strength of the extracellular osmotic conditions. At mild osmolarity, SAGA mutants only show very slight defects on RNA Pol II recruitment and gene expression, whereas at severe osmotic conditions, SAGA mutants show completely impaired RNA Pol II recruitment and transcription of osmoresponsive genes. Thus, our results define a major role for Rpd3, SAGA and Mediator in the Hog1-mediated osmostress gene induction.

INTRODUCTION

1. OSMOTIC STRESS

Single cell organisms living freely in nature, such as the yeast *Saccharomyces cerevisiae*, are exposed to highly variable environmental conditions that threaten their survival or at least prevent them from performing optimally. These environmental changes are commonly referred to as cell stress and can be of a physical or chemical nature, as changes in temperature, pressure, radiation, nutrient availability, access to oxygen and concentration of solutes and water (Hohmann, S. and W. H. Mager, 1997, Yeast stress responses. R. G. landes company, Austin, TX), (Hohmann, 2002),. In response to these stresses, cells are able to coordinate various intracellular activities to respond, adapt and maximize their probability of survival and proliferation.
One of the most common stresses is the change in water activity, defined as the chemical potential of free water in solution. To maintain an adequate cell volume and the appropriate conditions for biochemical reactions, the water activity of the cytosol and its organelles has to be lower than that of the surrounding medium. Two different situations can alter the water activity: an osmotic upshift (or hyperosmotic shock) and an osmotic downshift (or hypoosmotic shock). When cells experience a hyperosmotic shock, they rapidly lose water and shrink. On the other hand, a hypoosmotic shock increases the water concentration gradient and leads to a rapid influx of water, cell swelling and increased turgor pressure. In the yeast's natural environment, the water activity can change widely and rapidly. For this reason, yeast cells have developed mechanisms to ensuring survival to changes in water activity. Since passive flow of water occurs very fast, the survival mechanisms need to be activated within the first seconds after a sudden osmotic shift (Brown, 1976; Blomberg and Adler, 1992).

1.1. Hyperosmotic Shock

A hyperosmotic shock (osmotic stress) is characterized by water outflow, since cellular water follows its concentration gradient by passive diffusion, and shrinkage of the cells. This phenomenon results in an increased concentration of biomolecules and ions in the cell, which usually leads to an arrest of cellular activity. However, yeast cells have developed mechanisms to adjust to high external osmolarity. Adaptation to increased osmolarity is an active process that

comprises two distinct phases: detection of the osmolarity changes and development of the appropriate responses to maintain optimal cellular activity. In *S. cerevisiae*, production and accumulation of chemically inert osmolytes, such as glycerol, allows the cells to increase the internal osmolarity and plays a central role in the process of osmoadaptation (Hohmann, 2002; de Nadal et al., 2002; Gustin et al., 1998). Therefore, yeast cells can be metabolically active and proliferate over a range of external water activity. The time course of events upon shock depends on the severity of the shock. For instance, the time window is smaller after a mild shock and progressively longer after a more severe shock.

1.2. Signalling Pathways Involved in Osmoadaptation

Proteins that control signalling pathways in cellular responses to osmotic changes have been identified and studied at the molecular level. Several signalling pathways are activated upon osmotic stress in *S. cerevisiae*: the protein kinase A pathway, the phosphatidylinositol-3,5-bisphosphate pathway and the best characterized system, the HOG pathway (**H**igh **O**smolarity **G**lycerol response pathway).

The protein kinase A (cyclic AMP [cAMP]-dependent protein kinase) pathway affects expression of genes upon hyperosmotic shock (Norbeck and Blomberg, 2000). Moreover, 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 (Hohmann, S. and W. H. Mager, 1997, Yeast stress responses. R. G. landes company, Austin, TX), (Marchler et al., 1993; Ruis and Schuller, 1995). For this reason, protein kinase A most probably does not respond directly to osmotic changes. In fact, it is not clear how the activity of protein kinase A is regulated by stress.

It has also been observed that an osmotic stress stimulates production of phosphatidylinositol-3,5-bisphosphate, a molecule that might be a new type of phosphoinositide second messenger in an osmotic signalling system (Dove et al., 1997). However its actions have not been elucidated yet.

The HOG pathway is a mitogen-activated protein kinase (MAP kinase) cascade. It consists of a conserved eukaryotic signal transduction module, and its involvement in the process of osmoadaptation has been clearly established (Brewster et al., 1993; Gustin et al., 1998). Many basic principles of

osmoadaptation are conserved across eukaryotes, and therefore *S. cerevisiae* and the HOG pathway are an ideal model system for the study of these processes. The HOG pathway is the best-understood osmoresponsive system in eukaryotes. It is activated within less than one minute by a hyperosmotic shock and cells defective for this pathway or unable to activate it can not survive in high-osmolarity medium (Brewster et al., 1993). Thus, the role of the HOG pathway is to orchestrate a significant part of the response of yeast cells to high osmolarity.

2. MAPK PATHWAYS

Eukaryotic cells possess highly complex signalling pathways, each of which is preferentially recruited by distinct sets of stimuli, thereby allowing cells to quickly adapt to a changing environment. Prominent among signalling pathways are the mitogen-activated protein kinase (MAPK) signal transduction pathways, found both in higher and lower eukaryotic cells. Mammalian MAPK pathways can be activated by a wide variety of different stimuli, including hormones, growth factors, cytokines and environmental stresses such as osmotic shock, ionizing radiation and ischemic injury (Kyriakis and Avruch, 2001). The stress-responsive MAPKs (SAPKs), including the JNKs and the p38 MAPKs, are relatively new members in the MAPK family. Conservation of the stress MAPK cascades between yeast and mammals is very high.

2.1. Components of a MAPK signalling pathway: the central core

In MAPK cascades, signal transmission is achieved by the sequential activation of three protein kinases, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK (Figure 1). The MAPKKK consists of an N-terminal regulatory and a C-terminal catalytic kinase domain. In the inactive state, the regulatory domain locks the C-terminal kinase domain. Activation occurs by phosphorylation through an upstream protein kinase or through interaction with other proteins, a process that often involves small G-proteins. Once activated, MAPKKKs phosphorylate and thereby activate MAPKKs on serine and threonine

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

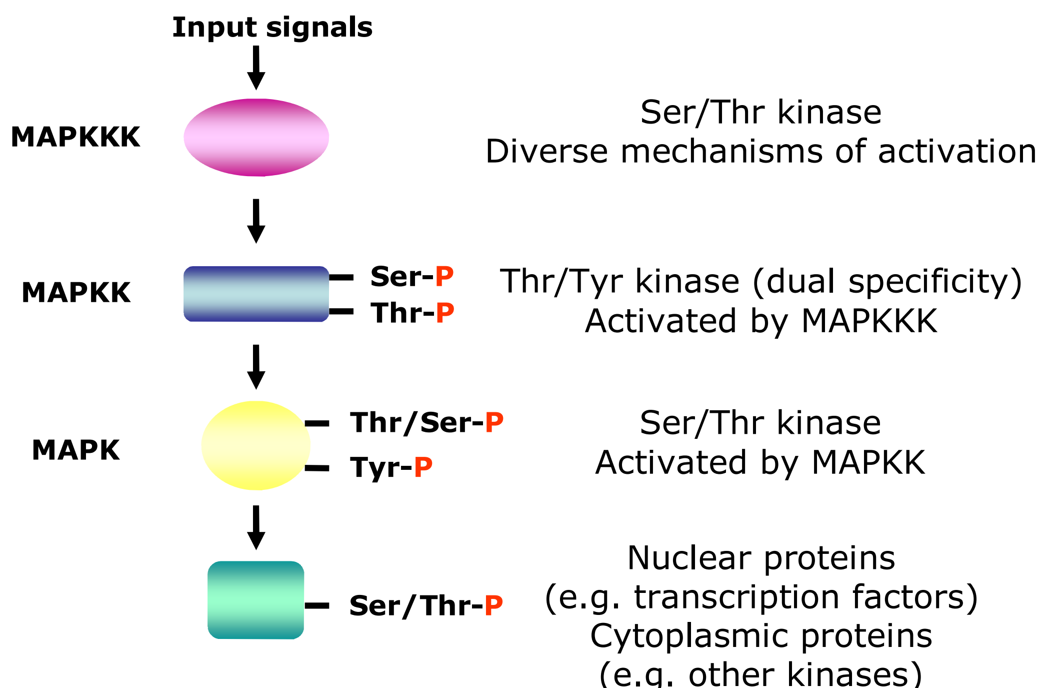

Figure 1. Schematic diagram of a MAPK pathway module. Core module of a MAPK pathway is composed of three kinases, MAPK kinase kinase, MAPK kinase and MAPK, that are sequentially activated by phosphorylation.

2.2. Signalling specificity of MAPK pathways

Different MAPK pathways can form interacting signalling systems. For instance, some individual elements can function promiscuously in several pathways. Conversely, some MAPK pathway components are often subjected to regulation by multiple inputs. Given the complexity and diversity of MAPK regulation and function, it is critical to preserve the efficiency and selectivity of MAPK pathways. For this reason, pathway organization is mediated by scaffolding proteins. Scaffold proteins bind and sequester select MAPK pathway components, and thereby they help to maintain pathway integrity allowing the coordinated and efficient activation and function of MAPK components in response to specific types of stimuli (Pawson and Scott, 1997). In some MAPK pathways, the signalling components themselves possess intrinsic scaffolding properties, such as the yeast MAPKK Pbs2 (see below). Alternatively, distinct proteins can act as scaffolding elements binding and segregating groups of signalling components, such as *S. cerevisiae* Ste5 (Printen and Sprague, Jr., 1994).

Despite the important role of scaffolding proteins in maintaining signalling specificity, other mechanisms may exist for this purpose. For instance, the MAPK substrate specificity. Although MAPKs are proline-directed kinases, substrate selectivity is often conferred by specific MAPK docking sites present on physiological substrates, often at considerable distance from the phosphorylation site in the primary sequence. This allows for a strong interaction which select MAPK subfamilies to the exclusion of others (Tanoue et al., 2000; Deacon and Blank, 1999; Yang et al., 1998; Kallunki et al., 1996). 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

Understanding of the *S. cerevisiae* MAPK pathways is more complete than that of MAPK pathways in other organisms. Extensive genetic and biochemical analysis revealed that *S. cerevisiae* contains five MAPKs on five functionally distinct cascades (Hunter and Plowman, 1997) (Figure 2). Four of these

pathways, the mating, the filamentation-invasion, the cell integrity and the high osmolarity pathways, are present in growing cells. The Smk1p MAPK, part of the spore wall assembly pathway, appears during sporulation and regulates that developmental process.

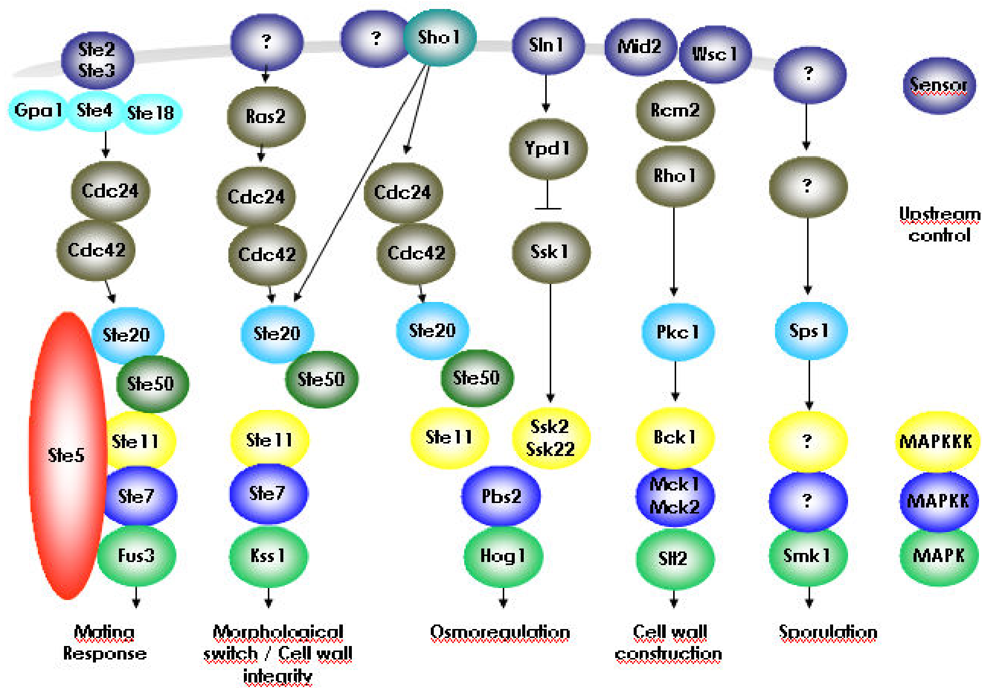

Figure 2. MAP kinase pathways in *S. cerevisiae*. *S. cerevisiae* contains five MAPK pathways: mating response, filamentation-invasion, osmoregulation, cell wall integrity and sporulation pathways. Some of the elements are shared between pathways.

3. THE HOG PATHWAY

The HOG pathway is the best-characterized osmoresponsive system in eukaryotes and hence serves as a prototype osmoregulating signalling pathway. In addition, it is one of the best-understood MAPK pathways. The HOG pathway specifically responds to increased extracellular osmolarity and is required for cell survival under these conditions. Activation of this pathway results in the initiation of a set of osmoadaptive responses, which includes metabolic regulation, cell cycle adaptation and gene expression regulation. As many other signalling pathways that exist in yeast, the HOG pathway has its equivalent

system in mammalian cells, the p38 and the c-Jun N terminal kinase (JNK) pathways. The conservation of function between the HOG pathway and the p38 pathway is indicated by the fact that the corresponding human gene partially complements the yeast mutants in the HOG pathway (Galcheva-Gargova et al., 1994; Han et al., 1994; Takekawa et al., 1997; Derijard et al., 1995).

The broad outline of the HOG pathway is fairly well described. The MAPK cascade consists of five protein kinases. Three MAPKKKs, Ssk2, Ssk22 (Maeda et al., 1995)) and Ste11 (Posas and Saito, 1997) activate a single downstream MAPKK, Pbs2, that in turn activates a single MAPK, Hog1 (Maeda et al., 1994; Brewster et al., 1993) (Figure 3). The pathway is activated predominantly by two independent mechanisms that lead to the activation of the MAPKKKs. The first mechanism involves a "two-component" osmosensor, composed of the Sln1-Ypd-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 GTP-ase Cdc42 (Posas et al., 1998; Hohmann, 2002) (Figure 3).

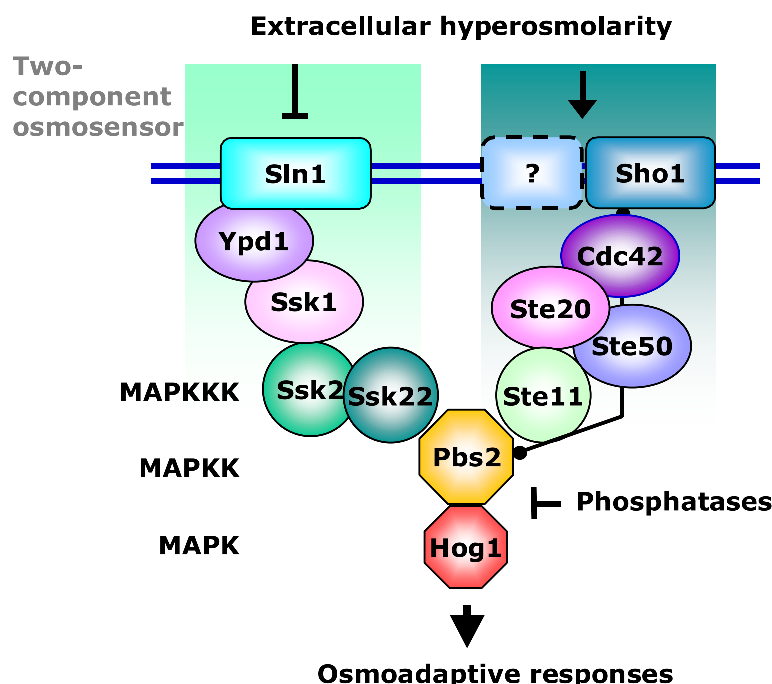

Figure 3. Schematic diagram of the yeast HOG pathway. Two major independent upstream osmosensing mechanisms lead to the activation of specific MAPKKKs and converge on a common MAPKK, Pbs2. Under osmostress, activated Pbs2 activates the MAPK Hog1, which induces a set of osmoadaptive responses.

3.1. Osmosensors of the HOG pathway: the Sln1 branch

The Sln1 branch involves a “two-component” osmosensor. A two-component system is composed of a sensor molecule and a response-regulator molecule. Typically, a sensor protein has an extracellular input domain and a cytoplasmatic histidine kinase domain. A typical response-regulator is a cytosolic protein containing the receiver domain and a DNA binding domain. When the sensor protein is activated, it 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 in the switching of its output function. However, the Sln1 branch is composed of three proteins: the primary osmosensor Sln1, the intermittent phosphorelay protein Ypd1 and the response regulator protein Ssk1, that functionally behave as two linked two-component systems. Sln1 corresponds to the first two-component system, since it contains both a histidine kinase domain and a receiver domain (Ota and Varshavsky, 1993). The Ypd-Ssk1 pair functions as the second two-component system. At normal osmolarity, the osmosensor Sln1 autophosphorylates itself and the phosphate is then sequentially transferred to the receiver domain, then to a specific His on Ypd1 protein and finally, to an Asp residue on 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 (Maeda et al., 1994). Genetic disruption of the SLN1 gene is lethal, due to the resulting constitutively activation of the HOG pathway (Maeda et al., 1994).
Even though two-component systems have not been found in animals, the extension of such a signalling unit by the HOG cascade in yeast is an indication of the increasing degree of sophistication of osmotic stress signalling in eukaryotes.

3.2. The Sho1 branch

In addition to the Sln1-Ypd-Ssk1 osmosensor pathway, *S. cerevisiae* has an alternative way of sensing hyperosmolarity independently of the Sln1 branch.

This pathway relies on the Sho1 osmosensor (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 formed appears to consist 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 (Posas and Saito, 1997; Maeda et al., 1995). 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 (O'Rourke and Herskowitz, 1998; Posas and Saito, 1997) 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 (Tatebayashi et al., 2006; Truckses 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 MAPKK 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); (Marcus et al., 1994b; Printen and Sprague, Jr., 1994). 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).

3.3. 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 way, while the Sln1 branch shows an approximately linear dose response up about 600 mM NaCl.

It has been speculated that the two branches may interpret osmotic changes via different physical stimuli. The finding that components of the Sho1 branch are localized or recruited to places of active cell growth (Raitt et al., 2000; Reiser et al., 2000) indicates that it plays a specific localized role in osmosensing. The precise subcellular localization of components of Sln1 branch has not been reported, but it could be proposed that the Sho1 branch is involved in the sensing of osmotic changes during cell growth and expansion, while the Sln1 branch would be implicated in sensing osmotic changes in the environment (Hohmann, 2002). Together, both branches of the HOG pathway may then orchestrate osmotic responses and integrate the need for cell expansion in response to osmotic signals generated by growth and by the environment.

Besides the Sln1 and the Sho1 branches, a third 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 *ssk1Δ sho1Δ msb2Δ* strain is more osmosensitive than an *ssk1Δ sho1Δ MSB2* strain. However, the biological importance of Msb2 is not clear yet.

3.4. Signalling through the HOG pathway

The MAPKK Pbs2 is activated by phosphorylation on Ser514 and Thr518 by any of the three MAPKKKs Ssk2/Ssk22 and Ste11. Since Pbs2 is a cytoplasmic protein, phosphorylation of its substrate, the MAPK Hog1, occurs in the cytosol. Dual phosphorylation on the conserved Thr174 and Tyr176 activates Hog1 (Brewster et al., 1993; Schuller et al., 1994). Phosphorylation induces 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). Both phosphorylation and nuclear localization of Hog1 are transient effects. The timing and the period of the response depends on the severity of the osmotic shock: under mild osmotic stress (0.4M NaCl) Hog1 phosphorylation peaks within 1 minute and disappears within about 30 minutes. In contrast, under severe osmotic shock (1.4M NaCl) Hog1 phosphorylation and nuclear accumulation is delayed compared to mild stress and is maintained up to a few hours (Van Wuytswinkel et al., 2000). Moreover, this delay correlates with a delay in stress-responsive gene expression.
Some observations indicate that interaction with the substrate and perhaps successful execution of a signalling program may determine nuclear retention. For instance, deletion of the genes encoding the transcription factors Msn2, Msn4 (Reiser et al., 1999), Hot1 and Msn1 (Rep et al., 1999b) has been shown to reduce the period of Hog1 nuclear localization.
Nuclear accumulation of Hog1 suggests that an important part of Hog1 actions take place in the nucleus. However, a portion of activated Hog1 remains in the cytosol, where it is clear that activated Hog1 also mediates regulatory effects. Among the best documented of such effects is activation of the protein kinase Rck2 (Bilsland-Marchesan et al., 2000; Teige et al., 2001), which controls translation efficiency (Teige et al., 2001), and the phosphorylation of ions transporters, which is crucial for the rapid reassociation of proteins, previously dissociated from chromatin due to the osmotic stress, with their target sites in chromatin (Proft and Struhl, 2004).

3.5. Modulation and feedback control of the HOG pathway

Hog1 phosphorylation and activation is a transient event (Jacoby et al., 1997; Maeda et al., 1994; Tamas et al., 2000) which means that the pathway is controlled by specific feedback mechanisms. One of these feedback mechanisms involves several phosphatases. Two phosphotyrosine phosphatases (Ptp2 and Ptp3) as well as three phosphoserine/threonine phosphatases (Ptc1 to Ptc3) genetically interact with the HOG pathway. Overexpression of any of these phosphatases suppresses the lethality caused by inappropriate activation of the pathway (Jacoby et al., 1997; Maeda et al., 1994; Mattison and Ota, 2000; Ota and Varshavsky, 1992) (Warmka et al., 2001; Wurgler-Murphy et al., 1997). 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.

3.5.1. Ptp2 and Ptp3

Overexpression of Ptp2 and Ptp3 diminishes the amount of tyrosine-phosphorylated Hog1. Moreover, Ptp2 and Ptp3 interact directly with Hog1. This interaction is not observed with active phosphatases, which suggests that binding occurs specifically to phosphorylated Hog1 and that the phosphatases 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). A negative feedback loop exists between Hog1 and Ptp2/Ptp3. Active Hog1 enhances the Ptp2 activity (Wurgler-Murphy et al., 1997) and induces Ptp3 expession (Jacoby et al., 1997). 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 even 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).

3.5.2. Ptc1

Among the serine/threonine phosphatases, Ptc1 seems to be the one that truly functions in the deactivation of the HOG pathway. In mutants lacking both *PTC1* and *PTP2* the inappropriate HOG pathway overactivation causes a growth defect. No other combination of deletion mutations between the two Ptps and the three Ptcs causes similar effects (Warmka et al., 2001). Ptc1 inactivates Hog1 and also dephosphorylates phosphothreonine *in vitro*. Furthermore, overexpression of Ptc1 inhibits Hog1 activation, as measured by its kinase activity, but does not affect the level of Hog1 tyrosine phosphorylation, indicating that Ptc1 inhibits the HOG payhway by dephosphorylating Hog1, rather than an upstream kinase (Warmka et al., 2001).
It has been described that Ptc1 is recruited to the Pbs2-Hog1 complex by the Nbp2 adapter (Mapes and Ota, 2004). Nbp2 contains separate binding sites for Ptc1 and Pbs2: the novel N-terminal domain binds Ptc1, while the SH3 domain binds Pbs2. As predicted for an adapter, deletion of *NBP2* disrupts Ptc1-Pbs2 complex formation (Mapes and Ota, 2004).

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 the metabolic adaptation (Figure 4). Among the targets of the HOG pathway are important inducible transcription factors such as Atf1 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 under hypertonic stress (Dihazi et al., 2004).

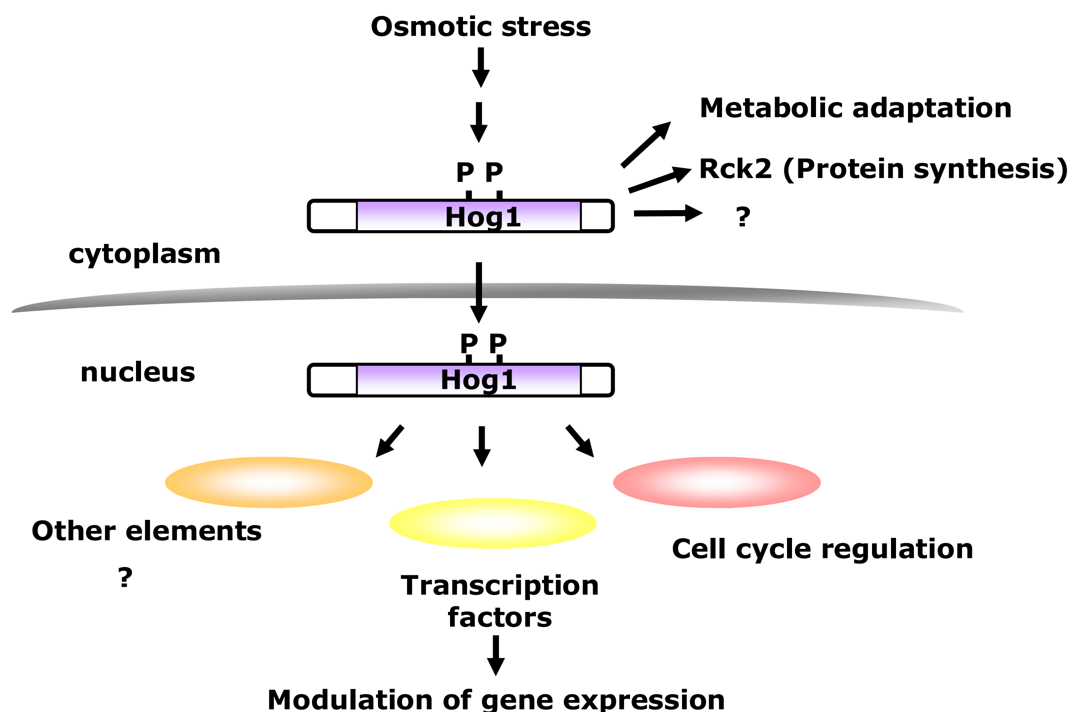

Figure 4. Functions of the MAPK Hog1. Once Hog1 is phosphorylated and activated, it controls several functions, such as cell cycle regulation, modulation of gene expression and metabolic adaptation.

4.2. Regulation of protein synthesis

Hog1 is also involved in the control of translation. There is evidence that under osmotic stress translation elongation is directly downregulated through a linear

signalling pathway (Teige et al., 2001). Rck2 is a member of the calmodulin protein kinase family (Melcher and Thorner, 1996) that phosphorylates and thereby inhibits translation elongation factor EF-2 (Bilsland-Marchesan et al., 2000; Teige et al., 2001). Rck2 is a direct target of Hog1 (Bilsland-Marchesan et al., 2000) and both Hog1 and Rck2 are needed for osmostress-induced inhibition of protein translation (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, the expression of genes encoding functions important 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.

4.3. Regulation of cell cycle progression

One of the most important roles of the MAPK Hog1 is the coordinated control of the progression of the cell cycle. In response to osmotic stress, the HOG pathway mediates a transient cell cycle arrest required for proper cell adaptation. For instance, during cell cycle arrest, cells are able to modify their transcriptional program.

The role of SAPKs in cell cycle control was first proposed in *Schizosaccharomyces pombe* for the Sty1 MAPK pathway (Shiozaki and Russell, 1995). Alteration of its components, resulting in either hyperactivation or signal abrogation, resulted in cell cycle defects. Later this pathway was related to environmental responses (Wilkinson and Millar, 2000). More recently, a role for p38 MAPK pathway in cell cycle progression has been reported in several organisms. Actually, different reports indicate that different type of mammalian cells arrest at several stages of the cell cycle (G1-S, G2 and mitosis) upon osmostress (de Nadal et al., 2002; Dmitrieva et al., 2001; Dmitrieva et al., 2002; Mikhailov et al., 2004).

Different mechanisms have been proposed for the control of cell cycle progression by the p38 SAPKs. It has been reported that control of G1-S progression is achieved by the differential regulation of specific cyclin levels (cyclin A or D1) as well as by phosphorylation of critical cell cycle regulators such as pRb, p53, p21, HBP1 or the Cdc25A phosphatase. Also, several targets

for the SAPK have been defined in G2 and mitosis. For instance, p38 targets the Cdc25B and Cdc25C phosphatases as well decreases Cdc2 activity in response to several stimuli (Ambrosino and Nebreda, 2001; Goloudina et al., 2003; Todd et al., 2004; Pearce and Humphrey, 2001; Xiu et al., 2003; Wilkinson and Millar, 2000). In such a complex scenario where several targets for the SAPKs have been described, it is still not clear whether specific mechanisms are used to respond to different stimuli and whether different cell types use different mechanisms to cope with stressful situations. In *S. cerevisiae* the mechanism by which Hog1 regulates cell cycle progression upon osmotic stress has been partially described by our research group.

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, the restriction point in mammalian cells, yeast cells decide if begin a new cycle, conjugate with another cell, or sporulate. The events of the cell cycle need to be properly regulated to obtain a successful division of an eukaryotic cell. For this reason, transitions between G1/S and between G2/M are strongly regulated. When one of these events is aborted, cells use diverse mechanisms, known as checkpoints, to monitor proper completion of each stage of the cell cycle. Thus, cell cycle progression can be delayed until the execution of an unfinished step allowing the cells to begin a new cycle. In *S. cerevisiae,* Cdc28 is the main coordinator of the yeast cell division cycle (Figure 5). Cdc28 is the unique CDK (Cyclin Dependent Kinase) in yeast that controls cell cycle progression and it is the equivalent to Cdk1 in mouse and human systems. Environmental effects that influence the decision to undergo cell division affect Cdc28 kinase activity. Activation of CDKs is dependent on two events: cyclin binding and stimulatory phosphorylation. Cyclins were discovered as proteins that appear and disappear in synchrony during the cycle. Cyclins are defined by their ability to bind and activate a CDK but are often recognized by the presence of a conserved domain, the "cyclin box" (Kobayashi et al., 1992). Cyclins confer a limited range of functions on Cdc28. Cdc28 cyclins have been classified into two broad groups: 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 are expressed in three successive waves from Start to mitosis and control the events between the DNA replication until the cytokinesis (Figure 5).

There are several different mechanisms for regulating Cdc28 activity in the cell:

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.

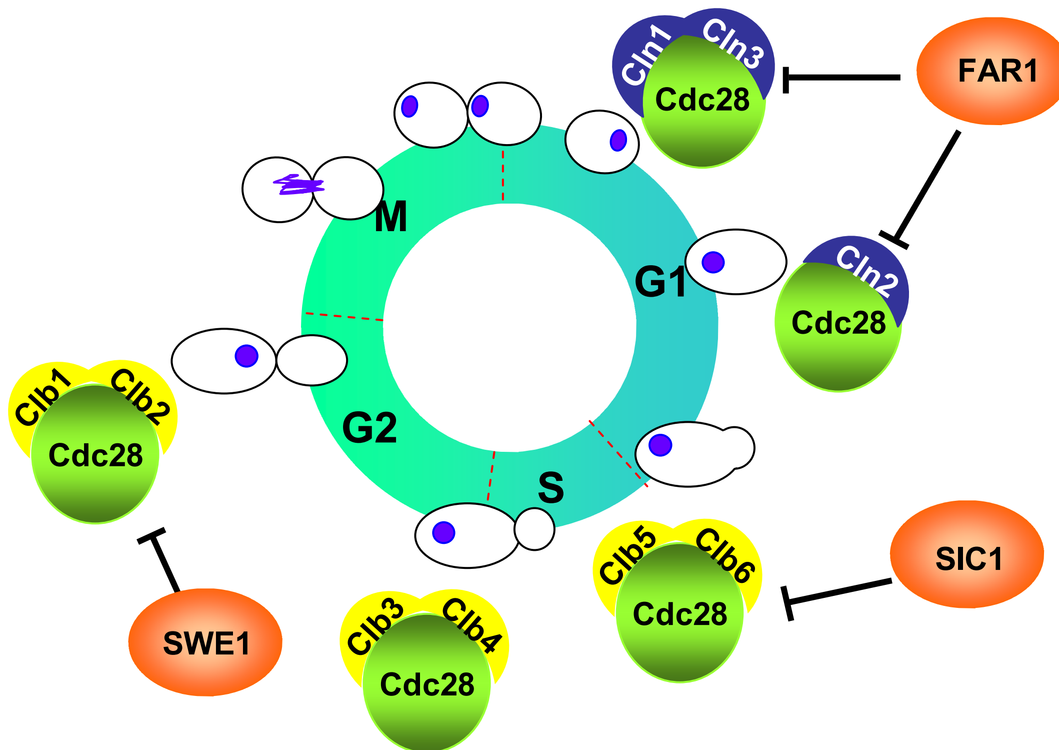

Figure 5. Schematic diagram of *S. cerevisiae* cell cycle. Cdc28 associates to different cyclins at different phases. It is illustrated the bud and position of nuclei along cell cycle.

4.3.1. Role of the CDK inhibitor Swe1 in the Hog1-mediated arrest at G2 phase

Survival to osmostress requires regulation of cell cycle at different levels. Previous reports suggested that osmostress induces a delay at G2 (Alexander et al., 2001; de Nadal et al., 2002). As mentioned above, entry into mitosis is controlled by the activity of the Clb2-Cdc28 complex, which is held in check by the protein kinase Swe1. The phosphorylation of Cdc28 at Y19 by Swe1 inhibits its activity (Lim et al., 1996) leading to a delay entry into mitosis until critical cell size has been reached (Rupes, 2002; Kellogg, 2003; Harvey et al., 2005) or

defects in bud formation or cytoskeletal function are monitored by the "morphogenesis checkpoint" (Cid et al., 2002; Lew, 2003). Swe1 phosphorylation is opposed by the Mih1 phosphatase (Russell et al., 1989).

Swe1 stability plays a crucial role in the progression of cell cycle and several requirements are critical for its regulation: phosphorylation of Swe1 by the Clb2-Cdc28 complex (Asano et al., 2005; Harvey et al., 2005) and also the activity of the Hsl1 checkpoint kinase together with Cdc5 (Hanrahan and Snyder, 2003; Asano et al., 2005). When bound to the septins (Versele and Thorner, 2005), Hsl1 tethers Hsl7 at the bud neck, which is in turn required for recruitment of Swe1 to the bud neck to facilitate spatially controlled Swe1 phosphorylation, prior to ubiquitin-mediated degradation (Sakchaisri et al., 2004) (Figure 6). Thus, timely phosphorylation and subsequent degradation of Swe1 are critical for proper activation of the Clb2-Cdc28 complex.

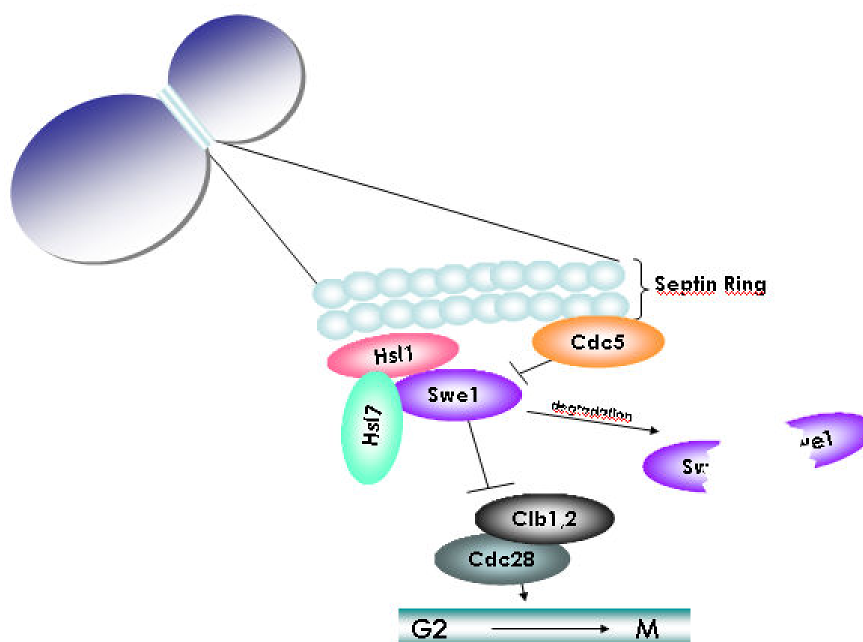

Figure 6. Model illustrating the Swe1 phosphorylation and degradation events during the cell cycle (adapted from (Sakchaisri et al., 2004)).

In (Clotet et al., 2006), it is proposed a novel regulatory mechanism of the Hsl1 morphogenesis checkpoint kinase that allow cells to integrate stress signals to modulate cell cycle. First of all, it is demonstrated that activation of the HOG pathway, either by the presence of osmostress or by expression of a mutated Sln1 osmosensor or a constitutively active MAPKK Pbs2 (Maeda et al., 1994; Maeda et al., 1995; Posas et al., 1996; Posas and Saito, 1997; Wurgler-Murphy et al., 1997), results in both G1 and G2 cell cycle arrest. The way by which Hog1 controls G2 progression involves 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 (situated in the middle of the Hsl7-binding domain in Hsl1 (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. This accumulation, together with the decreased levels of Clb2, is the responsible for the decreased activity of the Clb2-Cdc28 complex and for the arrest at G2 phase.
Thus, the coordinated action of the MAPK Hog1, modulating cyclin transcription and targeting specific cell cycle regulators, controls cell cycle progression upon osmotic stress to prevent entry into mitosis without the cells being adapted to the new extracellular conditions. An obvius advantage of this dual mechanism is the increase of the efficiency of the G2 arrest by establishing two converging (additive) mechanisms. Alternatively, this system could be envisioned as a two step mechanism. An initial step to cause immediate arrest by acting directly over the checkpoint kinase (Hsl1 phosphorylation) and then a decrease on Clb transcription that would be essential to maintain the arrest for a prolonged time (Clotet et al., 2006).

4.3.2. Role of the CDK inhibitor Sic1 in the Hog1-mediated arrest at G1 phase

In two recent reports from Dr. F. Posas' group (Escote et al., 2004; Zapater et al., 2005) (see Suplementary Articles), it is extensively described the mechanism by which Hog1 controls G1 transition in response to osmotic stress. Similarly to the mechanism explained for G2, this consists in a dual mechanism

that involves regulation of cyclin expression and the targeting of the cell cycle regulatory protein Sic1. For detailed explanation, see Supplementary Articles.

4.4. Regulation of gene expression

One of the main functions of MAPKs in response to osmostress is the regulation of gene expression. In mammalian cells, p38 controls the expression of more than 100 genes (Ono and Han, 2000). In *S. cerevisiae*, genome-wide transcription studies revealed that a large number of genes (~ 7%) show significant but transient 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). These osmostress-regulated genes are implicated mainly in carbohydrate metabolism, general stress protection, protein production and signal transduction. The role of Hog1 in the regulation of gene expression at transcriptional level will be extensively described in this Thesis.

5. TRANSCRIPTION

Like other eukaryotes, yeast has three different DNA-dependent RNA polymerases to mediate transcription:

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

Transcription regulation of eukaryotic protein-coding (class II) genes is an orchestrated process that requires the concerted functions of multiple proteins or transcription factors. These factors can be classified into three groups:

- **sequence-specific DNA-binding transcription regulators (i.e., activators and repressors),** which 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.

- **General/basal transcription factors,** which are ubiquitous and include RNA polymerase II (Pol II) and a set of accessory general transcription initiation factors (GTFs) that bind to core promoter DNA elements (e.g., TATA box) and allow specific recruitment of Pol II to the core promoter.
- **Transcription cofactors/co-regulators (coactivators and corepressors)**, which interact with regulators and play essential roles in mediating the effects of regulators on the general transcription machinery either via direct physical interactions with GTFs and/or Pol II or indirectly through modification of chromatin structure.

5.1. The transcription initiation machinery: complexity at the core

The general (or basal) class II transcription machinery is composed of Pol II and six GTFs, which include TFIIA (Transcription Factor IIA), TFIIB, TFIID, TFIIE, TFIIF and TFIIH. Most of GTFs and Pol II are complexes formed by the stable association of several proteins. Pol II itself is a large multisubunit enzyme (with a mass of about 0.5MDa) composed of 12 different proteins. 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).

Figure 7 sumarizes the different steps involved in PIC formation and transcription initiation at a core promoter. The first step 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. TFIIH is a complex of nine subunits one of which is a kinase (Cdk7 in mammalian cells, Kin28 in *S. cerevisiae*) that, during initiation or early elongation, phosphorylates the CTD (carboxy-terminal domain) of the largest subunit of Pol II (Feaver et al., 1994; Valay et al., 1995). The CTD consists of 52 repeats of the aminoacid sequence Y-S-P-T-S-P-S. Ser5 is the one phosphorylated by the kinase activity of TFIIH, a step that facilitates promoter clearance by disrupting interactions of the CTD with components of the PIC. The final PIC is thus a large nucleoprotein complex composed of more than 40 proteins with a total mass of over 2.2 MDa. After complex 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 and TFIIB, TFIIE and TFIIH are released (Zawel et al., 1995). However, some studies have shown that, after transcription initiation and Pol II scape, TFIIA, TFIID, TFIIE and TFIIH can remain associated with the core promoter forming a scaffold that is stabilized by activators. This suggests that the second and subsequent rounds of transcription initiation (i.e., re-initiation) may not require *de novo* recruitment of TFIIE, TFIIH or the Pol II holoenzyme, but may just need re-incorporation of TFIIB, TFIIF and Pol II (Yudkovsky et al., 2000).

In vivo, however, formation of the initiation complex and elongation of the transcript are much more complex processes, as DNA is “trapped” into chromatin. Admission of the transcription initiation complex will therefore be possible after modification and remodelling of the nucleosomal structure underlying the respective gene and its promoter (see below).

Recent data strongly suggest that PICs are composed of different sets of factors at distinct promoters, reflecting the spatiotemporal profile of gene expression, especially in multicellular organisms. Thus, transcription initiation is also regulated at the level of PIC formation at core promoters, and many distinct PICs with varying composition and distinct promoter recognition factors play important roles in transcriptional regulation (Muller and Tora, 2004).

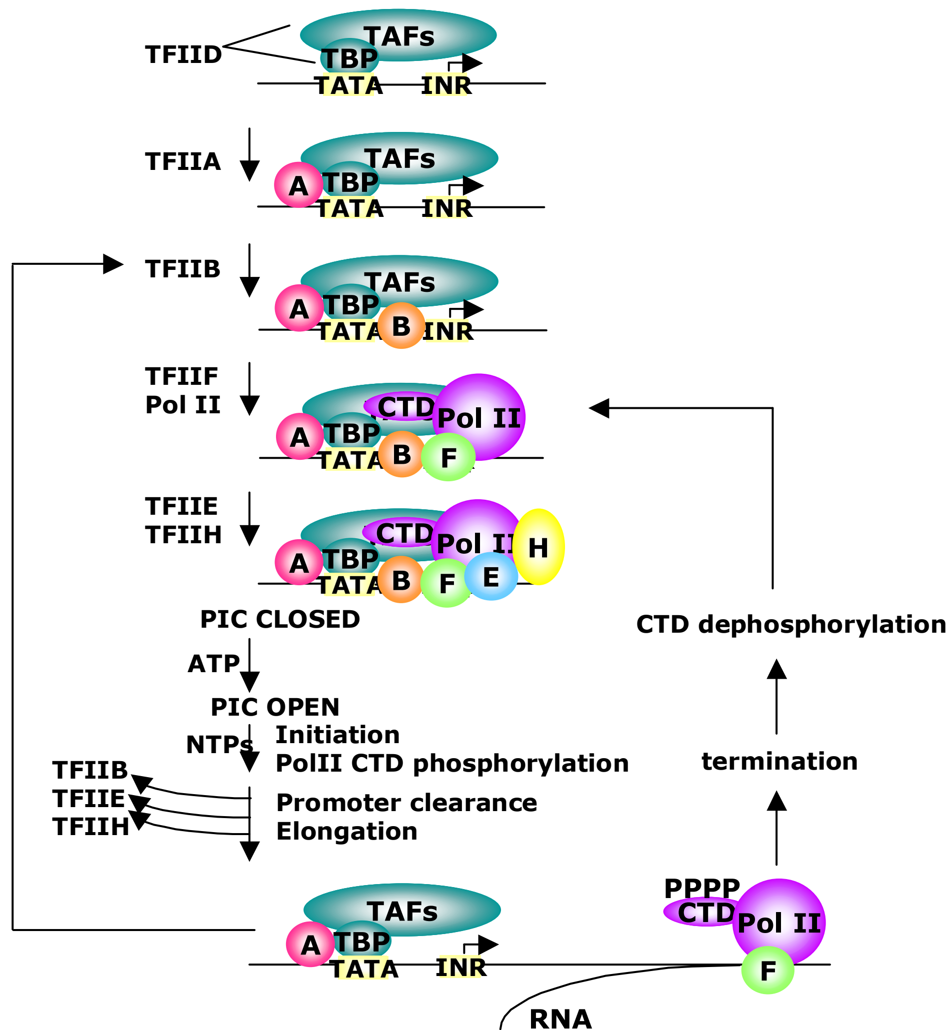

Figure 7. Model for a stepwise assembly and function of a pre-initiation complex (PIC) (adapted from Martinez, 2002).

5.2. Coactivators and chromatin remodelling

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. Some activators will be extensively described

later, among them, the Mediator, TFIID, the SAGA complex and the Rpd3 deacetylase complex.
Like in higher eukaryotes, the organization of DNA into chromatin and chromosomal structure plays a central role in many aspects of yeast cell biology. In accessing the genetic material during replication, transcription and repair, the respective cellular machineries (coactivators in the case of transcription) have to work on chromatin as the native DNA template. Generally, chromatin is repressive to extraneous access, and this inhibitory effect must be overcome by regulatory factors. Two main enzymatic activities are employed to regulate chromatin access: chromatin modifying complexes and chromatin remodelling complexes.

Chromatin modifying activities introduce posttranslational modifications on the histones, including histone acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP-ribosylation. Most of these modifications are observed on the N-terminal tails of histones, with the exception of ubiquitylation, which occurs on the C-terminal tails of H2A and H2B. Acetylation (with Acetyl-CoA as a donor enzyme) is carried out by histone acetyltransferases (HATs), which can be grouped into four families in yeast, based on homology:

- The Gcn5 family members are most similar to Gcn5 from *S. cerevisiae*, such as PCAF. Gcn5 is the catalytic subunit of the SAGA complex.
- The MYST familiy of HATs. Among its members, the Sas3 HAT, which is part of the NuA3 complex, and Esa1, the only essential HAT in yeast which is part of the NuA4 complex.
- The p300 family.
- The general transcription factor HATs include the TFIID subunit TAF1, TFIIC and Nut1, a component of the Mediator complex.

Acetylation of the lysine residues at the N terminus of histones H3 and H4 counteracts the tendency of nucleosomal fibers to fold into highly compact structures *in vitro* (Garcia-Ramirez et al., 1995; Tse et al., 1998) and acetylated chromatin is more accessible to interacting proteins *in vivo*, as illustrated by its increased sensitivity to DNaseI (Hebbes et al., 1994). Therefore, in most cases, histone acetylation enhances transcription.

Histone acetylation is a reversible process and, accordingly, histone deacetylases (HDACs) catalyze the deacetylation reaction. Deacetylation is generally associated with transcription repression. 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 (homolog of Sir2, also known as sirtuin) proteins. This group is structurally unrelated to the other two families and has the unusual property of requiring NAD^+ as a cofactor in the deacetylation reaction. Sir2 has been characterized as essential for all forms of silencing in yeast (Peterson, 2002; Denu, 2003).

Histone modifications may affect chromatin structure directly by altering DNA-histone interactions within and between nucleosomes, thus changing higher-order chromatin configuration. A recent model suggest that combinations of histone modifications present an interaction surface for other proteins, which translate this so-called "**histone code**" into a gene expression pattern (Jenuwein and Allis, 2001). This model could explain how the same chemical modification can have different functional consequences depending on the respective target residue. However, the "histone code" is still in discussion since similar marks in the same residue result in some cases to opposite transcriptional effects. Thus, this epigenetic marking system represents a fundamental regulatory mechanism.

Chromatin remodelling activities 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. The first such activity found was the Swi/Snf2 ATPase, whose main activity is to alter histone-DNA contacts within 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 (Peterson and Herskowitz, 1992; Laurent et al., 1991). Swi2/Snf2 is part of the SWI/SNF multiprotein complex, which possesses ATP-dependent remodelling activity. The SWI/SNF complex is highly related to

the RSC (remodels the structure of chromatin) complex and to the imitation switch (ISWI)-type ATPases, which were identified based on their similarity to Swi/Snf2.

5.2.1. Mediator

The Mediator complex was originally identified in *S. cerevisiae* in the early 1990s as a requirement for activator-dependent stimulation of Pol II transcription (Kelleher, III et al., 1990; Flanagan et al., 1991). Out of the 37 independent subunits that have been annotated to date in Mediators from different organisms, 22 are conserved in all eukaryotes. These conserved subunits most likely form the structural and functional core of the complex. Yeast Mediator comprises 25 subunits, 4 of which form a specific module (the Srb8-11 module), that is variably present in different Mediator preparations (Borggrefe et al., 2002; Samuelsen et al., 2003).
A combination of biochemical, genetic and structural data suggest that the yeast Mediator is composed of three functionally and physically distinct modules or subcomplexes (Figure 8): the head module, the middle domain and the tail module, presumably responsible for recognizing and binding to activators (Bhoite et al., 2001); (Guglielmi et al., 2004). The fourth distinct subcomplex contains the cyclin-kinase pair Cdk8 and CycC which phosphorylates the CTD at the serine 5 residue in the heptapeptide repeat (Hengartner et al., 1998).
The best structural information available on yeast Mediator is a ~35Å resolution three-dimensional structure that was generated from electron-microscope images (Asturias et al., 1999).
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. Thus, several subunits of Mediator are essential for general Pol II transcription in yeast cells. For instance, more than 5000 genes showed nearly the same dependence on Med17 (Srb4) as on the largest subunit of pol II (Holstege et al., 1998). Although the exact mechanism of Mediator-dependent activated transcription is not yet clearly defined, there have been many studies that aim to elucidate the

nature of its role in this process. One of the models proposed is based on the idea that Mediator is involved in the recruitment of RNA pol II to the preinitiation complex. In this model, the tail domain would recognize and bind to activators, leading to the subsequent recruitment of RNA Pol II by the head domain (Himmelfarb et al., 1990; Jiang and Stillman, 1992; Song et al., 1996).

Nevertheless, the promoter architecture (position and number of activation domains, chromatin state) affects the ability of an activator to recruit Pol II to the PIC, suggesting that the involvement of Mediator is more than just a physical bridge between activator and CTD. It implies that Mediator has roles beyond recruitment of RNA pol II.

In a recent issue (Wang et al., 2005) it is proposed that Mediator also functions at a postrecruitment step. According to this work, Mediator would have the ability to stimulate the rate of initiation by promoter bound Pol II through its interaction with activators.

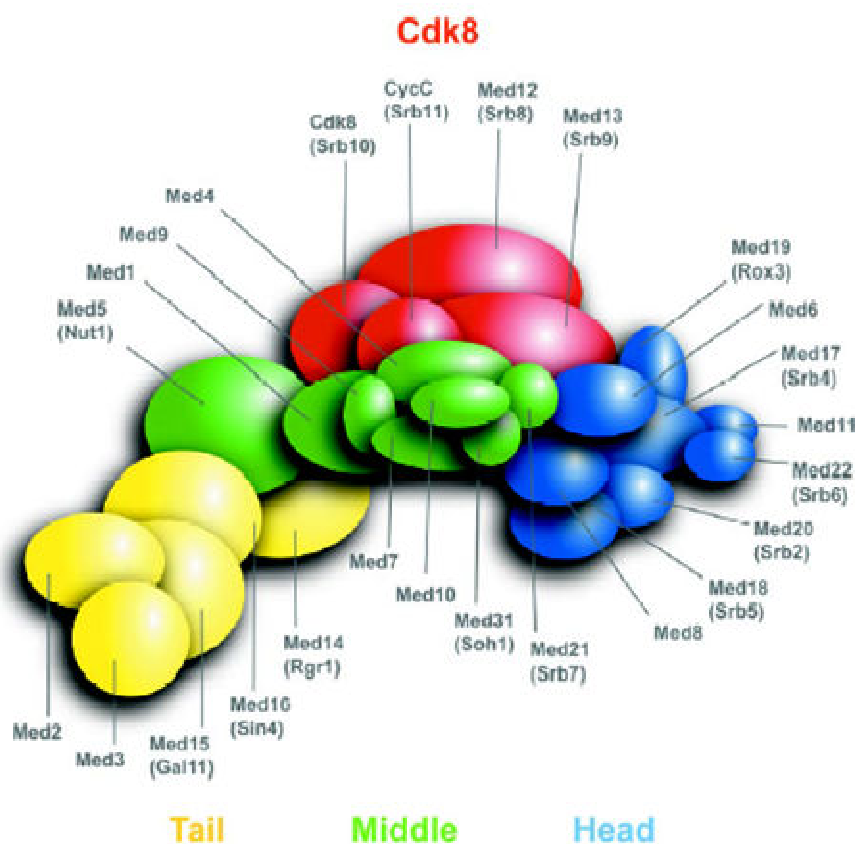

Figure 8. Topological organization of the yeast Mediator. Approximate sizes and positions of components are shown (Guglielmi et al., 2004).

Another function of Mediator, through its Cdk8 subunit, is to stimulate phosphorylation of the CTD by TFIIH (Kim et al., 1994). It was later shown that Mediator remains at the promoter after transcription initiation (Yudkovsky et al., 2000). Taken together, these results suggest a role for Mediator as a scaffold for PIC formation for a second round of transcription and a possible role in the CTD phosphorylation cycle (Biddick and Young, 2005).
Mediator may also negatively regulate transcription by marking activators for destruction. For instance, Srb10 phosphorylates the transcription regulator Gcn4, which signals the destruction of Gcn4 by ubiquitin-mediated proteolysis (Chi et al., 2001).
The more recent research on Mediator is generating surprising results. Two recent issues (Fan et al., 2006; Andrau et al., 2006) suggest that Mediator is not a stoichiometric component of the basic Pol II machinery but rather a complex selectively required by specific activators, since Mediator occupancy does not strictly correlate with Pol II occupancy *in vivo*. Moreover, Mediator is found on some inactive promoters prior to transcription to mark regulatory regions ahead of input stimulatory signals, and also on coding regions of some highly active genes.

5.2.2. TFIID

In S. cerevisiae, TFIID is composed of the TATA box-binding protein (TBP) and 14 TBP-associated factors (TAFs 1 to 14). Like all other components of the transcription machinery, TAFs have been highly conserved. Studies in yeast have shown that, with the exception of TAF14, all TAFs are essential for viability, which suggests that each essential TAF must perform at least one obligate, non-redundant function. In both yeast and human cells, TAFs have been found in large nuclear complexes besides TFIID. In yeast, the three histone-like TAFs (TAF6, TAF9 and TAF12) and two other non-histone-like TAFs (TAF5 and TAF10) are integral components of the SAGA complex (Grant et al., 1998a).
As mentioned before, binding of TFIID to the TATA box is the initial step of PIC assembly and it is critical for the rate and efficiency of this process. However, whereas TBP is a general transcription factor, a variety of *in vivo* studies have demonstrated that TAFs are highly promoter selective. Different mechanisms have been proposed to explain their mechanism of action.

The first mechanism proposes that TAFs are the targets of activators and that different activator-coactivator combinations selectively regulate transcription (Burley and Roeder, 1996; Naar et al., 2001). The second proposed mechanism is the promoter recognition; two types of promoters with different TAF selectivity have been identified in yeast: promoters whose transcription is highly TAF dependent and promoters whose transcription appears to be completely independent of all TAFs (Shen and Green, 1997; Apone et al., 1998; Li et al., 2002; Moqtaderi et al., 1998). A third proposal is that TAFs provide a catalytic activity required for transcription (for instance, histone acetyltransferase activity (Mizzen et al., 1996).

Although initially identified as a cofactor required for transcriptional activation *in vitro*, TFIID is now known to play a critical role in activation in a gene-specific manner. According to the different observations mentioned above, the TAF subunits would include direct targets of gene-specific activators as well as promoter recognition factors that interact to stimulate transcription (Chen and Hampsey, 2002).

5.2.3. SAGA

Yeast SAGA (Spt-Ada-Gcn5-Acetyltransferase) is a 1.8 MDa complex that represents a paradigm for multi-subunit transcriptional cofactors. SAGA is composed of several submodules:

- A subset of ADA proteins: Ada1, Ada2, Ada3 and the prototypical HAT coactivator Gcn5 (Ada4).
- The TATA-binding protein (TBP)-related set of Spt proteins (Spt3, Spt7, Spt8 and Spt20.
- A subset of TAFs.
- Tra1.
- A recently described submodule composed by the proteins Ubp8, Sgf11 and Sus1.
- Sgf29 and Sgf73 proteins, with unknown function.

Thus, SAGA incorporates multiple activities.

The 3D structure of SAGA has been revealed by electron microscopy (Wu et al., 2004) (Figure 9). According to this structure, SAGA is formed by five modular domains and shows a high degree of structural conservation to human TBP-free

TAF_{II}-containing complex (TFTC). The TAFs subunits, shared with TFIID, occupy a central region in SAGA and form a similar structure in both complexes. The SAGA-specific structural components Spt7 and Ada1 are also located within this central region. Three components that perform distinct regulatory functions, Spt3, Gcn5 and Tra1, are spatially separated, which underscores the modular nature of the complex.

Several functional submodules have been described in yeast SAGA. The ADA subcomplex contains Gcn5, the catalytic HAT subunit (Grant et al., 1997) regulated by the other two subunits, Ada2 and Ada3 (Balasubramanian et al., 2002; Sterner et al., 1999). This module links SAGA physically and functionally to activators and nucleosomal histones.

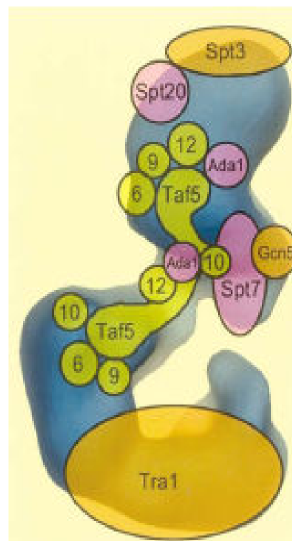

Figure 9. Schematic representation of the localization of the SAGA subunits on the 3D model of the complex (Wu, Mol Cell 2004).

A second functional module is composed by proteins that are essential for the structural integrity of the SAGA complex and includes Ada1, Spt7 and Spt20 (Grant et al., 1997; Sterner et al., 1999).

A third module contains the subunits Spt3 and Spt8, which have been shown both genetically and biochemically to be required for SAGA interaction with TBP and are involved in core promoter (TATA) selectivity *in vivo* (Eisenmann et al., 1994; Larschan and Winston, 2001; Sterner et al., 1999).

Mutations in the genes encoding components of the ADA subcomplex or the third complex have very mild phenotypes, whereas inactivation of components of the second module causes the broadest and more severe set of phenotypes (Horiuchi et al., 1997; Sterner et al., 1999). Consistent with this, genome-wide expression analyses of *gcn*5Δ and *spt3*Δ null mutants indicate that *GCN5* and *SPT3* are required, respectively, for the expression of only 4% and 3% of yeast genes, whereas a *spt20*Δ mutation affects 10% of the genome (Lee et al., 2000).

The TAF module is involved in the interaction of SAGA with nucleosomes (Grant et al., 1998a) and is also important for SAGA integrity and structure (Durso et al., 2001; Grant et al., 1998a; Kirschner et al., 2002).

Tra1 is a large (434 kDa) protein that is also a subunit of the H4-specific HAT complex NuA4 (Grant et al., 1998b). Due to its size, Tra1 is likely to have several functions, including a possible architectural role within the SAGA complex. Tra1 has also been shown to contact directly acidic activators *in vivo*, such as Gal4 (Bhaumik et al., 2004) and Gcn4 (Fishburn et al., 2005) as part of the SAGA and NuA4 complexes. In both cases, the interaction activator-Tra1 is required for recruitment of SAGA and for transcriptional activation of the target genes.

The last functional module has been recently described. It is composed by the subunits Ubp8, Sgf11 and Sus1. Ubp8 is a histone H2B-deubiquitylating enzyme (Daniel et al., 2004; Henry et al., 2003) that was described to associate to the SAGA complex through the newly identified Sgf11 subunit (Ingvarsdottir et al., 2005; Lee et al., 2005; Powell et al., 2004). More recently, it has been proposed that Sus1, a protein identified as a functional component of the SAGA complex and the nuclear pore-associated mRNA export machinery (Rodriguez-Navarro et al., 2004), forms together with Ubp8 and Sgf11 a functional module within SAGA (Kohler et al., 2006). Ubiquitylation of H2B is transient during gene activation, and ubiquitin is removed by Ubp8 acting within the SAGA complex (Daniel et al., 2004; Henry et al., 2003). Both ubiquitylation and deubiquitylation are required for optimal gene activation.

The striking structural and functional conservation in several subunits of both SAGA and TFIID suggest that these two complexes might perform similar functions *in vivo*. However, a recent genome-wide study (Huisinga and Pugh, 2004) reveals that ~90% of the genome shows a greater dependency on TFIID,

while SAGA plays a predominant regulatory role at a small fraction of the genome (~10%). The genes predominantly regulated by SAGA are genes commonly upregulated during general environmental stress. Thus, SAGA might be particularly geared for turning on genes that respond to stress, while TFIID might be more involved in regulating housekeeping genes, many of which are downregulated during stress.

5.2.4. HISTONE DEACETYLASES: RPD3

As mentioned previously, Rpd3 belongs to the class I of yeast histone deacetylases. Rpd3 was isolated genetically in a screen for mutants with reduced potassium dependency (rpd mutants) in a potassium transporter gene-
deletion background. Early studies demonstrated that Rpd3 was required to achieve maximum positive and negative transcriptional states, thus implicating it in transcriptional regulation (Vidal et al., 1990; Vidal et al., 1991). 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 (Lechner et al., 2000; Kasten et al., 1997; Rundlett et al., 1996): 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). For instance, Rpd3 is necessary for repression of genes involved in meiosis and metabolism (Vidal and Gaber, 1991; Kadosh and Struhl, 1997; Rundlett et al., 1998). 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 (Bernstein et al., 2000; Sun and Hampsey, 1999). Furthermore, the histone deacetylase Hos2, which also belongs to the class I of yeast histone deacetylases, 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 (Rundlett et al., 1998; Kadosh and Struhl, 1997). 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).

6. MAPKs AND TRANSCRIPTION

One of the main functions of MAPKs in response to different stresses is the regulation of gene expression. There is no unifying mechanism by which MAPKs modulate gene expression. The best-understood mechanism is the direct phosphorylation of promoter-specific transcription factor targets (Karin and Hunter, 1995; Kyriakis and Avruch, 2001; Treisman, 1996). This mechanism can regulate the target activity by several mechanisms, including control of protein levels, regulation of binding to DNA, nucleocytoplasmic shuttling and altering their ability to transactivate (Yang et al., 2003). Thus, p38 and JNK SAPKs target several transcription factors directly enhancing their ability to activate transcription (e.g. MEF2A/C, Elk1, Sap1a). However, it is increasingly obvious that MAPK cascades do not modulate transcription by targeting transcription factors alone. Recent evidence implicates co-regulatory proteins as targets of MAPK cascades. The finding that the downstream kinases MSK1 and MSK2 phosphorylate nucleosomal proteins (Clayton and Mahadevan, 2003; Thomson

et al., 1999) provided the first direct link between a MAPK cascade and chromatin modification during gene induction. On the other hand, acetylation of histones H2B and H4 by ATF-2 is stimulated by JNK phosphorylation (Kawasaki et al., 2000). Additionally p38 has been reported to phosphorylate the TATA-binding protein (TBP), a prerequisite for its binding to the TATA box (Carter et al., 1999).

7. ROLE OF Hog1 IN TRANSCRIPTION

As mentioned previously, increases in extracellular osmolarity result in changes in the expression of a large number of genes. Better understanding of the yeast response to osmotic stress has been achieved from the use of DNA microarrays, which have permitted to perform genome-wide analysis of the transcriptional response under any type of stress. In the last few years, several independent studies have analyzed the global transcriptional response of *S. cerevisiae* to osmotic stress (Causton et al., 2001; Gasch et al., 2000; Posas et al., 2000; O'Rourke and Herskowitz, 2004; Rep et al., 2000). From these studies two conclusions can be drawn. First, different stress conditions (such as time of exposure to salt and osmolyte concentration) result in a different pattern of expression. Thus, exposure to high concentration of salt results in a delayed transcriptional response. And second, the Hog1-mediated signalling pathway plays a key role in global gene regulation under saline stress condition, since the response in *hog1Δ* cells is different from that observed in wild-type cells. In fact, approximately the 75% of genes induced after 20 minutes of exposure to 0.8M NaCl are fully or strongly dependent on the Hog1 MAPK (Posas et al., 2000). A relationship between the intensity of the response and the involvement of the Hog1 kinase can be drawn from the observation that genes considered very highly induced are more dependent on Hog1 than genes only moderately induced. Thus, although in some cases the HOG pathway is not the unique relevant signalling pathway involved, its central role in the global response to osmotic stress is clearly established.

The main groups of genes highly induced after exposure to 0.4M NaCl for 10 minutes (Posas et al., 2000) are genes encoding proteins involved in

carbohydrate metabolism (mainly, sugar transport and phosphorylation, and glycerol, trehalose and glycogen metabolism), protein biosynthesis (mainly ribosomal proteins), ion homeostasis, signal transduction and aminoacid metabolism.

7.1 Transcription factors under the control of Hog1

As mentioned before, it has been reported that SAPK can modify gene regulation by direct phosphorylation of transcription factors, both activators and repressors. In yeast, five transcription factors have been proposed to be controlled by the Hog1 MAPK: the 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) and the MADS box protein Smp1 (de Nadal et al., 2003). 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. However, there remained the possibility that additional transcription factors are required for gene expression upon stress.

7.1.1. Smp1

Smp1 is a member of the MEF2C family of transcription factors. It had been previously reported in higher eukaryotes that this family of transcription factors can be targeted by the mammalian p38 MAPK (McKinsey et al., 2002). In fact, Smp1 activator is directly phosphorylated by Hog1 on several residues within its transactivation domain. This phosphorylation by the MAPK is essential for Smp1-mediated gene expression (de Nadal et al., 2003). Furthermore, it was also reported that Smp1 may play an important role not only in osmostress responses, but also in a new function for the Hog1 MAPK required for cell survival in the stationary phase (de Nadal et al., 2003).

7.1.2. Msn2/Msn4

Msn2 and Msn4 are Cys_2His_2 zinc finger proteins that activate the expression of a number of stress-inducible genes (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). Although often considered to be functionally redundant in part because they activate gene expression through a common site, the stress response element (STRE), they are differentially regulated and may play distinct roles under different environmental conditions (Garreau et al., 2000; Gasch and Werner-Washburne, 2002). The STRE element is characterized by the core sequence CCCCT in either orientation and it is usually found in two or more copies in front of Msn2/4 target genes (Treger et al., 1998; Moskvina et al., 1998). Msn2/4 activity is regulated by their subcellular localization, residing in the cytosol under standard growth conditions and translocating to the nucleus under stressful conditions. This translocation is controlled by their phosphorylation state (Gorner et al., 1998). Several signalling pathways are thought to influence the activity of Msn2/4. These include the target of rapamycin (TOR) pathway (Beck and Hall, 1999), the protein kinase C pathway (Heinisch et al., 1999; Nierras and Warner, 1999), the protein kinase A pathway (Gorner et al., 1998), and the HOG pathway (Rep et al., 2000). According to this, Msn2/4 are required for the induction of a subset of Hog1-dependent genes, among them *CTT1* and *ALD3* (Rep et al., 2000).

7.1.3. Hot1

Hot1 (**H**igh-**O**smolarity-induced **T**ranscription) is a transcription factor related to Msn1 that was identified in a two-hybrid screening for proteins interacting with Hog1 (Rep et al., 1999b). It has been reported that Hot1 controls a small subset of genes, such as *STL1*, *GPD1* and *GPP2*, involved in the transport and production of glycerol (Rep et al., 1999a). Interaction of Hot1 with Hog1 is critical for recruitment of the MAPK to the Hot1-dependent promoters and essential for their transcriptional induction upon stress (Alepuz et al., 2001). Hot1 is phosphorylated by Hog1 in response to osmotic stress, but this phosphorylation appears not to be a relevant event for transcriptional regulation (Alepuz et al., 2001; Alepuz et al., 2003).

7.1.4. Sko1

Sko1 is an ATF/CREB-related factor (Nehlin et al., 1992; Vincent and Struhl, 1992). Such factors possess a bZIP domain, i.e., a leucine zipper for dimerization, and a basic transcription activation domain. Sko1 inhibits transcription of several genes that are inducible by osmotic stress (Proft and Serrano, 1999; Garcia-Gimeno and Struhl, 2000) by recruiting the general corepressor complex Ssn6-Tup1. Release from Ssn6-Tup1 repression in response to osmotic stress requires direct phosphorylation of Sko1 by the Hog1 MAPK (Proft et al., 2001). Interestingly, Hog1 phosphorylation switches Sko1 activity from a repressing to an activating state, which involves recruiting of SWI/SNF and SAGA complexes (Proft and Struhl, 2002). Recent genome-wide studies have demonstrated that yeast cells contain approximately 40 Sko1 target promoters in vivo (Proft et al., 2005). Sko1 binds to a number of promoters for genes directly involved in defense functions that relieve osmotic stress. In addition, Sko1 binds to the promoters of genes encoding transcription factors, including Msn2 among others. Lastly, Sko1 targets PTP3, one of the phosphatases that negatively regulates Hog1 kinase activity, and it is required for osmotic induction of PTP3 expression.

In addition to the conventional MAPK role in regulating transcription factor activities by direct phosphorylation, it has been reported that upon osmotic stress Hog1 can associate with the chromatin at promoter regions of target genes through interaction with specific transcription factors (Alepuz et al., 2001). Thus, apart from the role of Hog1 in the modification of transcription factors, its specific chromatin association with stress-responsive promoters suggested a new dimension to gene regulation by signalling kinases (see Results and Discussion).

7.2 Function of Hog1 preceding its role in transcriptional activation

A new direct function for Hog1 that precedes its known role in transcriptional activation has been reported (Proft and Struhl, 2004). Most chromatin-

associated proteins dissociate from the DNA template within the first minute after osmotic stress, presumably due to increased ionic strength in the nucleus. A very rapid Hog1-dependent mechanism permits reassociation of these proteins with chromatin within a few minutes. This mechanism consists in the direct modulation of ion transport by Hog1-mediated phosphorylation of the Nha1 Na^+/H^+ antiporter. This Hog1-dependent function precedes, and indeed is required for, Hog1-dependent activation of gene expression. Thus, the MAPK Hog1 can mediate very rapid stress relief in a manner that precedes and facilitates the long-term change in gene expression patterns.

OBJECTIVES

The basic objective of the research in our laboratory is to understand the mechanisms used by stress-activated kinases to regulate adaptive responses. A major response controlled by the Hog1 SAPK is regulation of gene expression. Therefore we aimed to investigate the mechanisms by which Hog1 regulates gene transcription in response to osmotic stress.
The specific objectives of this PhD project were:

I. to characterize the molecular mechanisms by which transcription factors under the control of the Hog1 SAPK are regulated to modulate transcription upon osmotic stress.
II. To identify novel chromatin modifying and remodelling activities involved in the Hog1-mediated osmostress gene expression.

RESULTS AND DISCUSSION

Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II.

Paula M. Alepuz, Eulàlia de Nadal, Meritxell Zapater, Gustav Ammerer and Francesc Posas

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Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II.

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As mentioned in the Introduction, Mitogen-activated protein kinase (MAPK) pathways are conserved signalling modules in low and high eukaryotes. These pathways respond to different extracellular stimuli by regulating gene expression, metabolism, cell death and proliferation. Yeast cells respond to increases in osmolarity in the extracellular environment by activating the stress-activated MAPK Hog1, a relative of the mammalian p38 family of stress-activated MAPKs. Several genome-wide transcriptional studies have demonstrated that a large number of genes are regulated by osmotic stress in a Hog1-dependent manner, which suggests a central role for Hog1 in stress-induced gene expression (Rep et al., 2000; Posas et al., 2000). One of the most common mechanism by which Hog1 and other SAPKs regulate gene expression is the modification of specific transcription factors (Kyriakis and Avruch, 2001). To date, several transcription factors have been proposed to act under the control of Hog1: Msn2, Msn4, Hot1, Sko1 and Smp1. Each factor controls a small subset of the osmoresponsive genes. However, before the publication of this paper, it was only characterized the mechanism by which the MAPK Hog1 regulates the activity of the transcription factors Smp1 (de Nadal et al., 2003) and Sko1 (Proft et al., 2001); (Proft and Struhl, 2002). Basically, it consisted on the direct phosphorylation of the transcription factors by the MAPK Hog1.

The presence of Hot1 and active Hog1 at osmosresponsive promoters is required for recruitment of the transcription machinery and gene expression.

The aim of the present paper was to analyze the mechanism by which the Hot1 activator controled Hog1-mediated osmostress gene expression. Hot1 is a transcription factor that regulates a small subset of genes mainly involved in

the production of glycerol and it was described that interacted with Hog1 (Rep et al., 1999b). At the beginning of this work it was known that both Hot1 and Hog1 were able to bind to osmostress promoters in response to osmotic stress (Alepuz et al., 2001). Furthermore, the same study showed that the lexA-Hot1 fusion protein required Hog1 activity for effective activation of a lexA operator system. However, the underlying mechanism by which Hot1 and Hog1 induced transcription remained unknown. Therefore, we aimed to investigate this mechanism.

We utilized chromatin immunoprecipitation (ChIP) experiments to analyze the binding of several components of the transcriptional machinery to the *STL1* promoter, a prototypical Hot1-regulated gene, before and after osmotic stress. To analyze the role of the MAPK Hog1 in this process, these experiments were performed in both wild-type and *hog1*Δ strains. The results showed that the transcriptional machinery (Pol II, Mediator and general transcription factors) was recruited to the *STL1* promoter only in response to stress. Furthermore, this recruitment was abolished in the *hog1*Δ strain. Similar results were obtained for the *ALD3* promoter, a Msn2/Msn4-dependent osmostress promoter.

We then analyzed the role of the activators Hot1 and Msn2/4 in Pol II recruitment. For this purpose, binding of Pol II to *STL1* and *ALD3* promoters was analyzed by ChiP in wild-type, *hot1*Δ and *msn2/4*Δ strains. Binding of Pol II to *STL1* was dependent on the presence of Hot1 and independent of Msn2 and Msn4, whereas binding of Pol II to *ALD3* was completely dependent on the presence of Msn2 and Msn4. The results revealed that binding of Pol II to osmostress promoters depends on the presence of specific activators for each promoter.

These evidences pointed out that in reponse to osmotic stress both the MAPK Hog1 and the corresponding activators are essential for the recruitment of the transcriptional machinery to osmostress promoters. It was described that recruitment of the MAPK Hog1 to osmostress promoters was dependent on the presence of specific transcription factors and on the activity of the MAPK (Alepuz et al., 2001). We demonstrated that also recruitment of Pol II to different promoters depends on the presence of specific activators and the MAPK. In addition, our data suggested that the mechanism by which the MAPK Hog1 regulates gene transcription in response to osmotic stress would not be

restricted to Hot1-dependent genes. Thus, although we studied the *STL1* Hot1-dependent promoter, the results could also be extended to Msn2/Msn4-dependent promoters.

Phosphorylation of Hot1 by the MAPK Hog1 is not required for gene expression.

Then, we performed experiments to decipher the mechanism by which Hog1 was recruiting Pol II. A possible mechanism was the direct phosphorylation of the activator Hot1 by the MAPK Hog1. In a previous study (Alepuz et al., 2001), it was shown that Hot1 was hyperphosphorylated during acute osmotic stress. However, it was not demonstrated whether Hog1 was the direct responsible for this phosphorylation neither which was the relevance of the phosphorylation. Initially, we performed an *in vitro* phosphorylation assay with active Hog1 and wild-type Hot1 or a mutant allele of Hot1 mutated in all putative phosphorylation sites for the MAPK. Wild-type Hot1 was phosphorylated by Hog1, whereas the mutated allele of Hot1 was not. We then tested whether the previously described binding of Hot1 to Hog1 (Rep et al., 1999b) was affected by elimination of the phosphorylation sites in Hot1. For this purpose, we performed two-hybrid analyses with a Gal4 fusion protein containing wild-type Hot1 or the mutated allele of Hot1. The results clearly showed that binding of Hot1 to Hog1 was not affected by mutation of the phosphorylation sites in Hot1. The same results were obtained when the two-hybrid analyses were performed with an inactive mutant of Hog1 (Hog1-K/N). We then investigated the physiological relevance of the phosphorylation sites in Hot1 by analyzing the transcriptional activity upon osmotic stress in a *hot1Δ* strain carrying an empty vector or centromeric plasmids encoding wild-type Hot1 or the non phosphorylable mutant of Hot1. Surprisingly, the results of the northern blot experiments demonstrated that both the level and the kinetics of expression of the osmoresponsive gene *STL1* were not affected in the strain carrying the non-phosphorylatable Hot1. Taken together, all these results demonstrate that Hog1-mediated phosphorylation of Hot1 is not essential for regulating gene expression in response to osmotic stress. This clearly suggested that the mechanism by which Hog1 regulates gene transcription in

response to osmotic stress is not based on the phosphorylation of Hot1 and must involve another mechanism.

To further investigate the mechanism, we analyzed the contribution of both the activator Hot1 and the MAPK Hog1 in the recruitment of the RNA Pol II. However, this was a difficult task since both proteins are interdependent for their binding to osmoresponsive promoters. To solve this problem, we took advantage of the observation that overexpression of Hot1 leads to Hog1-independent promoter binding of Hot1, without inducing any increase in transcription. Furthermore, despite constitutive binding of Hot1, recruitment of the transcriptional machinery remained completely dependent on stress and the presence of active MAPK. We then investigated whether the phosphorylation of Hot1 was required for the binding of Hog1 and Pol II in the overexpression system. By ChIP analyses, we could observe that, upon overexpression, the non-phosphorylatable mutant of Hot1 was constitutively bound to the *STL1* promoter while Pol II and Hog1 were recruited only after osmotic stress. Thus, we can conclude that in a system where binding of Hot1 and Hog1 are independent on each other, recruitment of Pol II is independent on the presence and the phosphorylation state of the activator whereas it is still dependent on the activity of the MAPK, since the recruitment is effective only upon osmotic stress. To confirm that activity of Hog1 is needed for the recruitment of Pol II, we performed ChIP experiments with a *hog1*Δ strain expressing a catalytically inactive Hog1 (Hog1-K/N). The results showed that recruitment of Pol II depends on the activity of the MAPK. Therefore, activity of the MAPK Hog1 is needed for the recruitment of Pol II, but phosphorylation of Hot1 is not essential.

Hog1 interacts with and recruits RNA Pol II and general components of the transcription machinery to induce gene expression.

Despite these conclusions, the mechanism by which Hog1 was recruiting Pol II was still unclear. As the activity of Hog1 was necessary for Pol II recruitment, we hypothesized that the activated MAPK was the entity that directly recruited the transcriptional machinery to targeted promoters. First, we addressed this question by a biochemical approach: through the identification of direct Hog1 interacting proteins. We purified proteins that associated with the fusion

protein GST-Hog1DS, where DS was the hypothetical docking site present on the C-terminal domain. Once purified, the interacting proteins were identified by MALDI-TOFF mass spectrometry. The analyses yielded Rpb1 and Rpb2, the α and β subunits of the RNA polymerase, as two of the most abundant proteins present in the coprecipitates with GST-Hog1DS. *In vivo* coprecipitation experiments confirmed the binding of Hog1DS to Rpb1 and showed that full-length Hog1, but not Hog1ΔDS, also bound to Rpb1. Thus, binding of Rpb1 to Hog1 is mediated by the non-catalytic region that contains the MAPK docking site. Furthermore, we performed an *in vitro* binding assay and showed that binding of Rpb1 to Hog1 was direct. Then we tested, by *in vivo* coprecipitation experiments, whether Hog1 was also able to interact with other components of the transcription initiation complex apart from Pol II. The results showed that Hog1 is also associated with Kin28, the catalytic subunit of TFIIH, and with several subunits of the Mediator complex. Interestingly, coprecipitation experiments demonstrated that under the same conditions the activator Hot1 was not able to interact with the transcriptional machinery. In addition, the simultaneous deletion of several osmostress-induced activators (*MSN1*, *MSN2*, *MSN4* and *HOT1*) did not affect the interaction between Hog1 and Pol II. All these evidences strongly supported the direct role of Hog1, but not Hot1, in the recruitment of the RNA Pol II holoenzyme to promoters in response to osmotic stress. Thus, Hot1 was acting as an anchor to target the kinase to specific promoters, as expected from the previous described data that showed that recruitment of the MAPK Hog1 to osmostress promoters was dependent on the presence of specific transcription factors (Alepuz et al., 2001).

Once clearly established the direct binding of Hog1 to the RNA Polymerase II holoenzyme, we analyzed the functional relevance of this interaction. We asked whether selective recruitment of the Pol II holoenzyme by Hog1 was sufficient to induce transcriptional activation. If Hog1 was enough to recruit and activate the transcriptional machinery and the transcription factor was only needed to anchor the MAPK to specific promoters, then it could be possible to by-pass this requirement by tethering Hog1 to an heterologous DNA binding domain. Thus, we fused Hog1 to the LexA binding domain and analyzed expression of a *LexA-LacZ* reporter gene. Under this condition, LexA-Hog1 was able to induce gene expression in response to stress and the induction was dependent on the MAPK activation because it did not occur in a *pbs2*Δ strain. Importantly, active Hog1

protein was required for induction of transcription, since neither the LexA-Hog1DS (catalytically inactive) nor the LexA-Hog1ΔDS proteins were able to induce transcription. Moreover, a catalytically inactive Hog1 that could not be phosphorylated by Pbs2 (Hog1 TAYA) was also unable to active transcription upon osmotic stress. These results, together with the previous observation that inactive Hog1DS binds Pol II but Hog1ΔDS did not, indicated that both activation of the MAPK and the presence of the DS were required for transcriptional activation. This means that, in addition to its role in the recruitment of Pol II holoenzyme, Hog1 plays an active role in the activation of the holoenzyme.

To complement the biochemical approach, we performed a genetic screening to identify elements of the transcriptional machinery required for Hog1-mediated *STL1* gene expression. For this purpose, a reporter strain containing an integrated *STL1*::*LacZ* construct was generated and genes whose overexpression caused a constitutive *STL1* expression in the absence of stress were searched for. The genes found were *SRB9*, *SRB4* and *SRB10*, all of them components of the Mediator complex. Importantly, these effects were Hog1-dependent, since they disappeared in a *hog1Δ* strain. Furthermore, *SRB9* and *SRB10* were also required for Hog1-dependent gene induction upon stress in the artificial LexA promoter system previously described. Thus, these were new evidences of the functional relevance of the interaction of Hog1 with the Pol II holoenzyme.

The novel mechanism for regulation of gene expression by SAPK described for Hog1 might be conserved among eukaryotic cells.

The mammalian SAPK homolog of Hog1 is the p38 MAPK, which is able to functionally replace Hog1 in yeast, suggesting a high degree of functional complementation (Galcheva-Gargova et al., 1994). To analyze whether p38 was also involved in a similar mechanism to that described for Hog1, we tested whether p38 was also able to interact with the mammalian RNA pol II holoenzyme. Coimmunoprecipitation experiments with HeLa cells transiently transfected with a vector carrying an epitope-tagged p38 demonstrated that the MAPK was able to interact with RNA Pol II, irrespective of the stress conditions. Further experiments must be done to determine whether p38 is

recruited to promoters and whether the MAPK is able to induce gene expression by direct interaction with Pol II. Nevertheless, this first evidence suggested that the novel mechanism for gene expression regulation described for yeast in this paper could be conserved among eukaryotes.

Thus, we propose a novel mechanism for regulation of gene expression upon osmotic stress based on the anchoring of active Hog1 to specific promoters by the transcription factor, which is essential for recruitment and activation of Pol II. Before the publication of this paper, only two studies had proposed a similar function for a MAPK. One of these studies referred to the Snf1 kinase, which belongs to a family of conserved kinases that control gene expression and metabolism in response to stresses affecting the cellular energy supply (Kuchin et al., 2000). The study showed that Snf1 kinase also associates with several components of the Mediator complex and that artificial recruitment of Snf1 to a heterologous promoter induced gene expression depending on the catalytic activity of the kinase. A second study (Cosma et al., 2001) was related to the Cdk1 protein kinase, involved in the progression of cell cycle. The study showed by ChIP experiments that recruitment of Pol II, TFIIB and TFIIH to SBF-dependent genes was completely dependent on Cdk1 activity. However, neither of both articles demonstrated a direct interaction between the kinase and the Pol II holoenzyme. Unlike these articles, our study was the first one that clearly showed the direct interaction between Hog1 and the Pol II holoenzyme, and additionally, established the key role of the MAPK in the recruitment of the Pol II holoenzyme. A recent report (Pokholok et al., 2006) corroborates these results and proposes that most MAPKs and protein kinase A subunits become physically associated with the genes that they regulate in the yeast genome.
In addition, our results suggested that this novel mechanism of regulation of gene expression could be conserved among eukaryotes. Later, this was demonstrated in (Simone et al., 2004), where it was described that the mammalian homolog of Hog1, p38, occupies and activates the myogenin (*MYOG*) and muscle-creatine kinase (*CKM*) promoters during human myogenesis.
Despite the novel evidences showed in this study, several questions still remained unanswered. In response to osmotic stress, the MAPK Hog1

stimulates transcription by directly recruiting the Pol II holoenzyme. Additionally, since Hog1 is a kinase and its activity is required for the induction of transcription, Hog1 might be modifying some component of the transcription machinery presumably by phosphorylation. The holoenzyme is a likely candidate, based on its physical interaction with Hog1, and it will be interesting to identify the phosphorylated protein(s). On the other hand, it remains unclear whether other transcription factors under the control of Hog1 follow a similar mechanism to that described for Hot1. If the novel proposed mechanism is conserved among eukaryotic SAPKs, it is likely that Sko1, Smp1 and Msn2/4 are working like Hot1, by recruiting Hog1 and RNA pol II to induce gene expression. Several specific groups of genes under the control of these transcription factors are known. Then, further experiments should be done to confirm this possibility. Finally, it is unknown the function of Hot1 phosphorylation by Hog1. As shown in this study, Hot1 phosphorylation is not required for Hog1 binding to target promoters and is not critical for induction of gene expression. It might be that fine tuning of gene expression might rely on this phosphorylation. Our proposed mechanism is independent on Hot1 phosphorylation and further efforts should be directed towards the finding of new targets of Hog1 which phosphorylation is physiologically relevant.

My personal contribution to this work was focused on the experiments with mammalian cells, that led to the last figure of this article. However, I did a close following of the whole work.

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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One of the most common mechanisms by which Hog1 and other SAPKs regulate gene expression is by modification of specific transcription factors (Kyriakis and Avruch, 2001; de Nadal et al., 2003). However, it is increasingly obvious that MAPK cascades do not modulate transcription by targeting transcription factors alone. The first article discussed in this PhD work (Alepuz et al., 2003) demonstrates that the MAPK Hog1 tightly binds chromatin in response to osmostress and elicits gene transcription by a mechanism that involves recruitment of the RNA Pol II holoenzyme. Other evidences implicate co-regulatory proteins as targets of MAPK cascades both in yeast and mammalian cells. The finding that the downstream kinases MSK1 and MSK2 phosphorylate nucleosomal proteins (Clayton and Mahadevan, 2003; Thomson et al., 1999) provided a direct link between a MAPK cascade and chromatin modification during gene induction. The organization of DNA into chromatin and chromosomal structure plays a central role in many aspects of yeast cell biology. Generally, chromatin is repressive to extraneous access, and this inhibitory effect must be overcome by regulatory factors during transcription. Chromatin modifying complexes and chromatin remodelling complexes are the two main enzymatic activities employed to regulate chromatin access.
The objective of the work presented in this article was to investigate whether the MAPK Hog1 was able to affect chromatin organization through a chromatin modifying or remodelling activity during the process of transcription.

Mutations in the deacetylase *RPD3* render cells osmosensitive with impaired expression upon osmotic stress.

With the idea to test whether Hog1 was involved in chromatin organization, we screened for osmosensitive mutants with impaired osmostress gene expression. Mutation of specific transcription factors involved in osmostress do not render

cells osmosensitive because each factor is controlling a small subset of the osmoresponsive genes. However, we hypothesized that deletion of certain chromatin modifying factors that affect osmostress gene expression to a greater extent would render cells osmosensitive. Thus, we analyzed the osmosensitive phenotype of several mutants in genes involved in chromatin modification. Deletion of genes encoding the Rpd3 histone deacetylase, a member of the class I HDACs, and its interacting protein Sin3 rendered cells osmosensitive. However, deletion of genes encoding the Hos1 and Hos2 deacetylases, also members of the class I HDACs, and the Hda1 and Hos3 deacetylases, members of the class II HDACs, did not affect cellular osmosensitivity. We concluded that the effect of histone deacetylation on cell survival and gene expression upon osmotic stress was specific for the Rpd3 deacetylase.

Histone acetylases and deacetylases play an important role in transcriptional regulation. Thus, we tested whether expression of Hog1-dependent genes was affected by deletion of *RPD3* by performing northern blot experiments. Expression of several osmostress-inducible genes was seriously reduced in the *rpd3*Δ cells when compared to a wild type strain. To analyze the global effect of *RPD3* deletion in Hog1-mediated gene expression, we performed genome-wide analyses by using microarrays holding the whole genome of *S. cerevisiae*, and compared gene induction upon osmotic stress in wild type and *rpd3*Δ strains. The results showed that of the genes induced more than twofold in response to osmotic stress, more than 90% showed reduced expression in the *rpd3*Δ strain.

As described in the first paper of this PhD Thesis (Alepuz et al., 2003), the presence of Hog1 in osmoresponsive promoters is necessary for induction of gene expression because it facilitates the recruitment of the RNA Pol II holoenzyme. We demonstrated that, when artificially tethered to a promoter as a LexA fusion protein, Hog1 is able to recruit the Pol II complex and induce transcription from a LexA-*lacZ* reporter system upon osmostress. Based on this observation, we analyzed whether activation of transcription by LexA-Hog1 was impaired by deletion of the Rpd3 complex. Interestingly, gene induction by LexA-Hog1 was completely abolished in cells deficient in *RPD3* or *SIN3*, but not in cells deficient in the deacetylases Hda1, Hos1, Hos2 and Hos3.

Taken together, all these results showed that the histone deacetylase Rpd3 is necessary for inducing Hog1-mediated gene expression upon osmotic stress. This adds a new perspective to the transcriptional regulation field. The role of

Rpd3 as an activator of transcription is in sharp contrast to that traditionally accepted for deacetylases and Rpd3 itself. Before the publication of this article, only one report demonstrated a positive role for a deacetylase, Hos2, in gene expression (Wang et al., 2002). As Rpd3, Hos2 also belongs to the class I HDACs, and was initially associated with transcription repression. However, Wang and colleagues demonstrated that Hos2 is essential for inducing the expression of the genes *INO1* and *GAL1*. We propose a more general positive role for Rpd3 than that proposed for Hos2: Rpd3 is affecting transcription of nearly 200 genes induced by osmotic stress. Moreover, as discussed later, the mechanisms by which Rpd3 and Hos2 are inducing transcription seem to be different.

The function of Rpd3 in osmostress gene induction requires its histone deacetylase activity.

The most highly conserved region among the yeast deacetylases Rpd3, Hda1, Hos1, Hos2 and Hos3 is a 60-70 aminoacids region, which, interestingly, is also found in deacetylases of other eukaryotic species. This motif contains four histidine residues organized in two pairs separated by 37 residues. For Rpd3, it was reported that at least three of these residues (H150, H151 and H188) play a key role with regard to histone deacetylase activity, and moreover, their function is not redundant (Kadosh and Struhl, 1998).
Thus, to analyze whether the function of Rpd3 in osmostress gene induction required its histone deacetylase activity, we generated a point mutant allele in which two of the histidine residues found in this conserved region (residues H150 and H151) were mutated to alanine. We performed beta-galactosidase assays to analyze expression of the LexA-Hog1 system in the *rpd3*Δ strain and in the deleted strain transformed with centromeric plasmids expressing wild type *RPD3* or the catalytically inactive allele 150:151. The catalytically impaired Rpd3 mutant could not restore expression of the LexA-Hog1. Moreover, the mutated allele was unable to complement *rpd3*Δ osmosensitivity. These results indicate that Hog1-mediated gene induction upon osmotic stress requires Rpd3 catalytic activity. However, it was not clear yet which substrates were the targets for the deacetylase activity. Hos2 also requires its deacetylase activity for binding to target genes and inducing its expression (Wang et al., 2002).

Since Rpd3 mainly deacetylates sites of acetylation on histones H3 and H4 (Kurdistani and Grunstein, 2003), we then tested whether histone deacetylation was a key step for osmostress gene induction. For this purpose, we used strains containing modified histone H4 acetylation sites. It was reported that replacement of the lysines in histone H4 with arginine (K-R) mimics the deacetylated state of histone H4, whereas replacement with glutamine (K-Q) mimics the acetylated state (Durrin et al., 1991). Thus, we analyzed expression by LexA-Hog1 in the H4(K-R) and H4(K-Q) mutant strains. The results showed that expression by LexA-Hog1 was increased in the H4(K-R) strain and, more importantly, it was completely abolished in the H4(K-Q) strain. In addition, the H4(K-Q) strain showed a clear osmosensitivity phenotype. These results suggested that histone H4 deacetylation is necessary for Hog1-mediated gene expression. However, the fact that expression induced by LexA-Hog1 in the H4(K-R) strain was lower than in the wild type suggested that effects of Rpd3 were not restricted only to histone H4. Thus, although the article demonstrates that the deacetylase activity of Rpd3 in osmostress gene induction is exerted on histone H4, it might also be exerted on histones H3, H2A or H2B.
Moreover, although other substrates related to transcription apart from histones have not been described, the possibility that Rpd3 might deacetylate other non-histone substrates to induce Hog1-mediated gene induction should not be excluded. On the other hand, other non transcriptional substrates have been described. For instance, human HDAC6 was reported to deacetylate alpha-tubulin, and this is necessary to regulate microtubule-dependent cell motility (Hubbert et al., 2002).

Rpd3 directly binds and deacetylates promoters to facilitate Pol II binding.

As described in the first article of this PhD Thesis (Alepuz et al., 2003), Hog1 binds to osmoresponsive promoters to mediate gene expression. We therefore wanted to analyze whether Hog1 physically associates with Rpd3 to target the deacetylase to specific promoters. For this purpose, we performed *in vivo* pull-down experiments which demonstrated that Hog1 coprecipitated with Rpd3 and its interacting protein Sin3, but not with the Hda1 deacetylase. Furthermore, we

performed *in vitro* binding experiments using purified proteins and showed that interaction between Hog1 and Rpd3 was direct.

We next wondered whether Rpd3, as Pol II, was recruited to osmostress promoters in a Hog1-dependent manner. ChiP experiments showed that, only in response to osmostress, Rpd3 and Sin3 were recruited specifically to the promoter region of the *HSP12* gene, an osmostress responsive gene. No binding of Rpd3 was detected in the surrounding regions of *HSP12* (including the coding region). Furthermore, we demonstrated that binding of Sin3 and Rpd3 to the *HSP12* promoter completely depended on the presence of Hog1, since it was abolished in a *hog1*Δ strain.

As the function of Rpd3 in osmostress gene induction requires its histone deacetylase activity, we hypothesized that, once recruited to the *HSP12* promoter, Rpd3 would deacetylate histone H4. To check this hypothesis, we performed ChIP experiments using specific antibodies against acetylated histone H4. The results showed a decrease in histone H4 acetylation in the wild type strain in response to osmotic stress, whereas no changes in acetylation were detected in the *hog1*Δ strain. Taken together, these results demonstrated that in response to osmotic stress, Rpd3 is recruited to osmoresponsive promoters in a Hog1-dependent manner to deacetylate histone H4.

Finally, we tested whether the presence of the Rpd3 complex and the decrease in promoter H4 acetylation correlated with gene induction by the Pol II holoenzyme. For this purpose, we performed ChiP experiments to detect binding of Pol II to the *HSP12* promoter in wild type and *rpd3*Δ strains. The results showed that Pol II binding to the *HSP12* promoter is restricted to osmostress and depends on the presence of Rpd3. These results are consistent with northern blot experiments, that showed that expression of *HSP12* was reduced in a *rpd3*Δ strain.

Together, these data clearly indicated that Rpd3 has a positive role in osmostress gene expression. Although a positive role has also been described for Hos2, the mechanism by which Rpd3 induces gene expression differs from the one proposed for Hos2. We propose the following working model for Rpd3-mediated gene expression in osmostress. In response to osmotic stress, Hog1 is recruited to osmoresponsive promoters by specific activators (Alepuz et al., 2001). Hog1 binding promotes recruitment of the Rpd3 deacetylase complex to the promoters, leading to histone deacetylation, entry of the Pol II holoenzyme

and induction of gene expression. In contrast, Hos2 has been described to associate physically with the coding regions of genes during transcriptional activity, when it deacetylates specifically histones H3 and H4 *in vivo* (Wang et al., 2002). Most of the acetylation and deacetylation reactions focus on promoter-specific alterations. Coding-region-specific acetylation and deacetylation events are also known to occur, but their roles in transcription are still poorly understood.

The effects of histone acetylation and deacetylation on transcription have commonly been interpreted in a structural context. Histone hyperacetylation is thought to weaken DNA-histone contacts by neutralizing the positive charge of the histone tails and decreasing their affinity for negatively charged DNA, thereby allowing access of transcription factors to promoters in the chromatin. Conversely, histone deacetylation is believed to prevent access by restoring the positive charge and strengthening the interactions between DNA and histones. Although this charge-neutralization model has become popular, *in vitro* analyses of nucleosomal arrays have provided contradictory evidences. For instance, histone-DNA interactions in *in vitro* reconstituted nucleosomes are not weakened by increased acetylation (Mutskov et al., 1998). These evidences together with the novel role for Rpd3 proposed in our article should cause a reassessment of the traditional role of histone deacetylation in transcriptional repression. Accordingly, some previous studies suggested that, in some particular genes, a decrease in histone acetylation is associated with transcription induction. For instance, genome-wide studies of histone deacetylase function in yeast showed that *RPD3* deletion led to down-regulation of more than 200 genes (Bernstein et al., 2000). Among the most highly down-regulated was BNA1, which encodes a NAD-biosynthesis enzyme.

Thus, it is increasingly evident that acetylation and deacetylation might not merely regulate gene activity by altering chromatin structure, but might also use other mechanisms. An alternative mechanism might consist in providing specific binding surfaces for the recruitment of repressors and activators. It is known that the acetylation sites of the histone amino termini provide a binding surface for regulatory factors that contain the bromodomain protein module, which is found in several eukaryotic transcription factors and coactivators, such as the SAGA subunit Gcn5 (Marcus et al., 1994a). Rpd3 could activate osmoregulated

genes by facilitating the binding of activators through a similar mechanism. Further experiments should be performed to analyze this possibility.
Recently, it has been identified the subunit composition of the two Rpd3 complexes, the large complex (Rpd3L) and the small complex (Rpd3S) (Carrozza et al., 2005; Keogh 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). The identification of the Rpd3 complex involved in osmostress gene induction would be very useful to further complete the study of the mechanism by which Rpd3 activates transcription. According to the different functions described for both complexes, it is likely that the Rpd3 complex involved in osmostress-induced transcription is the large one, which is recruited to promoters.
To decipher the mechanism of action of Hog1 and Rpd3 on osmostress promoters, the kinase activity of Hog1 should be taken into account. As it was already discussed in the first article (Alepuz et al., 2003), since Hog1 is a kinase and its activity is required for the induction of transcription, Hog1 might be modifying some component of the transcription machinery presumably by phosphorylation. Despite facilitating their recruitment, Hog1 neither phosphorylates Rpd3 nor Sin3 (unpublished results of our group). However, Hog1 might phosphorylate other proteins contained in the Rpd3L complex in order to activate the cascade of events that lead to the assembly of the preinitiation complex. Further phosphorylation assays should be performed to test this possibility.

The Rpd3 deacetylase complex is also involved in the control of heat shock responses.

Several genes induced by osmotic stress are also induced by other stresses, such as the heat stress. This is the case of *HSP12* and *CTT1*. This induction, however, does not depend on Hog1. As demonstrated in this article, Rpd3 is essential for the induction of these genes upon osmotic stress. However, it was unknown whether Rpd3 was required for the heat stress induction of these

genes. We hypothesized that a similar mechanism of regulation could be utilized, since the promoters to study were the same. To answer this question, we performed northern blot experiments to analyze expression of *HSP12* and *CTT1* on heat stress in a wild type strain and in *hog1*Δ and *rpd3*Δ strains. As expected, expression of both genes was induced by heat stress in a *HOG1*-independent manner, but the induction completely depended on *RPD3* since it was abolished in the *rpd3*Δ strain. Thus, these results indicate that Rpd3 is required for both osmostress and heat stress gene induction, whereas Hog1 is only involved in osmostress gene induction. Consistent with these results, a *rpd3*Δ strain is temperature sensitive.

Rpd3 is present on the promoters that require Rpd3 for their induction upon osmotic stress. Thus, we analyzed by ChIP analyses whether Rpd3 was also present on promoters induced by heat stress. As expected, the results demonstrated that Rpd3 is recruited to *CTT1* promoter in response to heat stress. Finally, we hypothesized that recruitment of Rpd3 in response to heat stress would not be dependent on Hog1. We demonstrated this hypothesis by analyzing Rpd3 recruitment by ChiP in wild type and *hog1*Δ strains. In contrast to osmostress, recruitment of Rpd3 upon heat stress was independent on Hog1. Taken together, all these results demonstrate that Rpd3 has a positive role not only in osmostress-induced gene expression, but also in gene induction upon heat stress. Thus, the positive role of Rpd3 could be a general role at least upon stress situations. Some interesting questions arise from these results: which protein or signalling pathway mediates recruitment of Rpd3 in response to heat stress? Is this protein recruited to promoters like Hog1? Further research will be needed to answer them.

The direct role of Rpd3 in transcriptional activity demonstrated in this article adds a new perspective to the transcriptional regulation field and revises the common idea that HDACs function only as transcriptional repressors. Further research will serve to completely decipher the mechanism by which Rpd3 activates gene transcription.

Personal contribution to this work: I provided the first author’s work with technical assistance and support in the design of the experiments (especially the design and analysis of the microarray assays, the northern blots, and the coprecipitation experiments). The ChIP experiments were performed by other

members of the group. The microarrays assays were performed by Lauro Sumoy (CRG).

Selective requirement for SAGA in Hog1-mediated gene expression depending on the severity of the external osmostress conditions.

Meritxell Zapater, Marc Sohrmann, Matthias Peter, Francesc Posas and Eulàlia de Nadal.

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Previous results showed that in response to osmotic stress Hog1 is intimately recruited to chromatin and that, 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). However, transcription initiation is a highly regulated process that requires the coordinated activities of a large number of factors, such as the coactivators. Thus, the aim of the work presented in this article was to identify systematically novel coactivators, in addition to Rpd3, that were required for the Hog1-mediated initiation of transcription in response to osmotic stress.

A genome-wide genetic screening reveals novel activities required for gene expression upon osmotic stress.

To identify novel activities involved in Hog1-mediated gene expression, we performed a high-throughput screening looking for mutations that render cells osmosensitive. As mentioned previously, mutation in transcription factors involved in osmostress do not render cells osmosensitive, possibly because each factor is controlling a small subset of the osmoresponsive genes. However, we showed that deletion of *RPD3*, a chromatin modifying factor, renders cells osmosensitive because it is affecting expression of a larger number of genes (de Nadal et al., 2004). Thus, 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 some activities could be required only at severe 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. Among these mutants, several complexes that had not previously been related to the osmostress response were identified, such as the Gim complexes, involved in cytoskeleton protein binding, or the class C Vps protein complex, involved in vacuolar biogenesis. A signal that the screening was highly reliable was indicated by the identification of the members of the Rpd3 complex as osmosensitive mutants, as reported by our group previously (de Nadal et al., 2004). Interestingly, two more complexes related to regulation of gene expression were identified as essential for cell viability upon osmotic stress: the SAGA and Mediator complexes. To characterize in more detail the importance of SAGA in survival at high osmolarity, we analyzed the osmosensitive phenotype of a full set of SAGA mutants at high salt and sorbitol concentrations. The results showed that deletion of any of the three subunits responsible to maintain the structural integrity of SAGA (*ADA1*, *SPT7* and *SPT20*) strongly affected cell growth at high osmolarity, whereas deletion of other SAGA subunits had mild or no defects on cell growth at high osmolarity. The same analysis was performed with several mutants on key components of Mediator. The results showed that Mediator mutants were also sensitive to osmostress. These results suggested a possible role for SAGA and Mediator in osmostress-induced gene expression.

SAGA is required for gene expression mainly in response to severe osmolarity whereas Mediator is required at mild and severe osmolarity.

The involvement of SAGA in the osmostress response was already suggested in (Proft and Struhl, 2002). That study showed that Hog1-dependent phosphorylation converts Sko1 from a repressor into an activator that recruits Hog1 itself and both SAGA and SWI/SNF complexes to the promoters. However, the study did not show neither the role of SAGA in osmostress transcription nor how SAGA was targeted to the osmostress promoters. Moreover, whether SAGA was recruited to promoters different from the Sko1-dependent promoters remained also unclear. Thus, we analyzed the role of SAGA in expression of osmoresponsive genes by northern blot at mild (0.4M NaCl) and severe (1.2M NaCl or 1.8M sorbitol) osmostress conditions in a wild-type strain and in several SAGA mutants. At 0.4M NaCl, SAGA deficient strains did not show any clear

osmosensitive phenotype, accordingly, they only showed a slight delay on transcription and the global levels of gene expression were unaffected. However, since SAGA mutants were unable to grow under severe osmotic conditions, we analyzed expression of the same genes under high osmolarity conditions (1.2M NaCl). Under these conditions, expression of several osmoresponsive genes was strongly reduced by deletion of *SPT20*. These results demonstrated that the SAGA complex is critical for osmostress gene expression only under severe osmostress conditions. This is the first evidence that indicates that the strength of the stimuli perceived by the cell can define the requirement for a coactivator for full gene expression. In addition, the results suggested that other osmoresponsive promoters regulated by other factors than Sko1, such as *STL1* and *CTT1*, which are Hot1 and Msn2/Msn4-dependent respectively, also require SAGA for full gene expression.

SAGA contains multiple catalytic activities. However, deletion of *GCN5* gene, which encodes SAGA histone acetylase subunit, has a limited effect on the expression of the bulk of the genome (Huisinga and Pugh, 2004). Similarly, deletion of *GCN5*, *ADA2* and the *SPT8* subunits had only a minor impact on osmostress gene expression even at severe osmotic conditions. In contrast, deletion of subunits required for the structural integrity of SAGA, such as *SPT20*, led to impaired transcription upon severe osmostress. This suggests that full gene expression and survival upon osmotic stress require the structural integrity of SAGA rather than some of its specific catalytic activities. Further experiments are required to deeply investigate the function that SAGA is exerting to properly induce transcription in response to osmotic stress.

To study the role of Mediator in expression of osmoresponsive genes upon stress, we analyzed by northern blot expression of several genes at mild (0.4M NaCl) and severe (1.2M NaCl or 1.8M sorbitol) osmostress conditions in a wild-type strain and in several mutants on key components of Mediator. In contrast to SAGA, the results showed that in these mutant cells transcription was impaired already at mild osmotic conditions (0.4M NaCl). Correspondingly, these mutant cells were osmosensitive at mild osmostress. It is worth noting that, deletion of the non-essential subunits of Mediator resulted in impaired transcription already upon mild osmotic stress. Inactivation of *SRB4*, an essential gene, results in a global decrease in gene expression (Holstege et al.,

1998), and also results in a decrease of the osmostress gene expression. This illustrates the crucial role of Mediator in osmostress gene response.
SAGA and TFIID both function as transcriptional coactivators and share a common set of subunits. However, a recent genome-wide study (Huisinga and Pugh, 2004) revealed that both complexes regulate different classes of genes: TFIID is required for transcription of about 90% of the genome, while SAGA plays a predominant regulatory role at a small fraction of the genome (~10%). Genes predominantly regulated by SAGA are genes commonly upregulated during general environmental stress. We tested this possibility by performing northern blot experiments in a strain bearing a temperature sensitive mutation in the *TAF2* subunit of the TFIID complex (*taf2-ts*) and analyzed gene expression at high osmolarity. The results showed that mutation of TFIID did not alter gene expression upon osmotic stress. Thus, SAGA, but not TFIID, is required for osmostress-induced gene expression at high osmolarity. This result supports the hypothesis that SAGA plays a role especially on stress responses.

Hog1 mediates recruitment of SAGA, Mediator and Pol II to osmostress promoters upon stress.

Once shown the relevance of SAGA and Mediator in osmostress gene expression, we analyzed the presence of both complexes at osmoresponsive promoters and the role of Hog1 in their recruitment. For this purpose, we performed ChIP experiments and followed the binding of the transcriptional complexes before and after stress in a wild type and in a *hog1*Δ strains. The results showed that SAGA, Mediator and Pol II were bound to promoters only after osmotic stress and in a Hog1-dependent manner. Moreover, recruitment of these complexes occurred under mild and severe osmotic conditions. Thus, Hog1 is required for recruitment of SAGA, Mediator and Pol II upon osmotic stress.
We next analyzed the kinetics of recruitment of these complexes in response to severe osmostress by following their binding at the osmoresponsive promoters by ChIP analyses. The results showed that once the MAPK is bound to promoters, the transcriptional complexes are rapidly recruited and then Pol II binding is stimulated and gene expression is induced. Recruitment of Hog1 and the transcriptional complexes and induction of gene expression in response to high osmolarity occur about one hour after cells are subjected upon stress.

Thus, severe osmostress conditions lead to a retarded transcriptional response compared to the rapid response observed upon mild osmolarity. However, the transcriptional response remains for a longer time under severe osmostress conditions. This delayed kinetics of induction was already described in (Van Wuytswinkel et al., 2000). The effect of severe stress on the kinetics of induction of the osmoresponsive genes is surprising because an earlier adaptive response would be expected under severely stressed cells compared with mildly stressed cells. Unpublished data showed that under severe osmolarity (1.2M NaCl), Hog1 phosphorylation and translocation to the nucleus follow a rapid kinetics similar to that observed at mild osmolarity. In addition, it was reported that Hog1 kinase activity is not inhibited under severe stress conditions (Van Wuytswinkel et al., 2000). Therefore, the delayed induction of expression of the Hog1-dependent genes upon severe osmotic stress probably results from a signalling defect downstream of Hog1. Additional investigation is required to deeply analyze this question.

Recruitment of Mediator depends on Hog1 and SAGA only at severe osmostress conditions.

Once demonstrated that SAGA and Mediator were recruited to osmoresponsive promoters in response to osmotic stress, we wanted to establish the order of recruitment of such complexes at these promoters with respect to Hog1. We performed ChIP analysis using several mutant strains. First, we analyzed whether binding of Hog1 to promoters was dependent on SAGA or Mediator. The results from the ChIP experiments showed that Hog1 was able to bind to promoters independently of SAGA and Mediator. These results are consistent with the previous observation that recruitment of SAGA and Mediator was abolished in the absence of Hog1, which indicates that binding of Hog1 precedes that of SAGA and Mediator (see above).
Then we established the relationship between SAGA and Mediator by analyzing recruitment of SAGA in a Mediator deficient strain and recruitment of Mediator in a SAGA deficient strain. The results showed that SAGA was bound to osmostress promoters independently of Mediator under mild and severe osmostress conditions. In contrast, although Mediator was bound to promoters independetly of SAGA under mild osmostress, at severe osmostress, recruitment of Mediator

was partially dependent on SAGA. Thus, these results indicate that SAGA is required for efficient recruitment of Mediator only under severe osmostress conditions.

Our results, in combination with results from other laboratories, indicate that the interplay between SAGA and Mediator can vary greatly depending on the gene network and the nature of the activator. As shown in Figure 10, SAGA is required for optimal recruitment of Mediator at genes activated by the activators Gal4 (Bhaumik et al., 2004) and Gcn4 (Govind et al., 2005) but not at genes activated by Met4 (Leroy et al., 2006). In contrast, Mediator is required for optimal recruitment of SAGA at the Gcn4-activated genes but not at genes activated by Gal4 and Met4. Our results lead to the proposal of a new model (Figure 11): at genes activated by Hog1, the interplay between SAGA and Mediator complexes depends on the severity of the stress. Thus, at mild osmotic stress, recruitments of SAGA and Mediator are independent, whereas at severe osmotic stress optimal recruitment of Mediator requires SAGA. The molecular bases which that define these mechanistic differences still remain to be elucidated. Taken together, our results emphasize the variety of mechanisms of action of coactivators and underline the necessity to study different promoters.

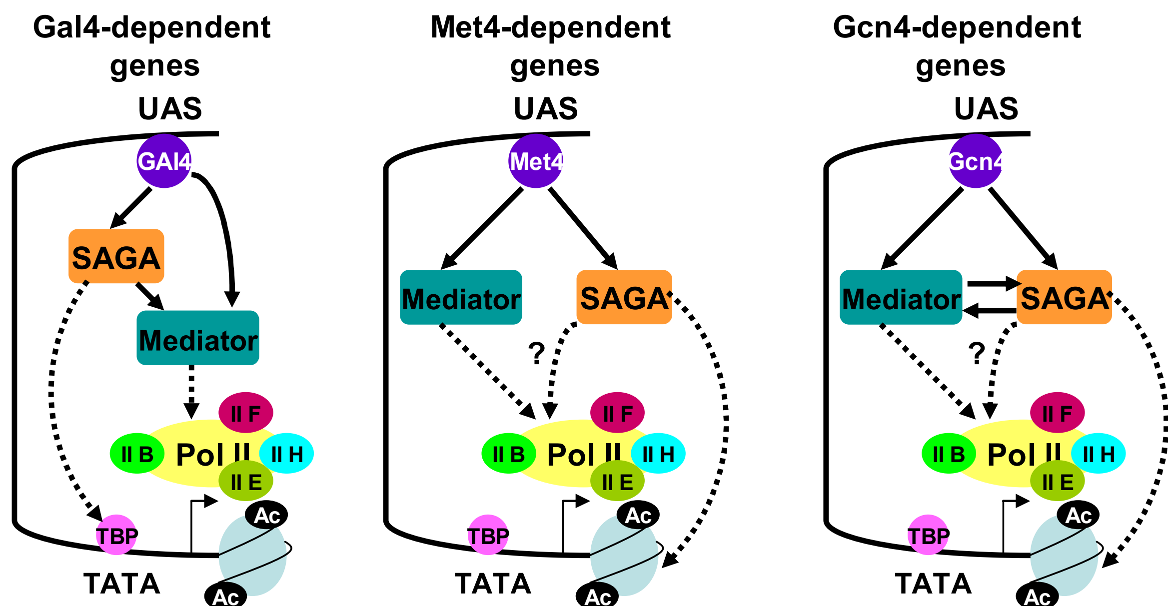

Figure 10. Model for Mediator and SAGA interplay in transcriptional activation (adapted from Leroy et al., 2006).

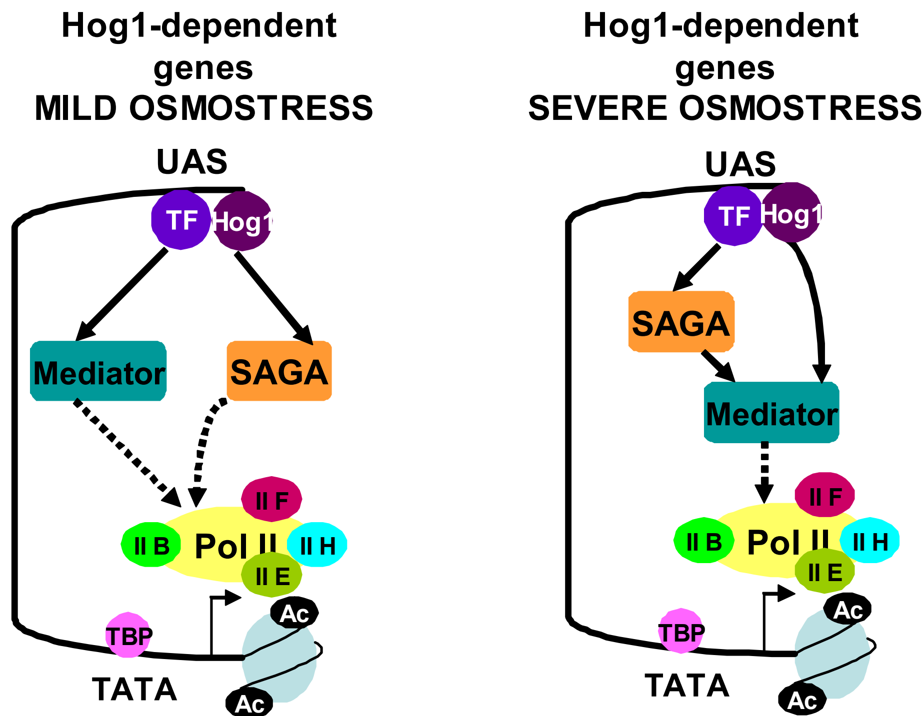

Figure 11. Model for Mediator and SAGA interplay in Hog1-dependent genes.

On the other hand, Figure 10 depictes some of +the functions of SAGA. At Gcn4 and Met4 promoters, the function of SAGA involves acetylation of histone H3 (Kuo et al., 2000; Leroy et al., 2006), whereas at Gal4 target promoters the function of SAGA consists in recruitment of TBP via interactions with Spt3/8 (Larschan and Winston, 2001; Sterner et al., 1999; Dudley et al., 1999). However, at osmoresponsive promoters, the function of SAGA is still unknown. As mentioned before, deletion of *ADA2* (a subunit involved in the histone acetyltransferase function) and *SPT8* did not affect osmostress gene expression under high osmolarity, which suggested that acetylation of histones and recruitment of TBP are not key functions of SAGA at osmoresponsive promoters. Therefore, further experiments are required to deeply investigate the function that SAGA is exerting to induce transcription in response to osmotic stress.

Recruitment of SAGA and Mediator also occurs at coding regions of stress-responsive genes upon induction.

Recent results from our group have shown that Hog1 is recruited to coding regions of osmoresponsive genes upon activation (Proft et al., 2006). Similarly, it has been proposed that Mediator is recruited to coding regions of specific active genes (Fan et al., 2006; Andrau et al., 2006). We then wondered whether Mediator and SAGA were also recruited to coding regions of osmoresponsive genes. To answer this question, we performed ChIP analyses, which showed that both Mediator and SAGA were recruited to the *STL1* coding region under mild and severe osmolarity. Additionally, as observed at the promoter, binding of Mediator to *STL1* ORF was reduced in a SAGA defective strain only under severe osmostic conditions. These results suggest that Mediator and SAGA might be exerting their functions not only on the promoters but also on the coding regions. It is still not known which is the role of Mediator and SAGA on these regions. This is a novel concept that requires additional investigation. It is also unknown whether general transcription factors also form part of the Mediator complex bound to coding regions. The insights provided here by the presence of SAGA and Mediator on coding regions open interesting areas for further studying the role of both complexes on coding regions of osmoresponsive genes.

Binding of Hog1 to Mediator and recruitment of RNA Pol II are reduced in SAGA deficient cells only at high osmolarity.

SAGA is recruited to different promoters through association to specific activators (Timmers and Tora, 2005). We then tested whether Hog1 was able to interact with the SAGA complex. For this purpose, we performed GST pulldown experiments in extracts from osmotically stressed cells expressing GST-Hog1 and tagged versions of several SAGA subunits. The results showed that GST-Hog1 (but not the GST control) coprecipitated all SAGA subunits tested. Thus, SAGA is recruited to osmoresponsive promoters through association to Hog1. However, it still remains unclear which specific SAGA subunit is the direct responsible for the interaction with Hog1.

Our ChIP data indicated that recruitment of Mediator to osmoresponsive promoters was reduced in a SAGA deficient strain only at severe osmostress conditions. This suggested that interaction of Hog1 with Mediator could be affected by deletion of SAGA at severe osmolarity. Thus we analyzed binding of Hog1 with the Mediator in wild type and SAGA defective strains expressing GST-Hog1 and a tagged Mediator subunit. Hog1 precipitated with Mediator, and as expected, at mild osmolarity, binding of Hog1 to Mediator was unaffected by deletion of SAGA. Interestingly, at severe osmolarity, binding of Hog1 to Mediator was dramatically reduced in a SAGA deficient strain. These results are in agreement with the ChIP data. We also showed, by coprecipitation assays, that interaction of Hog1 and SAGA is independent on Mediator. Thus, taken together, our results suggest that SAGA is required for promoting interaction of the Mediator with Hog1 at severe osmostress conditions, which explains the essential role of SAGA in osmostress-induced gene expression only at high osmolarity.

Finally, we analyzed the involvement of Mediator and SAGA in the recruitment of RNA Pol II to osmostress promoters. The fact that gene expression and cell survival were dramatically affected by inactivation of Mediator whereas were only affected under severe osmolarity by the deletion of SAGA subunits suggested that the recruitment of RNA Pol II could be impaired by inactivation of Mediator under mild osmolarity and in SAGA deficient cells only under severe osmolarity. This hypothesis was demonstrated by performing ChIP experiments. Pol II recruitment was analyzed in several mutants on key components of Mediator and the results showed that recruitment of Pol II was impaired even under mild osmostress conditions. In contrast to the requirement of Mediator, SAGA was only required for recruitment of Pol II at severe osmostress conditions, whereas at mild osmolarity the lack of SAGA only caused a slight delay in the recruitment of Pol II. Thus, we can conclude that Mediator is essential for osmostress Pol II recruitment, whereas SAGA is required for Pol II recruitment and gene expression selectively under severe osmolarity.

From the results exposed, we propose the following mechanism for the role of SAGA and Mediator in osmostress-induced gene expression. Recruitment of Hog1 to osmoresponsive promoters induces binding of SAGA and Mediator to these promoters. Under mild osmostress conditions, two redundant mechanisms to recruit Mediator must exist, one dependent on SAGA and a second one

independent on SAGA, which consists probably on the direct interaction of Mediator with Hog1-Pol II. This would explain the minor role of SAGA in the response to mild osmostress conditions. Under severe stress conditions, SAGA plays a major role in the recruitment of Mediator and its lack leads to a reduced amount of Mediator and Pol II on promoters and to impaired gene expression. Consistent with this model, binding of Mediator to Hog1 is strongly reduced in cells deficient for SAGA only under severe osmostress conditions.

Thus, our results define a major role for Mediator in osmostress gene expression and a selective role for SAGA under severe osmolarity conditions. This is the first evidence that the strength of the stimuli perceived by the cell can define the requirement for a coactivator to achieve full gene expression through the regulation of the interactions between transcriptional complexes.

However, despite the novel evidences showed in this study, several questions still remain to be ellucidated. The mechanism by which SAGA exerts its function in response to severe osmolarity conditions is one of the main questions to be answered. Thus, further efforts are being directed towards the finding of the SAGA activity responsible for its role in osmostress-induced expression.

Personal contribution to this work: except for the genome-wide screening which was performed by Eulàlia de Nadal during her stay in Matthias Peter’s lab, I have been fully involved in the design and execution of all the experiments described in this article.

GLOBAL DISCUSSION

The work presented in this PhD Thesis focus on the role of Hog1 MAPK in the regulation of gene expression upon osmostress. Several genome-wide transcription studies revealed that a large number of genes (7% of the genome approximately) showed significant changes in their expression levels after an osmotic shock in a Hog1-dependent manner (Causton et al., 2001; Gasch et al., 2000; Posas et al., 2000; O'Rourke and Herskowitz, 2004; Rep et al., 2000). This underlines the key role that the HOG pathway plays in global gene regulation under osmostress conditions.

One of the most common mechanisms by which Hog1 and other SAPKs regulate gene expression is by modification of specific transcription factors (Kyriakis and Avruch, 2001). Before the publication of the first article presented in this PhD Thesis, there was only known a mechanism by which the MAPK Hog1 regulated the activity of the transcription factors Smp1 (de Nadal et al., 2003) and Sko1 (Proft et al., 2001); (Proft and Struhl, 2002). This mechanism involved mainly the direct phosphorylation of the transcription factors by the MAPK Hog1.

Thus, the aim of the first article (Alepuz et al., 2003) was to analyze the mechanism by which the Hot1 activator controled Hog1-mediated osmostress gene expression. At the beginning of the work, it was known that Hog1 itself cross-linked with chromatin at several promoters in response to osmotic stress (Alepuz et al., 2001). This finding added a new exciting dimension to gene regulation by signalling kinases and indicated that Hog1 itself might take part in the activation process. The results obtained in the first article presented in this Thesis confirmed this hypothesis. It was demonstrated that a critical step to induce gene expression upon stress is the recruitment of the RNA Pol II complex to promoters by Hog1. The RNA Pol II machinery is recruited to osmoresponsive promoters only in response to stress and this association depends on the presence of both active Hog1 and the specific transcription factor. In addition, Hog1 interacts directly with the holoenzyme upon stress. Interestingly, it was demonstrated that the mammalian homolog of Hog1, the p38 MAPK, also interacts with the core of RNA Pol II in human cells. This result suggested that this novel mechanism directly involving Hog1 in the regulation of gene activation could be conserved among eukaryotic cells. This hypothesis is currently being tested by members of our research group, by analyzing recruitment of RNA Pol II to osmostress promoters in HeLa cells. Indeed, similar results have been observed for p38 during skeletal myogenesis, when p38 is recruited to

chromatin and selectively targets the SWI/SNF chromatin remodelling complex to muscle-regulatory elements (Simone et al., 2004).

This novel mechanism of gene activation described for Hog1 could be a general mechanism for protein kinases, since it has recently been described that several yeast MAPKs and protein kinase A become physically associated with the genes that they regulate in the yeast genome (Pokholok et al., 2006).

Thus, the first article presented in this PhD work (Alepuz et al., 2003) demonstrated that the MAPK Hog1 tightly binds chromatin in response to osmostress and elicits gene transcription by a mechanism that involves recruitment of the RNA pol II holoenzyme. This finding provided a putative link between the Hog1 MAPK cascade and chromatin modification during gene induction. Thus, the next aim was to investigate whether the MAPK Hog1 was able to affect chromatin organization through chromatin modifying or remodelling activities during the process of transcription. The second article (de Nadal et al., 2004) described the identification of the Rpd3 deacetylase complex as a chromatin modifying activity involved in the Hog1-mediated gene expression. It was demonstrated by microarray analyses that more than 90% of Hog1-dependent genes induced in response to osmostress have a significant reduction in their expression in a *RPD3* mutant strain. Moreover, Hog1 interacts physically with Rpd3 and, upon stress, targets the deacetylase to specific promoters, leading to histone deacetylation, entry of RNA Pol II and induction of gene expression. Although histone deacetylation has been traditionally associated to repression of gene expression (Robyr et al., 2002), we have demonstrated that the Rpd3 complex functions positively in induction of transcription in response to osmostress. Similar conclusions were drawn by a previous genome-wide study of histone deacetylase function in yeast. This study showed that *RPD3* and *SIN3* deletions lead to 2-fold down-regulation of 264 and 269 transcripts, respectively (Bernstein et al., 2000). The same study showed that genes down-regulated by trichostatin A (TSA), an HDAC inhibitor, corresponded with down-regulated genes in the *rpd3*Δ and *sin3*Δ profiles, which indicates that most of these genes may be direct targets of Rpd3 activation. In addition, a positive role for another class I histone deacetylase, Hos2, has also been described (Wang et al., 2002). Taken together, these results support the evidence of a positive role for histone deacetylases in induction of transcription. The study of the mechanism of action of HDACs is an emerging field, since

mammalian HDACs have emerged as molecular targets for the development of enzymatic inhibitors to treat human cancer. The molecular basis for the anticancer selectivity of HDAC inhibitors remains largely unknown. However, it has been speculated that the induction of histone acetylation produced by HDAC inhibitors might induce transcriptional activation of critical genes needed for tumor growth arrest (Cress and Seto, 2000; Marks et al., 2001).This hypothesis correlates with a transcriptional repressing role of HDACs, but the opposite results showed in this Thesis might contribute to decipher a new mechanism by which HDAC inhibitors would act. This mechanism might consist in inhibition of transcription of proliferative genes.

As transcription initiation is a highly regulated process that requires the coordinated activities of a large number of factors, it was likely that novel coactivators, apart from Rpd3, were required for the Hog1-mediated initiation of transcription in response to osmotic stress. The identification of these complexes was the aim of the third article presented in this PhD Thesis (Zapater et al., submitted). The work described in this article demonstrated that the SAGA and Mediator complexes are essential for the Hog1-mediated osmostress response. Several observations indicate that whereas Mediator is crucial for a proper gene induction upon any osmostress conditions, the role of SAGA at the promoters depends on the strength of the stimuli perceived by the cell, being essential only at severe osmolarity conditions. Moreover, Hog1 interacts physically with both complexes and the MAPK is required for their recruitment to promoters.

Thus, taken together, the results provided in this PhD Thesis have depicted a new scenario in the transcription regulation field. A signalling MAPK has been described to be directly involved in the initiation of transcription process by interacting with and recruiting several chromatin modifying complexes.

However, some key questions regarding the control of gene expression by the MAPK Hog1 still remain to be elucidated. A major issue is the identification of MAPK targets that are specifically modified to regulate transcriptional initiation. As discussed in the first article, since Hog1 activity is required for the induction of transcription and Hog1-mediated phosphorylation of Hot1 is not essential for regulating gene expression, it is expected that Hog1 phosphorylates other transcriptional proteins to regulate gene transcription in response to osmotic stress. As Hog1 facilitates the recruitment of the transcriptional complexes Rpd3, SAGA and Mediator it is likely that some subunits of these complexes are

targets of Hog1. Thus, we checked whether Rpd3 or Sin3, a subunit of the Rpd3 complex, were a target of Hog1. Neither of both was phosphorylated by Hog1. However, since the subunit composition of the Rpd3L and RpdS complexes has been recently identified (Carrozza et al., 2005), it should be checked whether Hog1 phosphorylates other proteins contained in the complex. In addition, we also tested phosphorylation of several SAGA and Mediator subunits by Hog1. We could not detect phosphorylation of any subunit, however, a more exhaustive analysis should be performed. Other transcriptional proteins, such as the nucleosomal proteins, should be taken into account as putative targets of the Hog1 MAPK. For instance, the mammalian p38 MAPK, through the downstream mitogen and stress-activated kinase 1/2 (MSK1/2), elicits phosphorylation of distinct pools of histone H3 at Ser10 and Ser28 concomitant with gene induction (Dyson et al., 2005; Soloaga et al., 2003). Thus, further research is required to identify transcriptional proteins as targets of Hog1 MAPK.

A second issue that remains open is the identification of novel transcriptional regulators or chromatin remodelling/modifying factors necessary for proper transcriptional response upon osmostress. In this PhD Thesis, three transcriptional complexes have been described to be required for Hog1-mediated gene expression: the Rpd3 deacetylase complex, SAGA and Mediator. In (Proft and Struhl, 2002) it was suggested that the SWI/SNF nucleosome-remodelling complex was important for activation of Sko1-dependent promoters upon osmotic stress. However, the relevance of SWI/SNF in osmostress transcription and how it is targeted to osmostress promoters remains unclear. This is an open question that requires further investigation.

Finally, it would be interesting to establish the relationship between the functions of Rpd3 and SAGA regarding the activation of transcription initiation upon osmotic stress. It is not clear whether recruitment of both complexes is independent or not. As discussed in the second article, an alternative mechanism by which acetylation and deacetylation might regulate gene activity, apart from altering chromatin structure, consists in providing specific binding surfaces for the recruitment of regulatory factors. Thus, it might be possible that Rpd3 deacetylation at osmoresponsive promoters facilitates the binding of other complexes involved in the osmoresponse, such as SAGA, Mediator, SWI/SNF or other putative proteins involved in this response. Further experiments should be perfomed to analyze this possibility.

The results provided in this PhD Thesis have demonstrated that the Hog1 MAPK regulates gene expression upon osmostress by several mechanisms. This MAPK not only modifies directly stress-dependent transcriptional regulators by phosphorylation, but also associates specifically to stress responsive promoters to recruit the Rpd3 histone deacetylase complex and the SAGA and Mediator complexes to induce gene expression. Moreover, it has been established a differential role of the SAGA coactivator depending on the strength of the stress perceived by the cell, which indicates that the transcriptional regulation is a fine tuned process. Moreover, the results obtained provide some evidences that the novel mechanism described for Hog1 MAPK might be conserved among eukaryotes. In addition, these results provide useful guidelines for studying the mechanism by which the mammalian homolog p38 kinase induces the initiation of transcription upon stress.

CONCLUSIONS

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

- The assembly of the transcriptional machinery to Hot1-dependent genes depends on the presence of Hot1 and active Hog1 MAPK, but not on Hot1 phosphorylation.
- Hog1 interacts with the RNA Pol II and with general components of the transcriptional machinery, which results in induction of gene expression.
- Anchoring of active Hog1 to promoters by the Hot1 activator is essential for recruitment and activation of RNA Pol II.
- The mammalian p38 kinase also interacts with the RNA Pol II.

- The SAGA, Mediator and Rpd3 complexes have been identified as novel regulators of osmostress-mediated transcription.
- Cells lacking the Rpd3 histone acetylase complex are osmosensitive and show compromised expression of osmostress genes.
- Hog1 interacts physically with Rpd3 and targets the deacetylase to specific osmoresponsive promoters.
- Binding of Rpd3 to specific promoters leads to histone deacetylation, entry of RNA Pol II and gene induction.
- Mediator is essential for proper gene induction upon any osmostress conditions.
- SAGA is essential for proper gene induction only upon severe osmostress conditions, whereas at mild osmolarity, SAGA mutants only show very slight defects on RNA Pol II recruitment and gene induction.
- Hog1 interacts physically with SAGA and Mediator and targets both complexes to specific osmoresponsive promoters.
- Recruitment of Mediator and RNA Pol II is mediated by SAGA at severe osmostress conditions.

SUPPLEMENTARY ARTICLES

Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1.

Xavier Escoté*, Meritxell Zapater*, Josep Clotet* and Francesc Posas.

Nature Cell Biology Vol 6 pp.997-1002, 2004.

*These authors contributed equally to this work.

Control of cell cycle progression by the stress-activated Hog1 MAPK.

Meritxell Zapater, Josep Clotet, Xavier Escoté and Francesc Posas.

Cell Cycle Vol 4 pp.6-7, 2005.

SUMMARY

As mentioned in Introduction section, the mechanism by which Hog1 controls G1 transition in response to osmotic stress was addressed in the following manuscripts.

In response to osmotic stress, the MAPK Hog1 elicits the program for cell adaptation that includes modulation of several aspects of cell biology, such as cell-cycle progression. Although the involvement of Hog1 in the cell-cycle regulation had already been reported (Alexander et al., 2001; Yaakov et al., 2003), the molecular basis of this regulation were not completely understood. The work of our group provided new insights into the knowledge of the mechanisms used by the SAPK Hog1 to regulate G1-S transition.

Activation of Hog1 by exposure of cells to high extracellular osmolarity or due to constitutive activation of upstream components of the MAPK pathway results in cells arrested at the G1 phase. In yeast, progression from G1 to S phase requires activation of the Cdc28 kinase by the G1 cyclins (Cln1, Cln2 and Cln3). The Cln-Cdc28 complex phosphorylates several sites at N-terminus of the CDK inhibitor Sic1, leading to its degradation by the SCF complex and consequent progression of the cell cycle into the S phase.

In (Escote et al., 2004) it is showed that Sic1 is involved in the Hog1-mediated G1 arrest and it is deciphered the mechanism used to exert this arrest. First it is shown that Sic1 is responsible for the G1 arrest induced by osmostress, since

deletion of *SIC1* abolishes this arrest. Activation of Hog1 results in a strong accumulation of Sic1 protein levels. This accumulation is explained, partially, by the down-regulation of Cln1 and Cln2 expression observed in response to osmostress, which reduces Sic1 phosphorylation and degradation. However, an additional mechanism independent of the decrease of Cln levels must exist to account for the Sic1-mediated G1 arrest, since activation of Hog1 in cells with sustained Cln2 levels does not prevent G1 arrest. The article demonstrates that this additional mechanism is based on the phosphorylation of Sic1 by Hog1. Hog1 interacts with Sic1 *in vivo* and *in vitro*, and Hog1 directly phosphorylates *in vivo* and *in vitro* one specific threonine at the C-terminus (Thr 173) of Sic1. As a result of this phosphorylation, its interaction with the Cdc4 E3 ligase of the SCF complex is reduced, and consequently, efficient Sic1 degradation is prevented and Sic1 is stabilized in response to osmotic stress. Thus, combination of both mechanisms, down-regulation of Clns expression and phosphorylation of Sic1, results in Sic1 stabilization and G1 arrest. Cells lacking Sic1 or that contain a Sic1 allele mutated in the Hog1 phosphorylation site are unable to arrest at G1 upon Hog1 activation, they progress into S phase without being properly adapted and thus, become sensitive to osmostress.

In (Zapater et al., 2005) the dual control exerted by Hog1 over Sic1 is discussed. Neither the decrease on Clns levels nor the phosphorylation of Sic1 by Hog1 can totally account for the G1 arrest upon osmotic stress. Therefore, it is proposed that the redundant mechanism over Sic1 to control cell cycle progression has an obvious advantage: the increase of the efficiency of the G1 arrest by establishing two additive mechanisms over Sic1. This increased efficency of the G1 arrest would explain why the maintenance of such a complex regulatory mechanism is an advantage.

However, alternative interpretations of the advantages of this dual regulatory mechanism may exist. Thus, this system can be envisioned as a two step mechanism. The phosphorylation of Sic1 would serve to cause an immediate arrest of the cell cycle, and then the decrease of Clns expression would be required to maintain the arrest for a prolonged time. This model would allow us to explain why cells at different stages of the G1 phase can be prevented from entry in S phase. Thus, cells at early G1 could be easily arrested by the simply decrease on Clns levels, whereas cells in advanced G1 would require a more direct effect over Sic1 to be arrested.

An obvious question formulated in (Zapater et al., 2005) is whether this dual mechanism that involves cyclin expression and targeting of cell cycle regulatory proteins is restricted to the G1 control or can be extended to other phases of the cell cycle. The article proposes that the G2 arrest induced by Hog1 is also mediated by a dual mechanism, as suggested by recent data of the group, in which Hog1 mediates a decrease on Clb2 transcription and a direct control of Cdc28-Clb2 activity by activation of Swe1. Recently, these data have been demonstrated and published (see (Clotet et al., 2006) in the Introduction section).

Finally, the article refers to the mechanisms proposed for the control of cell cycle progression by the mammalian p38 SAPK. Several targets for the SAPK have been described and it is not clear yet whether specific mechanisms are used to respond to different stimuli and whether different cell types use different mechanisms to overcome stressful situations. However, based on our yeast studies, it is proposed that in response to stress, the SAPKs might coordinate different mechanisms, probably involving modulation of cyclin levels together with the target of specific cell cycle regulators, to promote transient arrest at several steps of cell cycle to allow for proper cellular adaptation to extracellular stimuli.

Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1.

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