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Tesis Doctoral

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Summary

SUMMARY

Exposure of yeast to increases in extracellular osmolarity activates the stress-activated Hog1 MAP kinase, which is essential for cell survival upon osmotic stress. Activation of the Hog1 MAPK results in cell growth arrest, suggesting a possible role of the MAP kinase in the control of the cell cycle. Our results have shown that Hog1 activation resulted in accumulation of cells in the G1/S and G2/M transitions. At G1, Hog1 regulates the cell cycle progression by a dual mechanism that involves downregulation of G1 cyclin expression and direct targeting of the CDK-inhibitor protein Sic1. The MAPK interacts with Sic1, and phosphorylates a single residue of Sic1, which, in combination with the downregulation of cyclin expression, results in Sic1 stabilization and inhibition of cell cycle progression. Consistently, *sic1*□ cells, or cells containing a *SIC1* allele mutated in the Hog1 phosphorylation site, are unable to arrest at G1 phase after Hog1 activation, and become sensitive to osmostress. Together, our data indicate that Sic1 is the molecular target for Hog1 that is required to modulate cell cycle progression in response to stress at G1. On the other hand, activation of the Hog1 MAPK also results in an increase of cells in the G2 phase. Arrested cells displayed down regulation of the Clb2-Cdc28 kinase activity and consequently enlarged buds, defects in spindle formation and orientation. These effects were prevented by deletion of the *SWE1* gene. Thus, *swe1*Δ cells failed to arrest at G2, which resulted in a premature entry into mitosis and mislocalization of nuclei. Consistently, *swe1*Δ cells were osmosensitive. Swe1 degradation was reduced in response to activation of Hog1. Swe1 accumulation is mediated by the activity of the complex Hsl1-Hsl7. Hog1 phosphorylates a single residue at the regulatory domain of Hsl1, which leads to the mislocalization of Hsl7 from the bud neck, and consequent Swe1 accumulation. In addition, Hog1 downregulates G2 cyclin expression, reinforcing the inhibition of cell cycle progression at G2/M. These results indicate that Hog1 imposes a delay in critical phases of cell cycle progression necessary for proper cellular adaptation to new extracellular conditions.

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Introduction

Stress

Cells are exposed constantly to stress situations. Stress situations are variable environment modifications such as changes in temperature, pH, radiation, availability of nutrients, access to oxygen, (Hohmann, 2002). Cells are able to coordinate intracellular activities in order to respond to these stresses, which allow the cells adapt to the new environmental conditions. One of the most common stresses is water activity. Water activity is defined as the chemical potential of free water in solution. The water activity varies widely and rapidly. In *Saccharomyces cerevisiae* (*S. cerevisiae*), in order to maintain an appropriate cell volume, the cytosol water activity has to be lower than the water activity of the surrounding medium ((Blomberg et al 1992); (Wood 1999). Yeast cells have developed different mechanisms to survive a sudden change in water activity. The first seconds after an osmotic shock are critical because passive water loss or uptake occurs very fast. It is for this reason that cells need to have mechanisms to adapt as fast as possible within shift ((Blomberg et al 1992); (Brown 1976); (Brown , 1978)). The osmotic shock can develop in two different directions; a hyperosmotic or a hypoosmotic shock. Hyperosmotic shock on yeast cells is accompanied by rapid water outflow and cell shrinking. On the other hand, the hypoosmotic shock increases the water concentration gradient and leads to rapid influx of water, cell swelling, and consequently an increased turgor pressure. (Smits et al 1999). In our lab the main aim to understand is how the cells can respond and adapt to a hyperosmotic situation.

Hyperosmotic Shock

When cells are subjected to hyperosmotic shock, these cells lose cellular water because this water follows by passive diffusion a concentration gradient. This phenomenon results in an increase of the inner concentration of molecules, which alters some cellular processes. To adjust to high osmolarity external, yeast cells have developed a battery of mechanisms to adapt to extracellular changes. Adaptation to these new conditions is a process that is divided in two phases; sensing osmotic changes and the development of proper cellular responses. Accumulation of osmolytes has a central role in osmoadaptation, and in *S. cerevisiae* mainly this osmolyte is glycerol ((Brown 1978); (Yancey et al. 1982)).

Basically, there are two different aspects which are necessary to be considered in osmostress: survival to a quick change in the external osmolarity, and the acquisition of tolerance to this variable external osmolarity. Survival of a hyperosmotic shock and adaptation to high osmolarity are probably distinct mechanisms but both are at least in part overlapping: cells adapted to moderately high osmolarity medium survive a severe osmotic shock better than non adapted cells ((Schuller et al 1994); (Mager et al 1993)).

It was discovered the involvement in osmoadaptation of a mitogen-activated protein kinase cascade, a conserved eukaryotic signal transduction module, called the HOG pathway, ((Brewster et al 1993); (Gustin et al 1998)). A consensus idea of osmoadaptation is conserved across eukaryotes, and thus yeasts and the HOG pathway are an ideal model system for study these processes to understand higher eucharyotes.

Signalling Pathways

Variability in medium osmolarity affect diverse signalling pathways in *S. cerevisiae*: the cAMP-dependent protein kinase pathway, the phosphatidylinositol-3,5-biphosphate (IP3) pathway and the most important, the HOG pathway. Protein kinase A ([cAMP]-dependent protein kinase, PKA) has been described to modify expression of genes upon an osmotic shock (Norbeck et al 2000).

PKA mediates a general stress response essentially under all stress conditions, for instance: oxidative stress, heat shock, high ethanol levels, nutrient starvation, and osmotic stress ((Marchler et al 1993); (Ruis et al 1995)). Thus, PKA probably does not respond directly to osmotic changes. The activity of PKA is controlled by stress, it is no clear how is regulated. It has been described that an osmotic shock stimulates production of IP3, which could act as a second messenger in an osmotic signalling system (Dove et al 1997). However its actions have not been elucidated yet. Finally, there is the HOG pathway. The HOG pathway is the best-characterized system implicated in osmostress. The HOG pathway is activated within less than 1 min by osmotic shock (Brewster et al 1993) and cells lacking the pathway, or unable to activate it, can not survive in high osmolarity medium. Therefore, the HOG pathway coordinates a significant part of the cellular response of yeast cells to high osmolarity.

MAPK Signalling Pathway

MAPK cascades share a tier of three consecutively activated kinases: a MAP kinase kinase kinase (MAPKKK or MEKK (Figure 1), a MAP kinase kinase (MAPKK) or MEK, and a MAP kinase (MAPK).

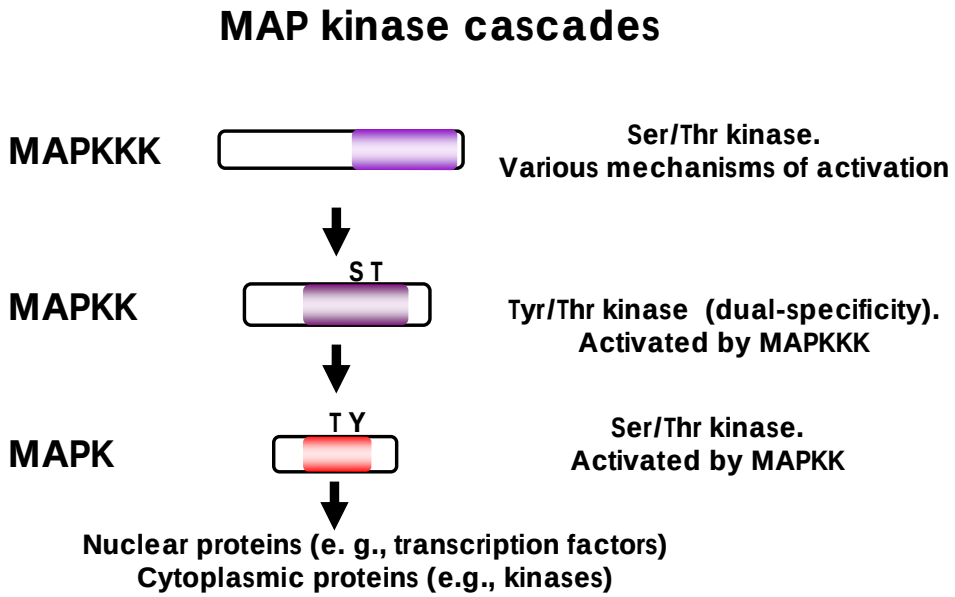

Figure1 MAPK pathways: schematic organization. All the MAPK cascades are composed of three consecutively activated tiers of kinases: a MAP kinase, a MAPKK and a MAPKKK. The MAPKKK phosphorylates and activates the MAPKK on serine and threonine at the N-terminal lobe of the kinase domain. Subsequently, the MAPKK phosphorylates the MAP kinase on a threonine (sometimes serine) and tyrosine residue.

The MAPKKK activates the MAPKK phosphorylating it on serine and threonine within a conserved N-terminal lobe of the kinase domain. MAPKKKs are composed by a N-terminal regulatory domain and a C-terminal domain which has the catalytic kinase activity. The regulatory domain blocks the accessibility of the proteins target to the C-terminal kinase domain. Activation can be achieved by two interrelationated ways: phosphorylation through an upstream protein kinase or by interaction with other proteins, allowing the interaction between the substrate and the catalytic domain. There are different systems of activation mechanisms and sensor upstream of MAP kinase and generally include receptor tyrosine-kinases (in animal systems), G-protein-coupled receptors, phosphorelay systems, and others. Different MAP kinase

pathways form interacting signalling systems. For instance, it has been described that a MAPKK may control several different MAP kinases, as is observed for example in yeasts. Therefore, the same kinase may be involved in different pathways. From *S. cerevisiae* to higher eukaryotes, this situation results in a complex network system of signalling pathways. After this activation, the MAPKK phosphorylates the MAP kinase on a tyrosine and threonine (sometimes serine), which are located adjacent to each other separated by a single amino acid (Thr/Ser-X-Tyr). These residues are located in the activation loop of the catalytic domain; this doble phosphorylation is a key step for a complete activation of the MAP kinase. Pathways of MAP kinase are negatively controlled by protein phosphatases acting on two levels: the MAPKK (serine-threonine phosphatases) or only on the MAP kinase (serine-threonine phosphatases and tyrosine phosphatases) (Keyse 2000). Once MAPK is phosphorylated, this phosphorylation leads to a transfer the MAPK from the cytosol to the nucleus, where it can phosphorylate its protein targets on serine/threonine followed by a proline (S/T-P). However, a portion of activated MAP kinase is retained in the cytoplasm to regulate cytosolic events (Reiser, Ruis, and Ammerer 1147-61). MAPK cascade targets can be divided in two major groups, transcription factors and other proteins (Alepuz et al 2003) (Alepuz et al 1997); (Andrews et al 2000); (Rouse et al 1994)).

Scaffold proteins could contribute to the signal specificity separating different MAPK modules. In the HOG pathway, Ste11 MAPKKK binds to Pbs2 MAPKK, which restricts Ste11 to activating only Pbs2, and not other MAPKKs ((Harris et al 2001); (Posas et al 1997)). Moreover, substrate specificity and negative feedback loops play an important role in signalling specificity too. For example, the role carried out by the protein phosphatases (Chang et al 2001). MAPK cascade targets can be divided in two major groups, transcription factors and other proteins ((Alepuz et al 1997) ; (Andrews et al 2000); (Rouse et al 1994)).

MAPK Pathways in yeast

MAP kinases have been implicated in different signalling processes: osmothyc stress, pheromone response, nutrient limitation, and filamentous growth … (Figure 2). In fact, MAP kinase (MAPK) cascades are common signalling modules found in both higher and lower eukaryotic cells. These pathways develop essential roles for cell survival, adapting to environmental changes or responding to the presence of cytokines, hormones, growth factors, controlling cell growth, morphogenesis, proliferation, and stress responses. ((Banuett 1998);(Chang et al 2001); (Gustin et al 1998); (Kultz et al 1998); (Kyriakis et al 2001); (Ligterink et al 2001)).

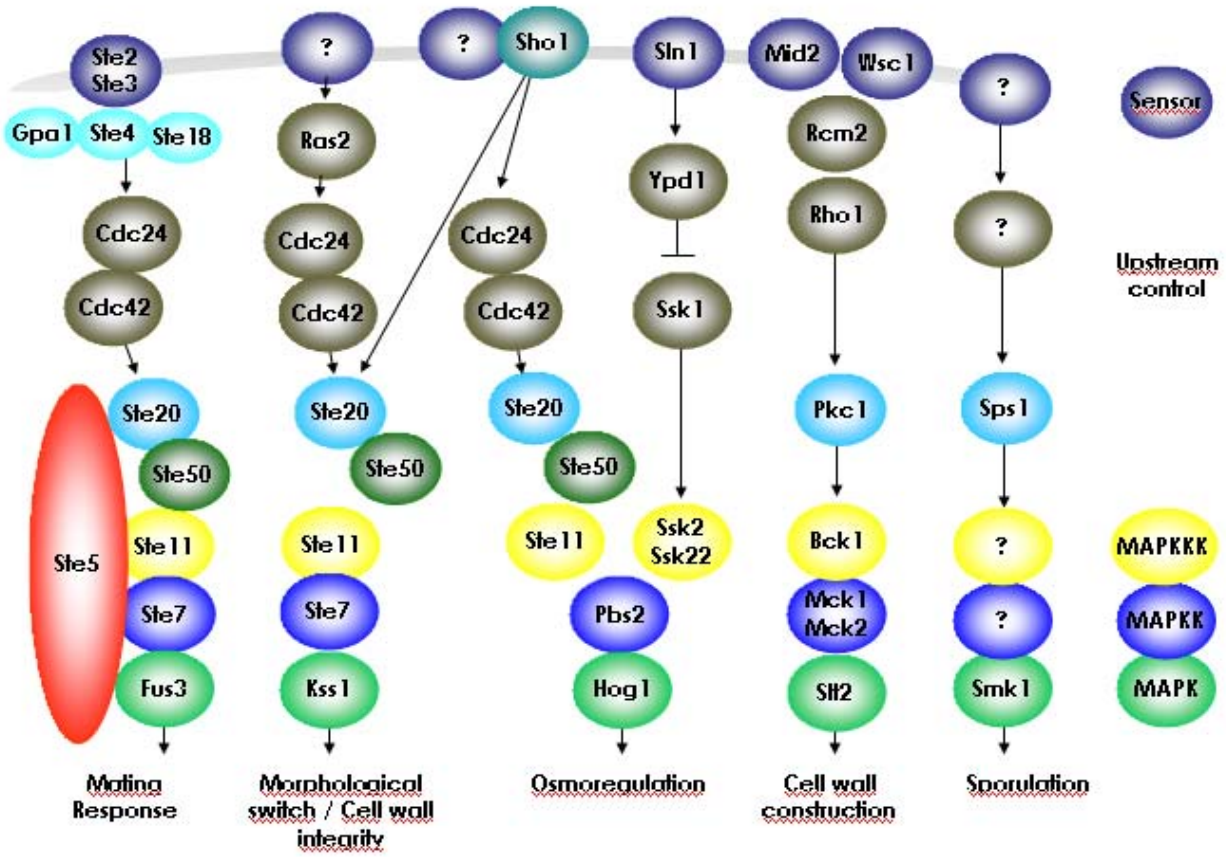

Figure 2. Outline of the *S. cerevisiae* MAP kinase pathways. There are five pathways in yeast, mating, cell wall integrity, osmoregulation, cell wall construction a sporulation pathway. Some of the components are shared between different pathways

There are a high conservation of MAPK pathways between humans and yeast suggest the fact that individual kinases in the yeast pathway can be replaced by the corresponding human enzymes(de Nadal et al 2002).

The HOG Pathway

The HOG pathway is the best-understood osmoresponsive system in eukaryotes and one of the best understood MAP kinase pathways. As it happens with other MAPK pathways, the central core of the yeast HOG pathway comprises a layer of three MAKKKs (Ssk2, Ssk22 and Ste11) which are responsible for the activation of the unique MAPKK of the pathway, Pbs2. Afterwards, Pbs2 activates by phosphorylation the Hog1 MAPK (Brewster et al 1993) (Figure 3). Hog1 is a protein homologue of the p38

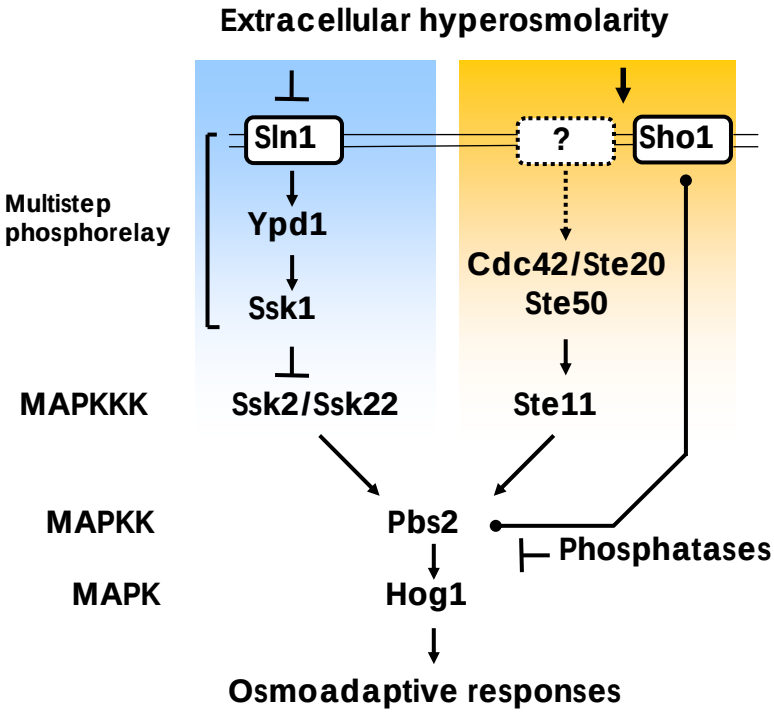

Figure 3. The HOG Pathway. The HOG pathway is a MAPK kinase pathway. It is constituted by a central core of MAPK kinases and two osmosensing branches Sln1 and Sho1. The signal converges at the MAPKK Pbs2. Once Hog1 has been activated different osmoadaptives responses are unleashed.

and c-Jun N-terminal kinase (JNK) families of stress-activated protein kinases (SAPKs). Hog1 responds to increased extracellular osmolarity and is required for cell survival under these conditions. Hog1 activation results in the iniciation of the osmostress response mechanism and adaptation to these new conditions. These mechanisms

involve cell cycle adaptation, the metabolic regulation, and gene expression regulation.

Two mechanisms activate the HOG pathway through the activation of either the Ssk2 and Ssk22 or the Ste11 MAPKKKs. These mechanisms are described as a branch, being the *Sln1 branch* and the *Sho1 branch* (Maeda et al 1995).
Different data suggest that the Sho1 and Sln1 osmosensing branches function independently and upstream of the MAPK. Exactly it is not clear the molecular mechanism by which these membrane proteins can detect osmotic changes. Possibly the sensors can able to detect changes in the physicochemical properties of the solvent due to an alteration of the water concentration or water structure; alternatively, they may sense mechanical stimuli that may occur as a consequence of the changes in water activity ((Gustin et al 1998); (Wood 1999)). Nevertheless, it has been accepted that osmosensors operate at the cell surface level as integrate membrane proteins, although other possibilities can not be discarded

Sensors of the HOG pathway

The Sln1 branch involves a "two-component" osmosensor, composed of the Sln1-Ypd1-Ssk1 proteins that conduces the signal to the Ssk2/22 MAPKKKs. Sln1 is a transmembrane protein divided in four regions: an N-terminal portion with two transmembrane domains, a linker region, a histidine kinase domain and a receiver domain (Ota et al 1993). Histidine kinases and receiver domains form a two-component systems, which are the prototype sensing and signalling units of prokaryotes ((Saito 2001); (Stock et al 2000)). In eukaryotic organisms histidine kinase signalling systems are much less frequent. Sln1 is the unique sensor histidine kinase in the *S. cerevisiae*. Sln1 senses osmotic changes, but it is not known how can sense this changes. Whatever it is, the signal is transduced using a phopho-relay mechanism which includes the Ypd1 and Ssk1 proteins. Unexpectedly, Sln1 acts as a negative regulator of the HOG pathway, therefore deletion of *SLN1* leads to a lethal phenotype because the pathway is activated constitutively (Maeda et al 1994). For that reason, the Sln1 histidine kinase is inhibited by hyperosmolarity (cell shrinking). In low osmolarity conditions, Sln1 autophosphorylates itself, then, this same phosphate is transferred to a specific His on Ypd1 in a receiver domain. Finally, this phosphate is

transferred to Ssk1 protein, on specific Asp, what inactivates Ssk1 ((Janiak-Spens, et al 2000);(Janiak-Spens et al 1999); (Janiak-Spens and West 2000); (Posas et al 2000)).

Upon an hyperosmotic shock, the Sln1 histidine kinase activity decreases leading to Ssk1 dephosphorylation and its activation ((Maeda et al 1995); (Maeda et al 1994); (Posas et al 1997);(Posas et al 1998);(Posas et al 2000)). Once dephosphorylated, Ssk1 binds to the N-terminal regulatory domain of Ssk2 leading to Ssk2 autophosphorylation and consequently its activation. Ssk2 directly phosphorylates and activates the MAPKK Pbs2 (Posas et al 1997).

There is a second mechanism for Pbs2 activation which involves several proteins; the transmembrane protein Sho1, the MAPKKK Ste11, the Ste11-binding protein Ste50, the Ste20 p21-activated kinase (PAK) and the small GTPase Cdc42 (Posas et al 1998). Pbs2 activation by Ste11 needs the interaction of Pbs2 with Sho1 ((Reiser, et al 1999) ; (Raitt et al 2000)).

Sho1 is composed by four transmembrane domains within the N-terminal half, a linker domain, and a SH3 domain for protein-protein interaction ((Posas et al 1997); (Raitt et al 2000)). Functional homologs of Sho1 have not been reported from higher eukaryotes. Sho1 is not an osmosensor by itself (Raitt et al 2000). Nevertheless, the Sho1 branch surely controls HOG pathway activation upon an osmotic shock, for that reason an unidentified sensor should exist.

Localization of Sho1 is on the cell surface, at places where growth and cell expansion occur ((Raitt et al 2000)); (Reiser et al 1999). It has been hypothesised that Sho1 could act as an anchor that directs signal transduction complexes to these areas. Anchoring of Sho1 to the cell surface is indispensable for signalling, but not is necessary in its specific localization. A possible role to explain existence of two branches could be that the Sho1 branch senses osmotic changes during cell growth, whereas the Sln1 branch monitors osmotic changes in the environment.

Sho1 branch activation includes a fast and transient formation of a protein complex at the cell cortex ((Raitt et al 2000); (Reiser et al 1999)). This complex consists of at least in Sho1 and Pbs2. These proteins interact via a SH3 domain located in the hydrophilic C terminus of Sho1 and a proline-rich region of the N terminus of Pbs2 ((Maeda et al 1995); (Posas et al 1997)). The intensity with which the SH3 domain interacts the Pbs2 proline-rich region correlates with the capacity to activate the

pathway and consequently the ability to survive at high osmolarity medium (Seet et al 2004). As well, the complex perhaps also includes, but not necessarily at the same moment, the PAK Ste20, the Rho-like G-protein Cdc42 and the MAPKKK Ste11 ((Raitt et al 2000); (Reiser et al 1999)) ((O'Rourke et al 1998); (Posas et al 1997)). In addition, Ste50, which is essential for Ste11 function (Posas et al 1998)). Recently, it has described a region in Sho1 C terminus that binds Ste11 independently of Pbs2. Thus, Sho1 has two independent contact regions: one that binds directly Ste11 and mediates its activation, and another region that binds Pbs2, directing Ste11 to proceed on Pbs2 (Zarrinpar et al 2004). Sho1 then binds Pbs2 recruiting it to the cell surface. As well, there is recruitment of Cdc42 plus the interacting Ste20 PAK kinase(s) and Ste11 MAPKKK. The assembly of the complex leads to activation of the PAK Ste20, phosphorylation of Ste11, and consequently the Pbs2 phosphorylation.

It has been expected that different sensitivities of the two branches of the HOG pathway may allow the cell to act in response a wide variety of osmolarity alterations (Maeda et al 1995). In a *ssk2*Δ *ssk22*Δ cells, which the viability in high osmolarity medium completely depends on the branch of Sho1, stimulation of Hog1 tyrosine phosphorylation needs at least 300 mM NaCl, is detectable after about 2 min, and achieves a maximum level at 5 min. In contrast, in *sho1*Δ cells, which are completely dependent on the Sln1 branch, Hog1 phosphorylation is already noticeable with 100 mM NaCl and reaches its maximal after 1 min with 300 mM NaCl. Therefore, these data imply that Sln1 osmosensor is more sensitive than the Sho1 branch sensor. It also appears that the Sho1 branch works in a switch fashion, whereas the branch of Sln1 shows approximately a linear dose response up to about 600 mM NaCl.

Higher eukaryotic cells do not have similar proteins to Sln1 which can act as a stress sensor. Therefore, determination of the sensor mechanism coupled to Sho1 branch could facilitate to discern the identity of the mammalian osmosensor.
A third osmosensing system working in parallel with the Sho1 branch was proposed. This alternative system would consist in the osmosensor membrane protein Mbs2 (O'Rourke et al 1998). This work is supported in the hypothesis that there is some residual signalling, which is *STE11* dependent; in the absence of Sho1 this fact still occurring, and that this signalling needs the action of Mbs2. Even though an *ssk1*Δ

*sho1*Δ *msb2*Δ strain is more osmosensible than an *ssk1*Δ *sho1*Δ, the biological significance of Mbs2 is not obvious.

Signaling Through the HOG Pathway

Ssk2, Ssk22 and Ste11 MAPKKKs phosphorylate and activate Pbs2 on Ser514 and Thr518. Then, Pbs2 is able to phosphorylate and activate Hog1 MAP kinase, which occurs in the cytosol. There is a double phosphorylation on the preserved Thr174 and Tyr176 allowing the activation of the Hog1 MAP kinase ((Brewster et al 1993); (Schuller et al 1994)). The timing and the period of the response depends on the rigorousness of the osmotic shock. When the shock is not strong, in the range of 0.4 M NaCl, phosphorylation of Hog1 reaches the maximum level within 1 min and almost disappears completely within about 30 min. In severe osmotic shock, such as 1.4 M NaCl, phosphorylation of Hog1 reaches the maximum level at about 30 min and is maintained high for several hours before it decreases ((Vandenbol et al 1989).

Hog1 phosphorylation promotes a fast and pronounced concentration of Hog1 in the nucleus, while in basal conditions distribution of Hog1 is between the nucleus and the cytosol ((Ferrigno et al 1998); (Reiser et al 1999)). After a hyperosmotic shock, nuclear accumulation of Hog1-GFP can be observed within less than 1 min. This fact is specific by osmotic shock , because a variety of other stresses do not cause Hog1 activation and thus do not mediate its nuclear accumulation ((Ferrigno et al 1998); (Reiser, et al 1999); (Schuller et al 1994)). When Thr174 and Tyr176 of Hog1 are phosphorylated by Pbs2, it is sufficient for nuclear concentration, because mutation of any of these sites avoids Hog1 concentration and therefore it is unable to respond to an osmotic shock ((Ferrigno et al 1998); (Reiser, et al 1999)). Hog1 phosphorylation is a key step for the transport into the nucleus, but this transport is not dependent on its protein kinase activity (Ferrigno et al 1998). On the other hand, the return to the cytosol is dependent on its kinase activity and matches with its dephosphorylation (Reiser et al 1999)).

Hog1 accumulation in the nucleus needs the presence of Gsp1, a RAN G-protein required for nuclear import of proteins containing nuclear localization signals, NLS (Ferrigno et al 1998); (Oki et al 1998). Hog1 import needs the karyopherin-beta Nmd5 too, but other nuclear import factors do not appear to be necessary for this import (Ferrigno et al 1998).

There are quite a lot of proteins that influence the time of residence of Hog1 in the nucleus, such as the phosphatases. Ptp2 functions as nuclear anchor for Hog1, whereas Ptp3 does the same role in the cytosol (Mattison et al 2000). For that reason, in a *ptp2*Δ cells Hog1 nuclear accumulation is reduced, correspondly overexpression of Ptp2 extends the period of nuclear residence, while an opposite effect was observed for *PTP3* (Mattison et al 1999).

Transcription factors are implicated in the MAPK nuclear retention too. Deletion of the genes encoding the transcription factors Msn2, Msn4, Msn1 and Hot1 reduce the time of Hog1 nuclear localization ((Reiser, et al 1999)); (Rep et al 1999)). Significantly, the more rigorous the osmotic shock, the longer it takes until Hog1 is phosphorylated. Once Hog1 is active, it is transferred into the nucleus, an observation that does not correlate with the apparent requisite to answer more quickly to hard stress (Van Wuytswinkel et al 2000). Even though, the transcriptional response dependent on Hog1 is delayed under these rigorous conditions((Rep et al 1999); (Van Wuytswinkel et al 2000). This fact proposes that some adaptation must happen before Hog1 was phosphorylated and translocated into the nucleus, and that translocation is specifically obstructed in an unknown manner until this process is concluded.

Hog1 phosphorylation and its nuclear localization are ephemeral effects. Depending on the osmotic shock hardness, Hog1 stays phosphorylated and localizated in the nucleus for quite a lot of minutes, even a few hours ((Brewster et al 1993); (Maeda et al 1994); (Mattisonet al 2000); (Tamas et al 2000) ; (Van Wuytswinkel et al 2000)). Hog1 phosphorylation and its nuclear accumulation has a good correlation ((Ferrigno et al 1998); (Mattisonet al 2000); (Reiser et al 1999)), which could signify a causal connection between dephosphorylation and nuclear export. Hog1 nuclear accumulation proposes that part of the activities of the MAPK take place in this organelle. Nevertheless, there is no doubt that Hog1 activated mediates regulatory effects outside the nucleus too. Thus, a portion of Hog1 activated remains in the cytoplasm.

Phosphatases Regulation of osmostress signalling:

Hog1 phosphorylation is a transient event. Hence, the HOG-pathway is regulated by specific feedback mechanisms that control its activity. One of these feedback mechanisms involves protein phosphatases. ((Jacoby et al 1997); (Maeda et al 1994); (Tamas et al 2000)). Two phosphotyrosine phosphatases (Ptp2 and Ptp3) and three phosphoserine / threonine phosphatases (Ptc1 to Ptc3) genetically interact with the HOG pathway. Overexpression of any of these phosphatases suppresses the lethality originated by unsuitable activation of the HOG pathway ((Jacoby et al 1997); (Maeda et al 1994); (Mattison et al 2000); (Ota et al 1992) ; (Warmka et al 2001); (Wurgler-Murphy et al 1997)). In the case of Ptp2, Ptp3, and Ptc1, there is direct proof that they affect the HOG pathway and act upon Hog1.

Overexpression of the phosphatases *PTP2* and *PTP3* suppresses unsuitable activation of the HOG pathway by deletion of *SLN1* or constitutive activation of Sln1, Ssk2, or Pbs2, ((Jacoby et al 1997); (Wurgler-Murphy et al 1997)). Overexpression of *PTP2* and *PTP3* reduces the amount of Hog1 tyrosine-phosphorylated. Ptp2 and Ptp3 directly interact with the MAP kinase ((Jacoby et al 1997); (Wurgler-Murphy et al 1997)). In a *ptp2*Δ and *ptp2* Δ *ptp3* Δ cells, Hog1 tyrosine phosphorylation upon osmotic shock is higher and more prolonged than in wild type cells. Furthermore, in the double mutant *ptp2* Δ *ptp3* Δ, this phosphorylation is observed without osmotic stress ((Jacoby et al 1997); (Wurgler-Murphy et al 1997)). Surprisingly, in *ptp2* Δ *ptp3* Δ cells the level of Hog1 tyrosine-phosphorylation is still able to respond to an osmotic shock, therefore other mechanisms of dephosphorylation must exist ((Jacoby et al 1997); (Wurgler-Murphy et al 1997)). The phosphatases might be part of a feedback loop because the expression of *PTP2* and *PTP3* is stimulated after osmotic shock, and this expression is in a HOG-dependent manner ((Jacoby et al 1997); (Wurgler-Murphy et al 1997)). On the other hand, this transcriptional effect can not account for the quick decrease of the Hog1phosphorylation state, because the enhance in phosphatase activity due, takes place after the level of phosphorylation of Hog1 has begun to reduce (Wurgler-Murphy et al 1997). As an alternative, there are some observations that propose the phosphatases might not act upon as specific regulators. The feedback loop, which is based in the expression of *PTP2* and *PTP3*, would denote that the pathway inactivates itself in an autoregulatory mechanism. Nevertheless, a negative feedback loop would do not take into account the success of the

response. Alternatively, Hog1 dephosphorylation involves its catalytic activity (Wurgler-Murphy et al 1997). This suggests that Hog1 activates the phosphatases to stimulate its own deactivation. More studies have to be done to shed some light on how the phosphates regulate the cascade.

Of the serine/threonine phosphatases, Ptc1 appears to be the only implicated in the deactivation of the HOG pathway. In *ptc1*Δ *ptp2*Δ cells, the inappropriate HOG pathway overactivation causes a cell growth defect. No other combination of deletion mutations between the phosphatases causes comparable effects (Warmka et al 2001). Deletion of *PTC1* provokes constant dual phosphorylation of Hog1 which is hardly responsive to osmotic shock (Warmka et al 2001). Possibly Ptp2 preferentially dephosphorylates Hog1 that already is dephosphorylated on Thr174.

Physiological Roles of Hog1

It is quite clear that the phosphorylation of Hog1 leads to its activation and rapid accumulation into the nucleus. Nuclear accumulations of Hog1 suggest that part of the actions that are regulated by the MAPK happen in this organelle (Figure 4). Gene expression control is one of these activities. In *S. cerevisiae*, genome transcription studies have revealed that a great number of genes (approximately 7%) show variations in their expression levels after a mild osmotic stress and that the Hog1 has a vital responsibility in much of this large-scale gene regulation. These genes are basically involved in a broad-spectrum stress protection, in signal transduction, in protein production or in carbohydrate metabolism (Figure 4).This large-scale modifications in transcription could explain for the metabolic adjustments which are necessaries for adaptation to an osmostress ((Hohmann 2002); (de Nadal, et al 2002)) (see below). Hog1 is involved in metabolic and in translation adaptation too. For example, the activation of the HOG pathway activates PFK2 (Dihazi et al 2004). Wild type cells accumulate three times more glycerol than *pfk2*Δ cells, and consequently, these cells are osmosensitives (Dihazi et al 2004).

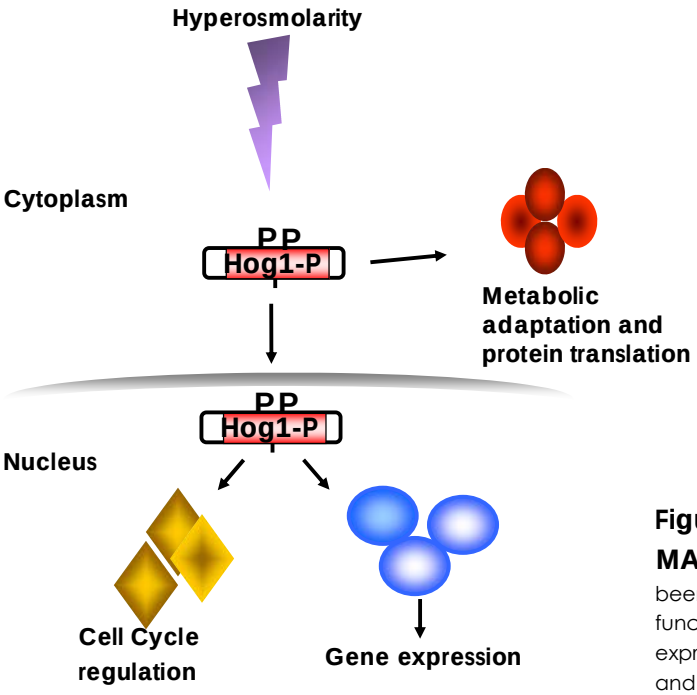

Figure 4. Functions of the MAPK Hog1. Once Hog1 has been activated, it develops different functions regulating cell cycle, gene expression metabolic adaptation and protein translation.

Hog1 is also implicated in regulation of translation. One example is the regulation of the kinase Rck2. Rck2 is a yeast Ser-Thr protein kinase homologous to the mammalian calmodulin kinases. In fact, Hog1-mediated Rck2 phosphorylation transiently increases during osmostress (Bilsland-Marchesan et al. 2000), which leads to the control of the translation elongation factor 2 (Teige et al. 2001).

Regulation of Gene Expression by the Hog1 MAPK

As mentioned previously, changes in environmental osmolarity provoke changes in the expression of a great number of genes. The use of DNA microarrays helped to understand how the yeast response to osmotic shock. There has been more than six independent studies in which yeast cells were exposed to osmotic stress and the global transcriptional response was analyzed ((Causton et al 2001); (Gasch et al 2000); (Posas et al 2000); (Rep et al 2000); (Yale et al 2001).

Transcription of genes that varies upon an osmotic shock have been proposed in diverse categories, such as; by type of response, or by biological function, or time of response (Posas et al 2000). Based on the onset of transcription and its biological function it has been described a possible list of extremely induced genes after exposure for 10 min to 0.4 M NaCl (Posas et al 2000): genes encoding proteins which are necessary for signal transduction, or genes encoding for components of the protein biosynthesis machinery, or by proteins involved in carbohydrate metabolism, or by proteins related to ion homeostasis, … An important quantity of the induced genes is regulated by other stress conditions too. This reflects a broad stress response controlled by Msn2 and Msn4 transcription factors. Several hypotheses can explain the large overlap between all these stress responses. One of them could be that in nature, diverse stress circumstances take place simultaneously, and as a result any sort of stress stimulates a broad response; and the specific stress disturbs cellular functions leading to another type of stress. Such as, osmotic shock may block electron transport leading to the production of reactive oxygen species. Thus, osmotic stress would indirectly induce an oxidative stress inducing genes implicated in oxidative protection. Genes encoding for proteins which are necessary for oxidative protection and for the heat shock response are induced very quickly upon osmotic stress.

Induction of a number of genes after osmotic shock is mediated, at least in part, by the Hog1 signal transduction pathway (Posas et al 2000). The analysis of the contribution of the HOG pathway has been done comparing the level of induction of responsive genes in wild type cells and in a *hog1*Δ strain. Analysis of these data indicates that, in many cases, the response in *hog1*Δ cells was different from that observed in wild type cells. However, from a quantitative point of view, the changes ranged from a weak effect to a virtual loss of the response. This indicates that the

Hog1 signal transduction pathway is certainly involved in the transcriptional response of most salt-responsive genes. The level of dependence on Hog1 differs upon the stress conditions. On the other hand, the group of HOG-dependent genes contains many of the most strongly induced genes and most highly expressed osmoregulated genes.

Hog1 regulates the expression of different genes through at least five transcription factors (Figure 4): the zinc finger proteins Msn2 and Msn4 (Schuller et al 1994); Hot1 (Rep et al 2000); the MADS box protein Smp1 (de Nadal et al 2003), and the bZIP protein Sko1 (Proft et al 2001).

As well to the usual MAPK role in regulating transcription factor activities by phosphorylation, it has been described that Hog1 can associate with the chromatin at promoter regions of target genes modifying their expression. Hog1 is recruited to the specific promoters through interaction with specific transcription factors (Alepuz et al 2001). In addition, the latest results support a model in which promoter-localized Hog1 stimulates transcription by direct targeting of specific components of the RNA pol II to osmoresponsive promoters (Alepuz et al 2001). Furthermore, Hog1 has been reported to bind to Sko1-dependent promoters through its direct interaction with Sko1, and then these proteins are necessary for the recruitment of the SAGA histone acetylase and SWI/SNF nucleosome-remodeling complexes in response to osmotic shock (Proft et al 2002).

Control of cell cycle by the Hog1 MAPK

Cell cycle in yeast

In budding yeast, cell cycle is divided basically in four phases, G1, S, G2 and M (Figure 5). At Start, the Restriction point in mammals, cells decide if begin a new cycle, conjugate with another cell, or sporulate. The transitions between G1/S and between G2/M are strongly regulated. The events of the cell cycle need to be properly regulated to obtain a successful division of a eukaryotic cell. When one of these events are aborted, cells use diverse mechanisms, known as checkpoints, that monitor proper completion of each stage of the cell cycle and can delay cell cycle progression, until the execution of an unfinished step, allowing the cells begin a new cycle.

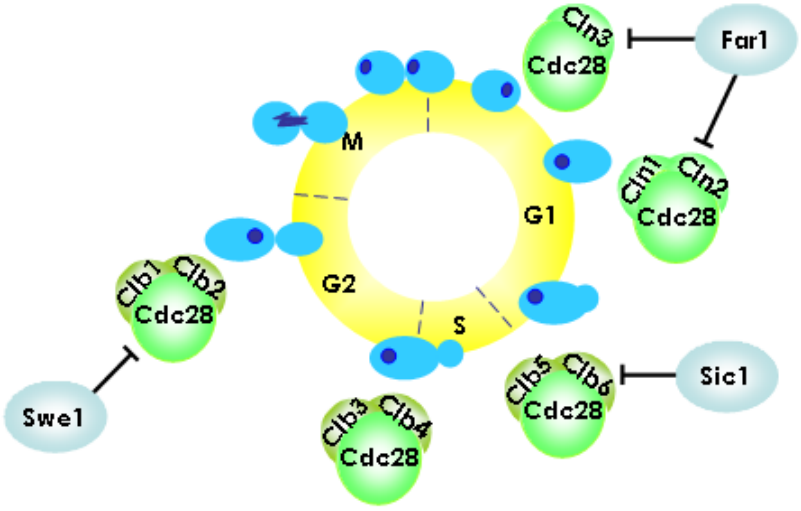

Figure 5. Schematyc diagram of *S.cerevisiae* cell cycle. Cdc28 associated to different cyclins at different phases. It was illustrated the bud and position of nuclei along cell cycle.

In *S. cerevisiae*, checkpoints block mitosis in response to defects in DNA replication (Longhese et al 1998); (Rhind et al 1998) in response to defects in spindle assembly or dynamics (Amon et al 1999); (Daum et al 2000) or formation of the bud (Madden et al 1998); (Chant et al 1999). Bud formation and septation require the actin cytoskeleton; these processes also rely on assembly of the septin filaments

(Trimble et al 1999). Defects or perturbations in either actin or septin assembly cause a G2 delay (McMillan et al 1998); (Barral et al 1999), (Shulewitz et al 1999); (Lew et al 2000).

Cell cycle control by Cyclin Dependent Kinases (CDK).

In *S. cerevisiae*, Cdc28 is the main coordinator of the yeast cell division cycle. Cdc28 is the unique CDK (Cyclin Dependent Kinase) in yeast that controls cell cycle progression. Environmental effects that influence the decision to undergo cell division affect Cdc28 kinase activity. *S. cerevisiae* Cdc28 is the equivalent to Cdk1 in mouse or human systems. CDKs are able to phosphorylate serine or threonine in S/T-P motifs (Langan et al 1989), (Shenoy et al 1989), but CDK-cyclin complexes have more stringent substrate specificities (Holmes et al 1996), (Kitagawa et al 1996), (Songyang et al 1994), (Songyang et al 1996).

Cdc28 have little or no protein kinase activity alone by itself, because the residues involved in the phosphate transfer are misaligned and the protein substrate binding site is obstructed (de Bondt et al 1993). Moreover, to obtain a full activation of CDKs generally requires two events; cyclin binding and stimulatory phosphorylation. There are many regulators of CDK activity which are under different types of control (transcriptional, translational, and proteolytic). A conserved feature is that some of the CDKs regulators are subjected to the proteolytic control generally mediated by an ubiquitin dependent mechanism (Hershko 1997).
Cdc28 itself seems to play a mostly passive role; hence, the regulators are able to activate and inhibit Cdc28. These regulators are produced by transcriptional waves and removed by specific proteolytic systems timed to match key cell cycle events (Figure 6).

If we consider the onset of the cell cycle, the transition between M/G1, it is necessary that Cln3-Cdc28 activates SBF and MBF. Once these transcription factors are activated, SBF and MBF are able to transcribe Cln1, Cln2, Clb5, and Clb6, which then form complexes with Cdc28. The activity of the Clb5-Cdc28 and Clb6-Cdc28 complexes is inhibited in G1 by Sic1, whereas the activity of Cln1-Cdc28 and Cln2-Cdc28 produces some events, such as to induce budding, SPB duplication, inhibition of APC activity and SCF-Cdc4 dependent destruction of Sic1. Destruction of Sic1, an inhibitor of Cdc28 associated to phase S Clbs, releases and activates Clb5-Cdc28 and Clb6-Cdc28, allowing to initiate S phase and SPB separation. Then, Clb3 and Clb4 are synthesized, which allows the spindle maturation and induce the synthesis of Clb1 and Clb2 and Cdc20 and Cdc5. Cdc28-Clb2 initiates the early events of mitosis and inhibition of SBF activity. Cdc20 stimulates the APC-mediated breakdown of Pds1, allowing sister chromatides separation, and Cdc5, counteracts the Cdc28-mediated inhibition of Cdh1-activated APC activity. Destruction of Clb1, Clb2, Cdc20, and Cdc5 follows immediately, leading to telophase, transcription of Sic1, Cln3 and Swi4 transcription. This returns the cell to the early-G1 state. Environmental influences and checkpoint regulators act at several positions to slow or stop all these processes.

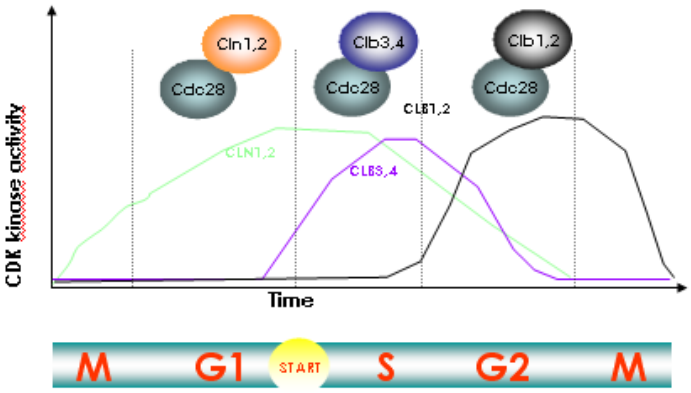

Figure 6. Scheme of cyclin transcripts along cell cycle and their correlation with CDK activity. During yeast cell cycle there are consecutives waves of cyclin expression that correlates with CDK associated activity.

Cdc28 regulators

Cdc28 activation: Phosphorylation of Cdc28 and binding to cyclins.

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). Full activation of most CDK requires phosphorylation at the position corresponding to T169 of Cdc28 (Desai et al 1992), (Ducommun et al 1991), (Gould et al 1991), (Solomon et al 1992). Cdc28 activation by Cak1 is essential for viability. The B-type cyclins require phosphorylated T169 to be fully active whereas it appears that is not necessary with G1 cyclins. Cak1 activity appears to be constitutive throughout the cell cycle (Espinoza et al 1996), (Sutton et al 1997).
The cyclins confer a limited range of functions on Cdc28. Cdc28 cyclins have been classified into two broad groups:

-the three G1 cyclins (Cln1, Cln2 and Cln3)

-the B-type cyclins (Clb1- Clb2, Clb3-Clb4 and finally Clb5-Clb6).

The G1 cyclins primarily regulate events during the cell cycle interval between mitosis and DNA replication. The yeast B-type cyclins receive their name from homology to the B cyclins of metazoans (Swenson et al 1989) and are expressed in three successive waves from Start to M, controlling the events between the DNA replication until the cytokinesis.

G1 Cyclins

Cln1 and Cln2 are 57% identical, cyclin A has been considered as a Cln2 homologue based in their similar structures. Cln1 and Cln2 directly bind to Cdc28 and activate its protein kinase activity (Tyers et al 1993). Cln1 and Cln2 and their associated protein kinase activities are maximal at Start (Wittenberg et al 1990). Cln-Cdc28 complexes stimulate DNA synthesis indirectly by accelerating the proteolysis of the Clb-Cdc28 inhibitor Sic1.

Cyclins levels are regulated in two separated ways; transcription and destruction. The transcription regulation of Cln1 and Cln2 is basically controlled by SBF transcription factor. The contribution of MBF transcription factor is probably negligible when SBF is functional. *CLN1* and *CLN2* transcription are essentially dependent upon SBF with a minor contribution by the MCBs. On the other hand, *CLN1* mRNA levels are dramatically reduced in a *swi4*Δ background (a specific component of SBF) (Nasmyth et al 1991), (Ogas et al 1991).

CLN3 transcription is not strongly periodic with respect to the others cyclins, but there is a small rise at the M/G1 border over its basal levels (Cross et al 1993). Cln3 is less abundant than the others G1 cyclins, and the specific activity of the protein kinase activity is lower than the values for Cln1 or Cln2 (Levine et al 1996), (Tyers et al 1993). It seems that Cln3-Cdc28 plays a unique role in G1 as an activator of *CLN1* and *CLN2* transcription through SBF.

B-Type Cyclins

The six B-type cyclins are commonly subdivided into three pairs based on sequence homology and transcriptional regulation (Clb1 and Clb2, Clb3 and Clb4 and Clb5 and Clb6). The functions of the members of this family are complex and partially overlapping.

Clb5 and Clb6 are 50% identical. *CLB5* and *CLB6* genes are coexpressed with *CLN1* and *CLN2* at M/G1 transition (Epstein et al 1992). At G1, Clb5 and Clb6 do not stimulate Start functions, because they are in inactive state by Sic1 until Sic1 destruction (Schwob et al 1994). Essentially, the primary roles for Clb5 and Clb6 are to initiate S phase in a timely fashion (Schwob et al 1994), preventing reinitiation (Dahmann et al 1995), and regulate negatively Cln-Cdc28 activity (Basco et al 1995). Moreover, Clb5 and Clb6 have an important a role in spindle formation (Schwob et al 1993), but Clb5 and Clb6 are not sufficient to form the bipolar spindles needed for mitosis, because are necessary the activity of the others B-type cyclins (Amon et al 1993).

CLB3 and *CLB4* are 62% identical to each other. The transcripts of *CLB3* and *CLB4* arise near the beginning of S phase (after the *CLN1* and *CLN2* peak) and remain with these high levels until late anaphase (Fitch et al 1992), correlating with their protein kinase associated Cdc28 activity. In asynchronous log phase cultures, the kinase activity associated to Clb3-Cdc28 constitutes the majority (67%) of all Cdc28 activity. Clb3 and Clb4 appear to play an essential role in spindle formation which can be accomplished by Clb1 and Clb2 (Richardson et al 1992).

CLB1 and *CLB2* are 62% identical to each other. Transcripts of *CLB1* and *CLB2* are strongly periodic, peaking about 10 min before anaphase (Ghiara et al 1991), (Surana et al 1991), and their protein kinase activity associated to Cdc28 has a similar periodicity (Grandin et al 1993). Clb2-Cdc28 constitutes the majority activity (85%) in arrested cells at mitosis. Clb2-Cdc28 constitutes the yeast MPF homologue (Fitch et al 1992.). Clb2-Cdc28 is important for spindle elongation (Lew et al 1993). Another significant function of Clb2 is that negatively regulates SBF-promoted transcription, avoiding the expression of G1 cyclins until the appropriate moment, and bud emergence (Booher et al 1993) and promotes the switch from tip-directed growth to isotropic growth in buds (Lew et al 1993).

Cdc28 INHIBITORS: CKIs and inhibitory phosphorylation

The mammalian CKIs (CDK inhibitors) have received extensive attention due to their roles as tumor suppressors and developmental regulators. These CKIs are divided into two groups. The first group is the INK4 class, which are characterized by the presence of multiple "ankyrin repeats" (Bork 1993). The INK4 class are able to inhibit CDKs free or in complex with a cyclin, that are primarily responsible for promoting passage through G1 (Serrano et al 1993). The second class of mammalian CKIs are general CDK inhibitors and recognize both CDK and cyclin components, such as p21 Cip1/Waf1. In yeast, Cdc28 phosphorylation at T18 and Y19 inhibits the activity CDKs (Krek et al 1991), (Norbury et al 1989). Inhibitory phosphorylations on Cdc28 are associated with checkpoints, in order to prevent a premature entry into M phase due to a DNA synthesis incomplete (Enoch et al

1990), or a DNA damage (Jin et al 1996), (Kharbanda et al 1994), (Ye et al 1997), or some others cell requirements such as cell size (Edgar et al 1989).

In *S.cerevisiae*, Far1 is a specific inhibitor of Cln-Cdc28 complexes, inhibiting its activity by substrate exclusion (Peter et al 1994). Far1 is regulated at multiple levels. Its transcription is cell cycle regulated, with a peak near the M/G1 transition (McKinney et al 1993), suggesting that Far1 may have a cell cycle function independent of its role in mating. Moreover, Far1 is not expressed in diploids (Chang et al 1990). Exposition of yeast cells to mating pheromone induces additional Far1 transcription and this induction is necessary but not sufficient for pheromone-induced cell cycle progression arrest (Chang et al 1992.).
Far1 protein is stable in G1 and is degraded rapidly following Start, in a ubiquitin dependent manner (McKinney et al 1993). Activation of the pheromone response pathway stimulates increased association of Far1 with all three Cln-Cdc28 complexes (Peter et al 1993), (Tyers et al 1993).

When yeast cells are exposured to mating pheromone forces an arrest at Start. Arrest at Start is achieved by shutting down Cln-Cdc28 activity. This is accomplished by repressing the transcription of Start-specific genes and inducing the synthesis and activation of Far1. These effects are mediated through the pheromone response pathway, a kinase cascade with two MAPK, Fus3 and Kss1. Fus3 and Kss1 are both capable of activating the induction of pheromone-responsive mRNAs, (Bardwell et al 1994), (Cook et al 1996), (Elion et al 1993), (Tedford et al 1997). Moreover, inhibition of Cln-Cdc28 activity is Far1-dependent, and from the Fus3 phosphorylation-dependent of Far1.

Sic1

Sic1 is an inhibitor of Cdc28-Clb complexes (Mendenhall 1993), (Schwob et al 1994) in contrast with Far1 that inhibits Cdc28-Cln complexes. Inhibitory activity associated to Sic1 is due to its ability to exclude substrates from the Cdc28 active site, and its inhibitory domain is in the C-terminal half of Sic1 (Verma et al 1997). This domain has a considerable similarity to the inhibitory domain of Rum1, an *S. pombe* CKI that has many functional and regulatory homologies with Sic1 (Sanchez-Diaz et al 1998). Expression of Sic1 protein is limited to the G1 phase

(Donovan et al 1994), (Mendenhall et al 1987). This pattern of expression is due its production and its degradation. Sic1 shows a periodic transcription that peaks at the M/G1 phase transition, whereas Sic1 protein is degraded via proteolysis, a step where is necessary the activity of Cln-Cdc28 at Start. The N-terminal domain of Sic1 is sufficient for its own ubiquitination and therefore it regulates Sic1 stability (Verma et al 1997). *SIC1* is a nonessential gene (Donovan et al 1994) but there are a high percentage of cells permanently arrested in G2 in *sic*1Δ cultures (Nugroho et al 1994). The main function of Sic1 is to prevent a premature S-phase initiation until after the formation of the bud initiation and spindle pole body duplication has been finished (Schwob et al 1994). This function is carried out by inhibiting Clb5-Cdc28 and Clb6-Cdc28 complexes until Sic1 is destroyed, which is, in turn, initiated by Cln-Cdc28- dependent phosphorylation of Sic1. Other function of Sic1 is to assist in the down-regulation of Clb-Cdc28 activity in late anaphase to telophase (Toyn et al 1996).

Inhibitory Phosphorylation on Y19: Swe1 and Mih1.

Cdc28Y19 is phosphorylated, reaching maximal levels of phosphorylation between S and G2 and becoming practically undetectable in M and G1 (Amon et al 1992), (Sorger et al 1992). The tyrosine phosphorylation of Cdc28 inhibits its activity, leading to a delay entry into mitosis (Lim et al 1996). Phosphorylation on this tyrosine increases when cells are exposed to a DNA damage. Y19 phosphorylation is an essential part of a different checkpoint system used to delay nuclear division when bud formation is prevented or delayed (Lew et al 1995). Swe1 is a kinase that directly phosphorylates this Y19, and it is homologous to *S. pombe* and higher eukaryotes is *wee1* (Booher et al 1993), (Russell et al 1987). The action of Swe1 is cyclin specific, because Swe1 is able to phosphorytable Cdc28-Clb2 but not Cdc28-Cln (Booher et al 1993). Overexpression of *SWE1* causes a premitotic cell cycle arrest avoiding entry to M phase, which can be suppressed by the *CDC28-Y19F*, a non phosphorylable allel (Lim et al 1996). The arrested cells have either a short mitotic spindle (Booher et al 1993) or duplicated but

unseparated spindle pole bodies (Lim et al 1996), and duplicated DNA, indicating that nor Cln-Cdc28 neither Clb5/6-Cdc28 complexes are inhibited (Figure 7).

Generally, Wee1-related kinases function in a highly conserved mechanism that controls the timing of entry into mitosis. Loss of Wee1 function causes fission yeast and budding yeast cells to enter mitosis before sufficient growth has occurred, leading to formation of daughter cells that are smaller than normal. In *S.pombe* recent studies suggest that Wee1 forms part of a cell-size checkpoint that prevents entry into mitosis before cells have reached a critical size. Recent experiments in fission yeast and budding yeast have provided new support for this idea. In addition, some studies have exposed the existence of vastly intricate signaling networks that are required for regulation of Swe1 (Sreenivasan et al 2002).

The *SWE1* promoter possesses an SCB box and its transcription peaks at about the time of Start with kinetics similar to *CLN2* and *CLB5* (Ma et al 1996). Swe1 accumulates in S phase and becomes hyperphosphorylated in G2 which leads to ubiquitin-mediated degradation.

On the other hand, Swe1 action is opposed by Mih1 (Russell et al 1989), (Russell et al 1986). Deletion of *MIH1* has no clear effect. When polarized cell growth is inhibited, the subsequent mitotic delay is exacerbated in a *mih1*Δ cell (Sia et al 1996).

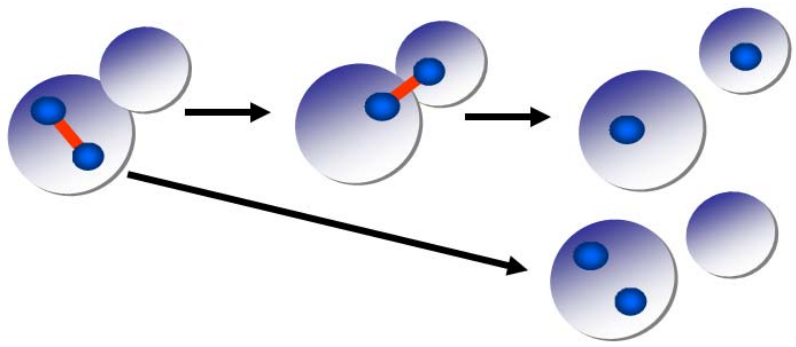

Figure 7 Spindle orientation in yeast. Spindles (red) and chromosomes (blue) are shown. In budding yeast the spindle orientation checkpoint causes cells that have not oriented their spindles along the mother-bud axis to block in anaphase until correct orientation is achieved. Checkpoint failure results in binucleate and anucleate cells. (Adapted from McCollum 2002).

Swe1 regulators: Hsl1, Hsl7 and Cdc5

The septin filaments of mitotic cells are composed of five gene products forming a double-ring structure at the neck between a mother cell and its bud. Swe1 is negatively regulated by Hsl1 and Hsl7 (Ma et al 1996). Hsl1 is a protein kinase, homologous to *S. pombe* Nim1 (Russell et al 1987), and Hsl7 contains a domain arginine methyltransferases, but it is unknown its role. Hsl7 interacts with both Hsl1 and Swe1 (Shulewitz et al 1999). Hsl7 and Hsl1 colocalize at the bud neck during most of the cell cycle and require septin function for this localization (Longtine et al 2000). Hsl1 associates directly with the septin filaments and acting as a tether to localize Hsl7 (Barral et al 1999). Septin ring built a mode of platform for Hsl1-Hsl7 complexes, which allow anchoring Swe1. This localization permits that the polo like kinase Cdc5 recognizes and phosphorylates Swe1 (Cid et al 2001) (Sakchaisri et al 2004), which leads to its further destruction. This event is a crucial step in the cell cycle progression because it allows entry into mitosis (Shulewitz et al 1999). Accumulation of Swe1 prevents separation of duplicated SPB, which is a necessary action to form a short premitotic spindle, (Lew et al 1993), (Lim et al 1996),(McMillan et al 1999), (McMillan et al 2002). Hsl7 localizes to the SPB during G1 before being redistributed to the bud neck. This movement suggests that Hsl7 participates in localized depletion of Swe1 in the surround of the SPB as well as in the Hsl1-Hsl7 -septin-dependent destruction of Swe1 that precedes the entry into mitosis.

Several environmental insults, including rapid changes in ambient temperature or osmolarity disrupt actin polarity causing delays in bud formation. Defects in actin organization or septin filament assembly at the bud neck result in hypophosphorylation and stabilization of Swe1 and consequently Clb-Cdc28 activity is inhibited (Booher et al 1993), (Edgington et al 1999).

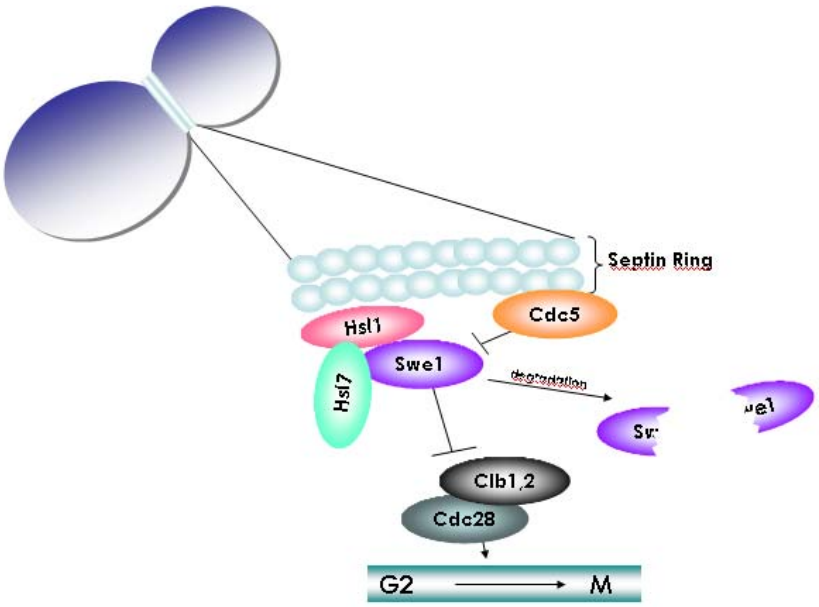

Figure 8. Model illustrating the Swe1 phosphorylation and degradation events during the cell cycle
(adapted from Sakchaisri et al 2004).

The morphogenesis checkpoint responds by delaying mitosis forbidden nuclear division before a bud has been constructed, preventing the formation of binucleate cells (Lew 2003), (Lee et al 2003), (Neef et al 2003), (Song et al 2001). Swe1 is stabilized and the resulting of this protein accumulation, leads to a G2 arrest (Song et al 2000) with elongated cells because the cells fail to switch from polarized to isotropic growth during budding (Geymonat et al 2002). Hsl1 and Hsl7 act in some manner to lower the level of Swe1 activity (Ma et al 1996).

During a checkpoint response, Hsl1 and Hsl7 do not target Swe1 for degradation, suggesting that the checkpoint mechanism may stabilize Swe1 by inhibiting Hsl1 or Hsl7 function. Hsl1 activity and localization depend on septin filament assembly at the neck (Edgington et al 1999), (Gladfelter et al 2001), (Gruneberg et al 2000). Hsl1 acts in concert with Hsl7 (Booher et al 1993), (Hu et al 2001) and both are required for efficient recruitment of Swe1 to the same location, (Hu et al 2001). The septin collar serves as an organizing platform to permit Cla4 and Cdc5 to sequentially phosphorylate Swe1 and that these modifications are critical for Swe1

degradation and therefore for proper onset of mitosis once Cdc28-Clb2 is active (Figure 8).

CELL CYCLE REGULATION BY PROTEOLYSIS

Many activators and inhibitors of Cdc28 activity are unstable proteins. Their have short half-lives. Mutations that stabilize these proteins usually have phenotypic consequences, which are toxic for the cell viability. For instance, Sic1 stabilization arrests the cell cycle between Start and avoiding the initiation of DNA synthesis (Schwob et al 1994), (Verma et al 1997). In Start, the ubiquitination machinery is constitutively active. The substrates must be activated for destruction by phosphorylation, which is usually dependent upon Cdc28 complexes. In contrast, at anaphase, the ubiquitination machinery is cell cycle regulated but the substrates appear to be active for degradation.

Ubiquitination of proteins is a conserved mechanism to target proteins for cellular proteolysis. Ubiquitin marks proteins for degradation when it is attached to a lysine residue. Once attached, the ubiquitin is used as a target for further ubiquitination, forming multiubiquitinated proteins, which are recognized by the 26S proteasome for degradation. The attachment of ubiquitin to the target protein requires a series of ubiquitin activators. First, ubiquitin is activated by an E1 enzyme (i.e, ubiquitin-activating *UBA1*). Then, ubiquitin is transferred to an E2 enzyme (ubiquitin-conjugating *CDC34*). From the E2 enzyme, the ubiquitin can be transferred to the target protein. The ubiquitination reactions involved in cycle regulation require an E3 or ubiquitin ligase, which specifically recognizes the substrate. One of these E3 is SCF, which have two common subunits, Skp1 and Cdc53, but differ in a third subunit, known as the F box protein (Bai et al 1996); (Figure 9). Different SCF complexes have different substrate specificities that are determined by the F-box component, such as Cdc4, Grr1 or Met30. Other E3 related in cell cycle regulation is APC.

Cln1, Cln2, Cln3, Cdc6, Far1, and Sic1 are unstable proteins. Their half-lives vary between 3 to 30 min and their instability is often Cdc28-dependent. The instability of all these proteins is generally accepted in an ubiquitin dependent manner.

SCF complex: Sic1 is ubiquitinated by Cdc4 and Cln1-Cln2 by Grr1.

SCF Cdc4 regulates the turnover rates for Sic1, Far1, and Cdc6. Mutations in SCF Cdc4 components generally arrest in G1 with a multibudded phenotype, which can be partially suppressed by deletion of *sic1* (Bai et al 1996), (Schwob et al 1994). Cdc28-Cln phosphorylates Sic1, and this step is a prerequisite for SCF Cdc4 recognition, because their interaction fails when Sic1 is not phosphorylated (Feldman et al 1997), (Skowyra et al 1997). Far1 ubiquitination appears to have a similar dependence upon Cdc28 phosphorylation. Correspondly, in mating-pheromone-arrested cells, conditions that severely reduce Cdc28-Cln activity, Sic1 is stabilizated (Mendenhall et al 1987), because Cdc4 is not able to recognize Sic1 non-phosphorylated.

SCF Grr1 mediates Cln2 ubiquitination. Cln1 and Cln2 must be phosphorylated to interact with SCF Grr1 (Skowyra et al 1997), (Willems et al 1996).

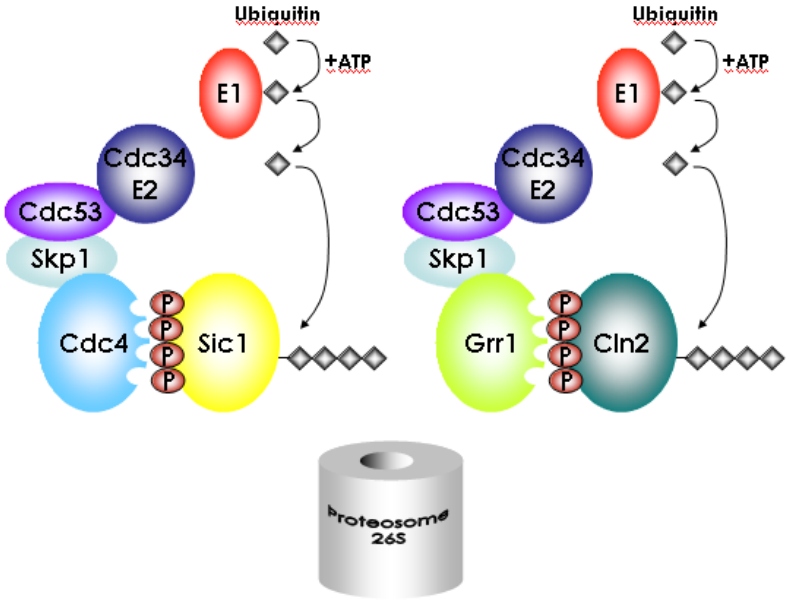

Figure 9. Schematic representation of budding yeast SCF. These ubiquitin ligases are called SCFs because each is formed by three major subunits: Skp1, Cdc53 and one of many F-box proteins. SCF Cdc4 binds phosphorylated Sic1through the F-box protein Cdc4 (which also targets Cdc6, Far1, and Gcn4 for degradation). SCF Grr1 binds phosphorylated Cln1 through the F-box protein Grr1 (which also targets Cln2 and Gic1/2 for degradation. (Adapted from DeSalle et al 2001).

Clb protein levels are periodic, with maximum accumulations occurring in the post-G1 phase of the cell cycle and declines in anaphase (Amon et al 1994), (Ghiara et al 1991), (Surana et al 1993). Destruction of the CDC28/CLB mitotic kinase is not required for the). The Clb2, Clb3, and Clb5 half-lives are 1 to 2 min during the G1 phase when its proteolysis is active. Throughout S and G2, Clb2 and Clb3 are stable (Amon et al 1994), (Irniger et al 1997) but Clb5 continues to turnover; however, it has a longer half-life, of 10 to 15 min (Amon et al 1994.). Clbs are proteolyzed via ubiquitinated intermediates. In vitro ubiquitination with crude cell lysates has been demonstrated for Clb2 and Clb3.

Transcription regulation of cell cycle components

Control of Cdc28 activity at Start is perhaps one of the most critical events in the life cycle of a yeast cell, since this determines whether the cell will begin a new round of mitotic division. Phase-specific transcription at this point in the cell cycle is a key event, involving Cdc28 regulators that include Cln1, Cln2 (Wittenberg et al 1990), Clb5 (Epstein et al 1992), (Kuhne et al 1993), (Schwob et al 1993), and Clb6 (Kuhne et al 1993), (Schwob et al 1993). Others cell cycle components such as Cdc6 (Bueno et al 1992), (Piatti et al 1995), (Zhou et al 1990.), Swi4 (Breeden et al 1991), and Swe1 (Ma et al 1996.) are strongly influenced by events at Start. Two related transcription factors, SBF and MBF, are responsible for most of the periodic, late-G1 -specific mRNA production. Transcription at Start is also affected by many environmental influences. The factors involved in Start-specific transcription and the controls over their activity are strongly regulated and there are environmental events that could alter this regulation.

SBF controls the expression of *CLN1* and *CLN2* (Nasmyth et al 1991), (Ogas et al 1991). It has been described a consensus promoter sequence reconigzed by SBF is known as the SCB (Swi4-Swi6-dependent cell cycle box), and the Swi4-Swi6 complex is known as the SBF (SCB binding factor). The DNA sequence specificity of SBF is somewhat plastic.

Osmostress and regulation of cell cycle in S.cerevisae

It has been described that exposure of yeast cells to an increase in external osmolarity induces a temporary growth arrest at G2, caused by a decrease in *CLB2* mRNA, phosphorylation of Cdc28, and inhibition of Clb2-Cdc28 kinase activity. The osmotic stress-induced G2 delay seemed dependent upon the kinase Swe1. Hog1 contributes to proper nuclear segregation after hypertonic stress in cells that lack Swe1, suggesting that hypertonic stress-induced cell cycle delay in G2 phase which is mediated in by Swe1 in cooperation with Hog1 (Alexander et al 2001).

Moreover, it was also reported that hyperosmotic stress on *Saccharomyces cerevisiae* cells produces a temporary delay at the G_1 stage of the cell cycle. This is accompanied by transitory downregulation of *CLN1, CLN2 and CLB5* transcript levels, although not of *CLN3*, which codes for the most upstream activator of the G_1/S transition. (Belli et al 2001).

In this PhD Thesis we aimed to characterize the molecular mechanisms which are regulated by Hog1 to control cell cycle progression upon stress.

Objectives

It was described that exposure of yeast cells to increases in extracellular osmolarity promotes a defect in cell growth. Our group was interested to study if this cell growth defect was associated to the regulation of the cell cycle progression by the Hog1 MAPK. Therefore, we decided to investigate how changes in osmolarity can affect the cell cycle machinery in the yeast *S. cerevisiae*.

Objectives:

- To characterize the phases of cell cycle those were under the control of Hog1.

In previous studies, it has been described a possible conection between stress and the regulation of G1 and G2. Therefore; our objectives were:

- Characterize the molecular and physiological significance of the role at G1 of Hog1.

- Determine the molecular mechanism and the physiological consequences of the Hog1 activation at the G2 phase.

Results

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Control of cell cycle progression bythe stress-activated Hog1 MAPK.
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Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1.
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Multiple checkpoint activation by the Hog1 MAPK is required for cell survival upon osmostress

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Activation of stress-activated protein kinases (SAPKs) is essential for proper cell adaptation to extracellular stimuli. Exposure of yeast cells to high osmolarity or mutations that lead to activation of the Hog1 SAPK results in arrest of cell cycle progression (Maeda et al 1994). Activated Hog1 leads to G_1 arrest by the direct targeting of the Sic1 CDK-inhibitor[5]. Here, we show that survival to osmostress also requires regulation of G_2 progression. Hog1 activation leads to Swe1 accumulation and downregulation of Clb2-Cdc28 activity. Cells lacking Swe1 fail to arrest at G_2 upon Hog1 activation and are osmosensitive. It is known that the Hsl1-Hsl7 complex regulates Swe1 stabilisation in response to morphogenetic defects[6-8]. Hog1 interacts and directly phosphorylates the Hsl7-docking site in Hsl1. Hsl1 phosphorylation by Hog1 results in delocalisation of Hsl7 from the septin ring which leads to Swe1 accumulation. Upon Hog1 activation, cells containing an Hsl1 allele mutated in the Hog1 phosphorylation site are unable to prevent Hsl7 localisation, fail to arrest at G_2 and become sensitive to osmostress. Together, our data indicate that a single MAPK can co-ordinately modulate different cell cycle checkpoints to allow cells to survive upon stress.

Activation of the stress-activated Hog1 kinase is essential for cell survival in response to high osmolarity, because the MAPK elicits an extensive program required for cell adaptation that includes regulation of gene expression, translation and cell cycle progression(Hohmann 300-72;de Nadal, Alepuz, and Posas 735-40). In response to stress, the Hog1 SAPK mediates a transient cell cycle arrest [2,4,5], which is necessary for proper generation of cell adaptation responses. Hog1 regulates cell cycle progression at the G_1 phase by a dual mechanism that involves down-regulation of cyclin expression and direct phosphorylation of the CDK-inhibitor protein Sic1. This combination results in Sic1 stabilization and inhibition of cell cycle progression[5,11]. Previous reports suggested that activation of Hog1 also results in a delay at G_2 [2,10](de Nadal, Alepuz, and Posas 735-40). Here, we show that Hog1 prevents G_2 progression by direct phosphorylation of the Hsl1 kinase protein which leads to Swe1 accumulation and decrease on Clb-associated Cdc28 activity. Taken together, we propose that a single MAP kinase is able to modulate different cell cycle checkpoints co-ordinately to prevent cell cycle progression in the presence of stressful conditions.

To understand the role of the SAPK Hog1 in cell cycle regulation we analysed cell growth arrest caused by sustained activation of the Hog1 MAPK (i.e., caused by mutations in the *Sln1* osmosensor or the *Pbs2* MAPKK) (Maeda, Wurgler-Murphy, and Saito 242-45;Posas et al. 865-75;Wurgler-Murphy et al. 1289-97;Maeda, Takekawa, and Saito 554-58;Posas and Saito 1702-05)and by osmostress.

Initially, we analysed the effect of Hog1 activation on cell cycle progression in cells allowed to proceed into S phase after release from alpha factor arrest. As expected (figure 1a)[5], direct release of alpha-factor arrested cells carrying a temperature sensitive allele of *SLN1* (*sln1^{ts4}*) at non-permissive temperature resulted in accumulation of a large number of cells in G_1 (unbudded cells with 1C DNA content). As previously shown, this accumulation was not observed in wild type cells and was prevented by deletion of *HOG1* and *SIC1*[5], obtaining similar results when cells were treated with osmostress (figure 1c)[5]. Interestingly, when *sln1^{ts4}* cells synchronised at G_2 were incubated at non-permissive temperature, a high percentage of cells remained in G_2 phase (~76% after 120 min, budded cells with

2C DNA content), whereas only a small proportion of wild-type cells remained in G_2 (~39%, after 120 min) (figure 1a). Osmostress treatment of wild type cells synchronized at G_2 also resulted in delayed G_2 exit when compared to non-treated cells (83% of cells in G_2 upon 80 min of stress vs 30% without stress) (figure 1d). Thus, activation of Hog1 results in both G_1 and G_2 arrest.

Activation of the HOG pathway at G_2 resulted in accumulation of cells with an elongated bud, which is reminiscent of altered Clb2-Cdc28 activity (figure S1a). Activity of Cdc28 at G_2 can be controlled by its state of phosphorylation and by the levels of Clb2. Both, Clb2 protein levels and Clb2-Cdc28 activity, diminished upon expression of the active Pbs2 allele ($Pbs2^{DD}$) (Figure S1b,c) as reported to occur in osmostress[2]. Phosphorylation that regulates Cdc28-Clb2 activity is driven by the activities of the Swe1 kinase and the Mih1 phosphatase[16]. Deletion of *MIH1* only enhanced the effect of Hog1 activation (not shown), whereas deletion of *SWE1* prevented bud enlargement (figure S1a) and partially bypassed the G_2 arrest imposed by the MAPK (figure 2a). Thus, Swe1 plays an important role in the G_2 arrest caused by activation of the Hog1 MAPK.

It was reported that stability of Swe1 is critical to regulate its activity towards Cdc28-Clb2 [17,18]. Swe1 degradation is stimulated by protein phosphorylation. We then analysed the phosphorylation of Swe1 in synchronised cells in the presence or absence of stress (figure 2b). Osmostress induced a delay in Swe1 phosphorylation suggesting that Swe1 stability could be affected by Hog1 activation. We then analysed whether Swe1 accumulates in response to Hog1 activation. Expression of $Pbs2^{DD}$ or the addition of NaCl (as reported previously[18]) resulted in a strong accumulation of Swe1 protein (figures 2c and 2d). Therefore, activation of Hog1 results in accumulation of the Swe1 CDK-regulator. Thus, as in G_1, where the MAPK mediated cell cycle arrest by a dual mechanism that involves downregulation of cyclin expression and control of Sic1 stability[5], in G_2, also a dual mechanism involving downregulation of Clb2 levels and stabilization of a cell cycle regulator such as Swe1 mediates cell cycle progression.

Failure of *sic1*Δ cells to arrest at G_1 upon osmostress causes premature entry into S phase and cells become partially osmosensitive[5]. Consistent with this observation, *swe1* deficient cells do not properly arrest at G_2 upon stress and also become

partially osmosensitive. These cells enter prematurely into mitosis which results in a high percentage of binucleated cells (figure 2e and data not shown). Synthetic *sic1* and *swe1* mutations render cells even more osmosensitive than the single mutations, resulting in sensitivity similar to the *hog1* mutation (figure 2b). Therefore, modulation of cell cycle progression by different checkpoints is required for cell survival upon stress.

It is known that localisation of the septin cytoskeleton, the activity of the checkpoint protein kinase Hsl1 and its interacting protein Hsl7 in the bud neck regulate Swe1 stability. Actually, Hsl7 interacts directly with both Hsl1 and Swe1 and mutations that impair either of these interactions, or change the localisation of Hsl1 or Hsl7, stabilize Swe1 protein[6-8,19-22]. We then followed the localisation of septins (i.e., Cdc11 and Cdc12), of the Hsl1 kinase and the Hsl7 protein fused to GFP. Localisation of the septins Cdc11 and Cdc12 (not shown) and Hsl1 kinase in the bud neck was not affected by Hog1 activation, nor by osmostress or expression of $Pbs2^{DD}$ (figure 3a,c). However, Hsl7 localisation dramatically changed upon Hog1 activation. Cells exposed to osmostress showed a dramatic delocalisation of Hsl7 from the bud neck. Hsl7 delocalisation was transient and correlated to the level of Hog1 activation (figure 3a,b). In addition, whereas Hsl7 was recruited at the bud neck in more than 70% of control cells, only 15% of these cells displayed Hsl7 in the bud neck when expressing $Pbs2^{DD}$ (figure 3c). It was reported that when delocalised from the septin ring, Hsl7 is rapidly dephosphorylated[19,23]. Correspondingly, activation of Hog1 results in rapid Hsl7 dephosphorylation (figure S2a). Thus, activation of the MAPK results in the delocalisation of Hsl7 from the bud neck, which promotes Swe1 stabilisation.

Hog1 interacts and phosphorylates Sic1 to regulate its activity at G_1. We then tested whether Hog1 was able to interact with and phosphorylate Hsl1 or Hsl7 to regulate Hsl7 recruitment to the bud neck. *In vivo* coprecipitation experiments showed that Hog1 is able to interact with Hsl1 (figure 4a) but not with Hsl7 (not shown). In addition, *in vitro* phosphorylation experiments showed that whereas Hsl7 or Swe1 were not phosphorylated by the MAPK (not shown), Hsl1 was phosphorylated in its C-terminal region (aminoacids 711-1517) but not in the N-terminal domain (aminoacids 1-900) (figure 4c). Moreover, exposure of yeast cells to osmostress induced a rapid phosphorylation of Hsl1, as seen by a shift on Hsl1

mobility in a SDS-Polyacrilamide gel. Interestingly, stress-induced Hsl1 phosphorylation was not observed in a *hog1*Δ strain (figure 4b).

It was described that Hsl7 recruitment to the bud neck requires the binding of Hsl7 to the Hsl1 C-terminal region[6,21]. *In vitro* kinase assays testing several Hsl1 fragments for direct phosphorylation pointed at the Ser1220 as the Hog1 phosphorylation site in the Hsl1 C-terminal region. Mutation of Ser1220 to Ala abolished phosphorylation of Hsl1 by Hog1 (figure S2b). Interestingly, Ser1220 is situated in the middle of the Hsl7-binding domain in Hsl1[6,21]. To asses the relevance of the phosphorylation of Hsl1 Ser1220 in the localisation of Hsl7 in response to Hog1 activation, we analysed Hsl7 localisation in cells expressing wild type Hsl1 or the unphosphorylatable mutant of Hsl1, Hsl1 S1220A ($Hsl1^{SA}$). Whereas Hsl7 localisation changed dramatically in response to osmostress or in response to $Pbs2^{DD}$ expression in cells containing wild type Hsl1, Hsl7 localisation did not change in cells expressing the unphosphorylatable $Hsl1^{SA}$ protein (figure 4d, e). It is worth noting that a high percentage of cells carrying the Hsl1 S1220E allele ($Hsl1^{SE}$, which can not be phosphorylated by Hog1, but the acid residue mimics the phoshorylated state) did not localise properly Hsl7 under non-stress conditions and, showed an abnormal morphology reminiscent to *hsl1* deficient cells (data not shown). Thus, phosphorylation of Hsl1 by Hog1 is a key determinant for Hsl7 localisation in response to high osmolarity. Correspondingly, cells containing the $Hsl1^{SA}$ allele were unable to arrest as efficiently as wild type cells in response to Hog1 activation (figure 4f) and were more sensitive to osmostress than cells carrying wild type Hsl1 (figure 4g).

Activation of the Hog1 MAPK is a key step on the generation of adaptive responses that allow for cell survival upon osmostress. Here, we show that in response to stress the MAP kinase is able to regulate G_2 progression by the dual targeting of different components of G_2 regulation, cyclin B levels as well as the direct phosphorylation of the Hsl1 kinase. Hsl1 is known to be a key regulator of the morphogenetic checkpoint and here, we propose that this checkpoint kinase can also integrate stress signals to modulate cell cycle control upon stress. Thus, the MAPK is able to directly modulate cell cycle progression through its coordinate action over two-independent checkpoints, the control of Sic1 at G_1, and Swe1 at G_2, to allow cells to recover before they progress into S phase and mitosis.

Methods

Yeast strains and plasmids. Strains used: W303 (*MATa*, *his3 leu2 trp1 ura3 ade2 can1*) and its derivatives YPC88 (*hog1::LEU2*), YPC65 (*sic1::KanMX*), YPC166 (*swe1::KanMX*), YPC343 (*sic1::KanMX swe1::URA3*), YPC410 (*SWE1-HA::TRP1*), YPC253 (*HSL1-GFP::KanMX*), YPC251 (*hog1::LEU2 HSL1-GFP::KanMX*), YPC361 (*HSL7-GFP::KanMX*), YPC357 (*hog1::LEU2 HSL7-GFP::KanMX*), YPC425 (*hsl::URA3 HSL7-GFP::KanMX*). TM141 (*MAT***a** *ura3 leu2 trp1 his3*) and its derivatives YPC38 (*sln1-ts4*), YPC29 (*sln1-ts4 hog1::LEU2*), YPC60 (*sln1-ts4 swe1::KanMX*), YPC130 (*SWE1-9xMyc::TRP1*). The P_{GAL1}-Pbs2^{DD} express a *PBS2* allele that contains two mutations that replace both the phosphorylation sites required for Pbs2 activation (Ser514 an Thr518 to Asp). Plasmid expressing Cdc11::GFP under the control of his own promoter was a generous gift from Drs. A. Casamayor and M. Snyder. The last 800 aminoacids from wild type Hsl1 (pJCE 1060) or the corresponding mutant S1220A (pJCE 1096) were cloned into pGEX-3X plasmid. Centromeric plasmid pYB117 expressing HSL1-HA from his own promoter were a generous gift from Dr. Y. Barral. To express haemagglutinin (HA)-tagged mutated versions of Hsl1 in yeast, a S1220A or S1220E substitutions were introduced in pYB117 generating pJCE1090 and pJCE1099 respectively.

Growth conditions, cell synchrony and cytometry analyses. Cells were grown in YPD or SC medium without uracil (URA) supplemented with either 2% dextrose, or 2% raffinose, when indicated. Galactose induction was accomplished by initial growth in URA plus raffinose, followed by addition of galactose to 2%. Cell synchrony was accomplished by treatment of cells with 40 μg/ml of pheromone for the indicated times. For flow cytometry analyses, cells were fixed in ethanol, treated with RNAse A, stained with propidium iodide and analized in a FACScan flow cytometer (Becton Dickinson).

Binding assays. *In vivo* interaction of GST-Hog1 with chromosomal HA-tagged Hsl1 was determined by coprecipitation. Exponential growing cells were subjected to a

brief osmotic shock (0.4 M NaCl, 10 min). Yeast extracts (3 mg) were prepared as in[20] and incubated with glutathione-sepharose beads. Beads were washed extensively and proteins precipitated proteins detected using anti-HA and GST antibodies.

Kinase assays. The Hsl1 proteins were expressed in *E. coli* and purified using glutathione-Sepharose beads, mixed with 1□g of purified GST-Hog1 or Hog1(KN) activated with GST-PBS2^{DD} and radioactive ATP. Clb2-associated Cdc28 kinase activity assays were performed on immunoprecipitated Clb2-HA. Clb2-Cdc28 complexes were immunoprecipitated using anti-HA antibodies from 1 mg of total cellular protein and assayed essentially as described in Belli et al 2001, using histone H1 as substrate. Phosphorylated histone H1 was assessed by using a Phosphoimager and referenced to the time 0 wild type activity.

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Competing Interests statement The authors declare that they have no competing financial interests.

Figure Legends

Figure 1 Activation of the HOG pathway results in G_1 and G_2 cell cycle arrest. (**a**) Cells arrest at G_1 in response to Sln1 inactivation. The *sln1^{ts}* or wild type strains were synchronized with α-factor for 2h, shifted at 37ºC for 1h and then released into YPD medium at 37ºC. Total DNA content was assessed by FACS analysis and presented as cell counts (y axis) versus 1C and 2C DNA content (x axis). The percentage of budding cells is depicted on the right side of each graph. (**b**) Cells arrest at G_2 in response to Sln1 inactivation. The *sln1^{ts}* or wild type strains were synchronized with α-factor for 3h, released into fresh media at 25ºC and shifted to 37ºC after 50 min in YPD (time 0). Total DNA content was analysed as in a. (**c**) Cells arrest at G_1 in the presence of osmostress. Wild type cells were synchronized in G_1 phase with α-factor and released into YPD medium containing 0.4 M NaCl. Total DNA content was assessed as in a. (**d**) Cells arrest at G_2 in the presence of osmostress. Wild type cells were synchronized with α-factor and released into YPD medium. After 50 min, NaCl was added to 0.4 M (time 0). Total DNA content was assessed as in a.

Figure 2 Swe1 mediates the G_2 arrest caused by Hog1 activation. (**a**) Deletion of *HOG1* or *SWE1* abolishes cell cycle arrest at G_2 caused by Sln1 inactivation. *sln1^{ts}*, *sln1^{ts} hog1Δ*, *sln1^{ts} swe1Δ* strains were synchronized with α-factor for 3h, released into fresh media at 25ºC and shifted to 37ºC after 50 min in YPD (time 0). Total DNA content was analysed as in figure 1a. (**b**) Swe1 phosphorylation is affected by osmostress. Cells expressing epitope tagged Swe1 from its chromosomal locus were synchronised by pheromone treatment and 50 min after release, cells were subjected (NaCl) or not (control) to 0.4 M NaCl. Swe1 and Cdc28 (loading control) were detected from cell extracts by immunoblotting using specific antibodies. Control strain (without tagged Swe1) was wild type W303 (-). (**c**) Swe1 accumulates in response to osmostress. Tagged Swe1 was expressed from its chromosomal locus in wild type cells (YPC130). Cells were synchronized as in c

and subjected (NaCl) or not (control) to 0.4M NaCl. Swe1 was detected from yeast extracts using monoclonal anti-myc specific antibodies. Control strain was wild type W303 (-). (**d**) YPC130 strain (which expresses Swe1-myc) containing either control vector (vector) or P_{GAL1}-Pbs2^{DD} plasmid were grown in SD-Ura plus raffinose and after addition of galactose cells were collected at the indicated times. The presence of myc-tagged Swe1 was detected as in c. (**e**) Deletion of *SWE1* and *SIC1* results in cells osmosensitive. The wild type (W303) strain and its derivatives *hog1*Δ, *sic1*Δ, *swe1*Δ and *sic1*Δ *swe1*Δ mutants were spotted on YPD plates or YPD plates containing 0.4 M or 0.8 M NaCl. Growth was scored after 3 days at 30ºC.

Figure 3 Localisation of Hsl7 is affected by activation of the HOG pathway. (**a**) Localisation of Hsl7 but not Cdc11 or Hsl1 is affected by osmostress. Wild type, *hog1*Δ or *hsl1*Δ cells containing chromosomal GFP tagged Cdc11, Hsl1 or Hsl7 were grown in YPD and subjected (NaCl) or not (control) to a brief osmostress (10 min at 0.4M NaCl). GFP proteins were visualised by direct fluorescence. (**b**) Time course of Hsl7GFP localisation upon osmostress. Wild type cells containing Hsl7GFP were subjected to 0.4M NaCl at the indicated times. Hog1 phosphorylation was followed using specific antibodies against phosphorylated p38 in whole cell extracts. (**c**) Localisation of Hsl7 is affected by Hog1 activation. Wild type cells expressing P_{GAL1}-Pbs2^{DD} or a control vector and GFP tagged Cdc11, Hsl1 or Hsl7 were grown in minimal media containing galactose.

Figure 4 Phosphorylation of Hsl1 Ser1220 by Hog1 determines Hsl7 localisation. (**a**) Hog1 interacts with Hsl1 *in vivo*. Yeast extracts containing GST or GST-Hog1 and untagged or tagged HA-Hsl1 (from its chromosomal locus) were precipitated using glutathione beads and precipitated Hsl1 was probed using anti-HA antibodies. GST proteins were detected using anti-GST antibodies. (**b**) Hsl1 is phosphorylated

upon osmostress in a HOG1-dependent manner. Wild type or *hog1Δ* cells carrying untagged or tagged HA-Hsl1 were synchronised with pheromone treatment and 50 min after release, cells were subjected (+) to a brief osmotic shock (0.4 M NaCl, 10 min). Extracts were treated (+) with alkaline phosphatase (AP). The presence of Hsl1 and Cdc28 was probed. (**c**) Hog1 directly phosphorylates the C-terminal region of Hsl1 *in vitro*. Hog1 and the constitutively activated Pbs2 allele ($Pbs2^{EE}$) purified from *E. coli*, were incubated in presence of kinase buffer and ATP. Then catalytically inactive Hsl1 kinase domain (1-600), a Hsl1 fragment (from aa 600 to 900) or the C-terminal domain of Hsl1 that contains from aa 717 to 1517, purified from *E. coli* were added in the presence of radioactive ATP. Phosphorylated proteins were detected by autoradiography (P32) or coomassie staining. Position of Hsl1 fragments is indicated on the left. (**d**) Mutation of Hsl1 Ser1220 affects Hsl7 localisation. The *hsl1Δ HSL7GFP* strain transformed with plasmids containing wild type Hsl1 or the mutated alleles Hsl1 Ser1220A ($Hsl1^{SA}$) or Hsl1 Ser1220E ($Hsl1^{SE}$) and P_{GAL1}-$Pbs2^{DD}$ or a control vector were grown as in figure 3c. Hsl7GFP was visualised by direct fluorescence. (**e**) Cells as in d were grown in minimal media and subjected (NaCl) or not (control) to a brief osmostress (10 min, 0.4M NaCl). Hsl7GFP was visualised as in d. **(f)** Cells containing an unphosphorylatable allele of Hsl1 become osmosensitive. *hsl1Δ* cells containing wild type Hsl1 or the Hsl1 Ser1220A mutant were spotted on YPD plates containing NaCl or Sorbitol. Growth was scored after 3 days.

Supplementary Information

Figure legends.

Figure S1. Activation of Hog1 causes a decrease on Clb2-Cdc28 activity. (**a**) Wild type, YPC89 (*hog1*Δ) and YPC166 (*swe1*Δ) cells containing an empty vector or P_{GAL1}-Pbs2^{DD} were grown for 7 hours in SD-Ura plus galactose. Cells were visualized by differential interference contrast. (**b**) Cells as in a carrying the P_{GAL1}-Pbs2^{DD} was transformed with a plasmid expressing HA-tagged Clb2 under its own promoter. Cells were grown in raffinose and then galactose (time 0) was added. Cells were collected at the indicated times and Clb2 was visualized by immunoblotting with monoclonal antibody 12CA5 to HA. Wild type cells without the Clb2-HA plasmid is shown as negative control (-). (**c**) Cells as in b; wild type (●) and *hog1*Δ (▼) were grown in the presence of galactose and collected at the indicated time points. Protein extracts were prepared and Clb2-Cdc28 kinase activity was assessed by an *in vitro* kinase assay of immunoprecipitated Clb2-Cdc28 using Histone H1 (HH1) as a substrate. Kinase activity was normalized to that of wild type, time 0. Data ± SD from 3 independent experiments is shown.

Figure S2. Hog1 phosphorylates the Hsl7 binding site in Hsl1. (**a**) Hsl7 is dephosphorylated upon Hog1 activation. Wild type, *sln1^{ts}* or *sln1^{ts} hog1Δ* strains containing HA-tagged Hsl7 were synchronized with pheromone for 3h, released into fresh media at 25ºC and shifted to 37ºC after 50 min in YPD (+). Extracts were treated (+) with alkaline phosphatase (AP). The presence of Hsl7 was probed using anti-HA. (**b**) Hog1 directly phosphorylates the Ser1220 of Hsl1 *in vitro*. Hog1 and the constitutively activated Pbs2 allele (Pbs2^{EE}) purified from *E. coli, were* incubated in presence of kinase buffer and ATP. Several fragments of Hsl1 (aa indicated in brackets) purified from E. coli were tested for phosphorylation. Hsl1 S1220A contains a mutation in Ser1220 in Hsl1. Purified proteins were added in the presence of radioactive ATP. Phosphorylated proteins were detected by autoradiography (P32) or coomassie staining. Position of Hsl1 proteins is indicated on the left.

Figure 1
Clotet *et al.*

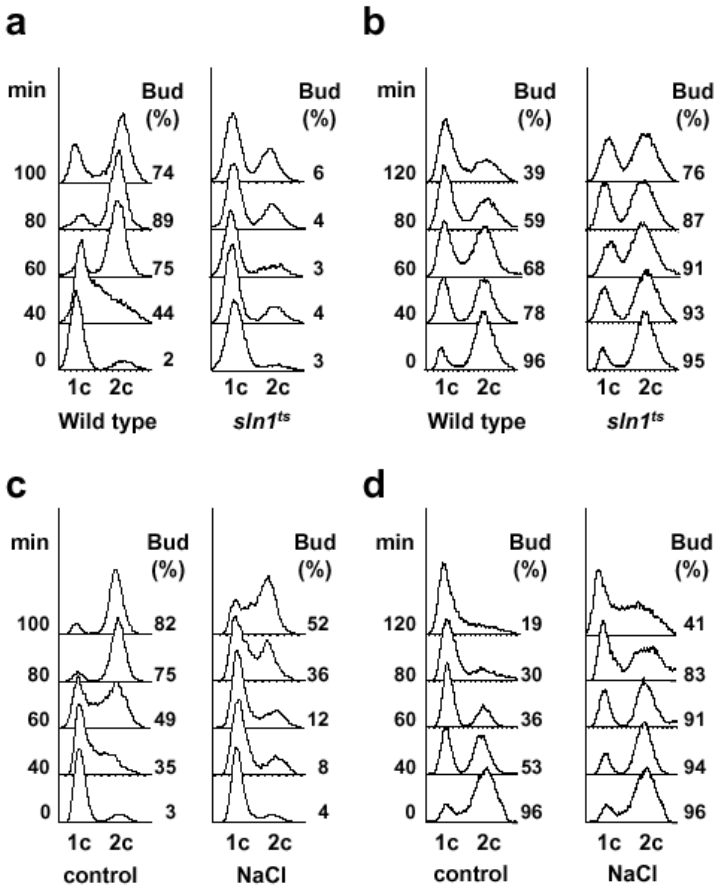

Figure 2
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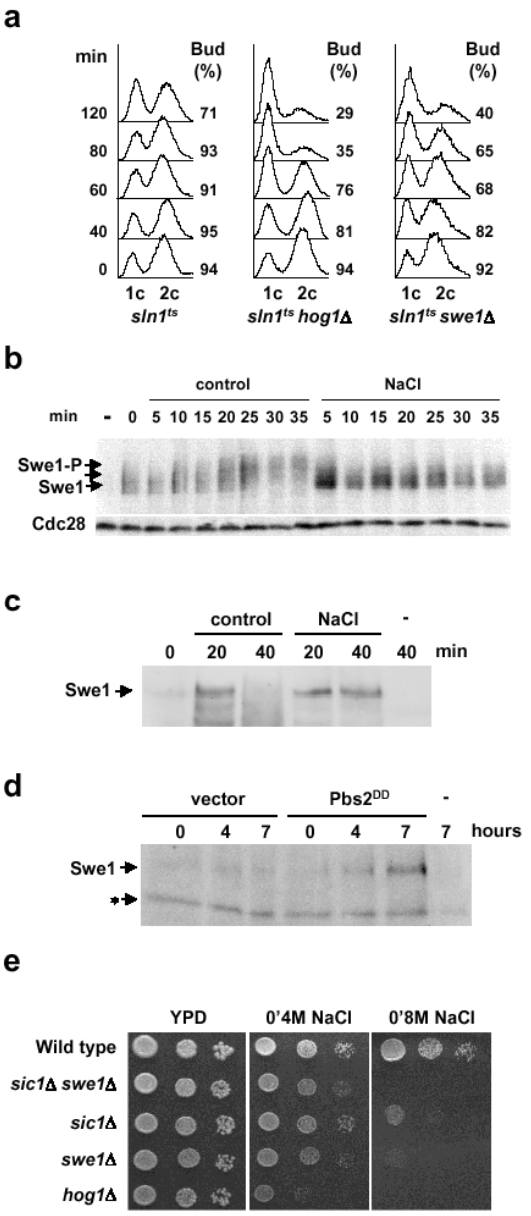

Figure 3
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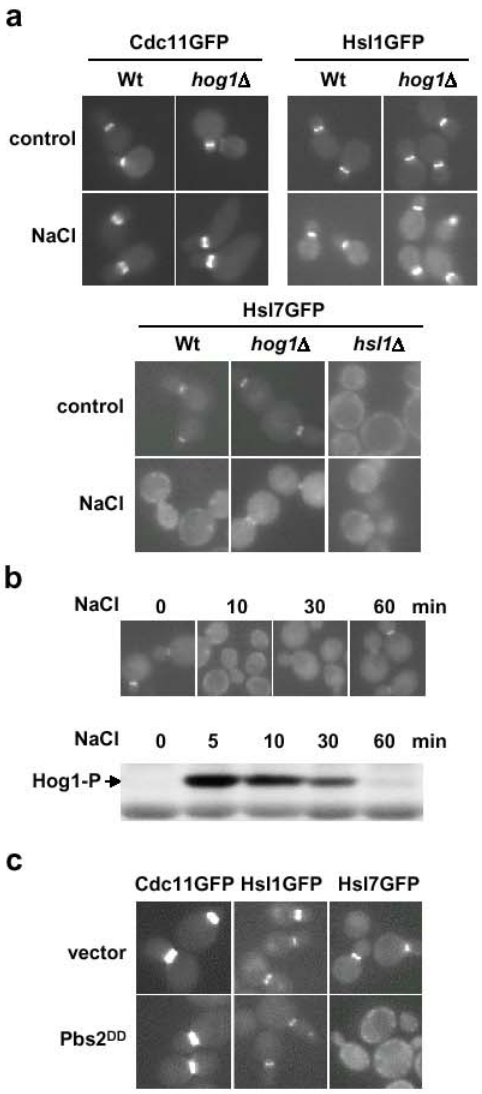

Figure 4
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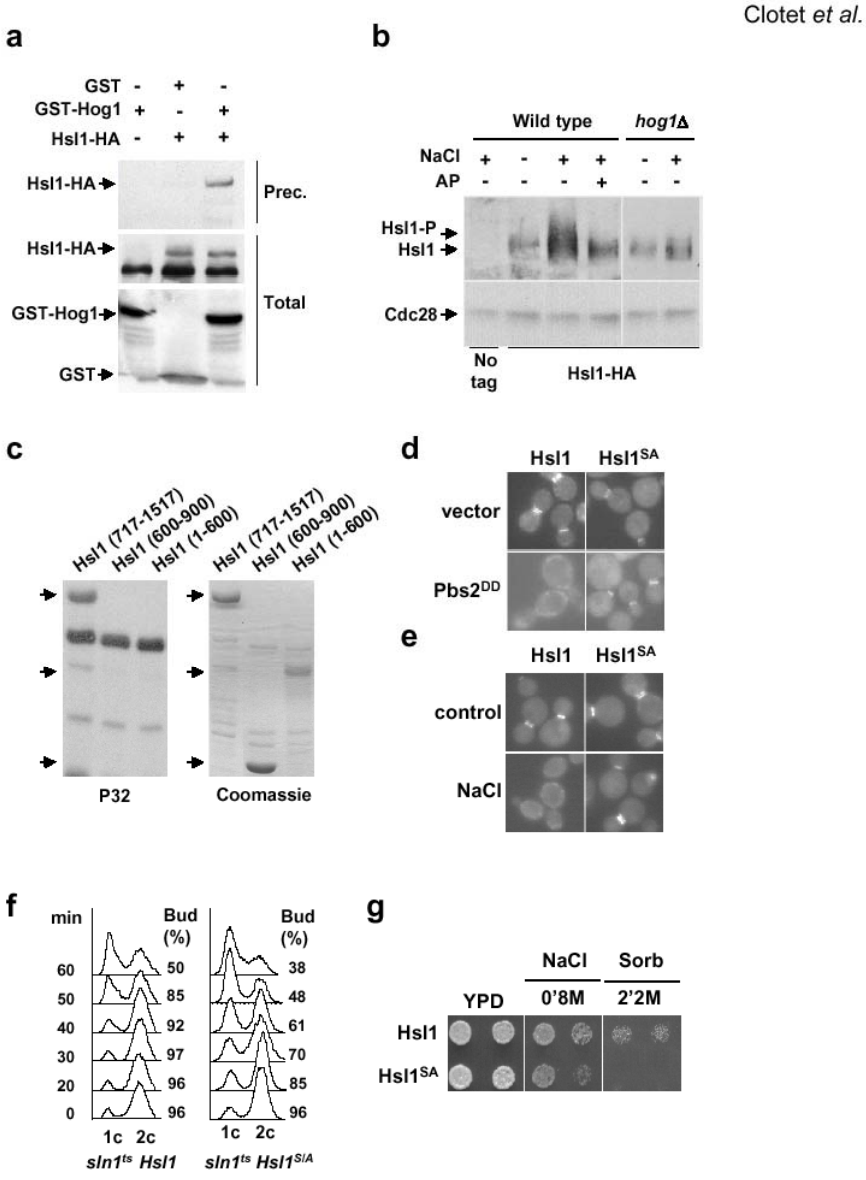

Figure S1
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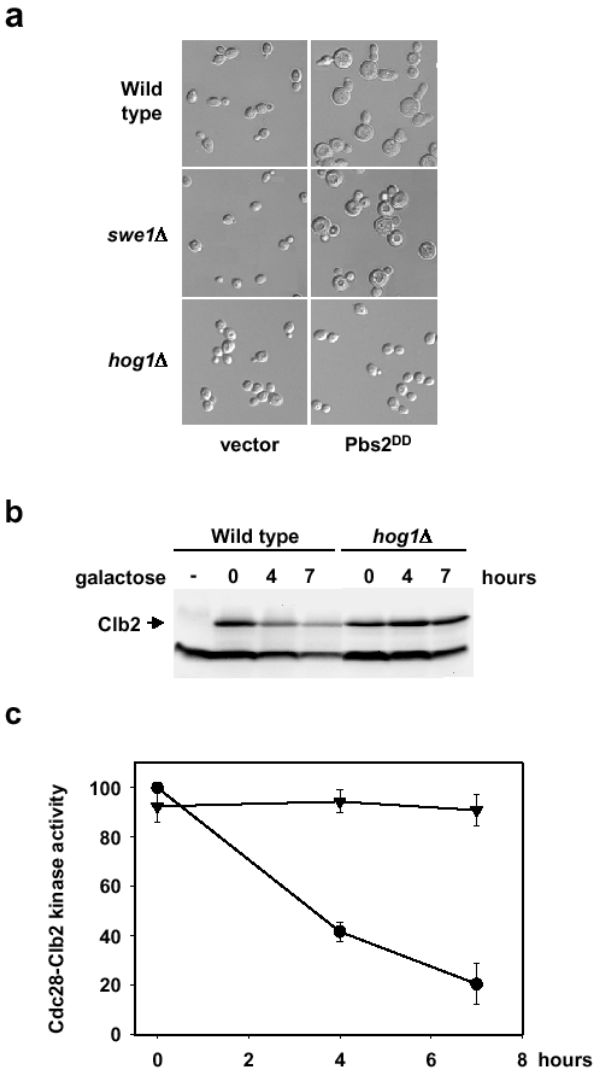

Figure S2
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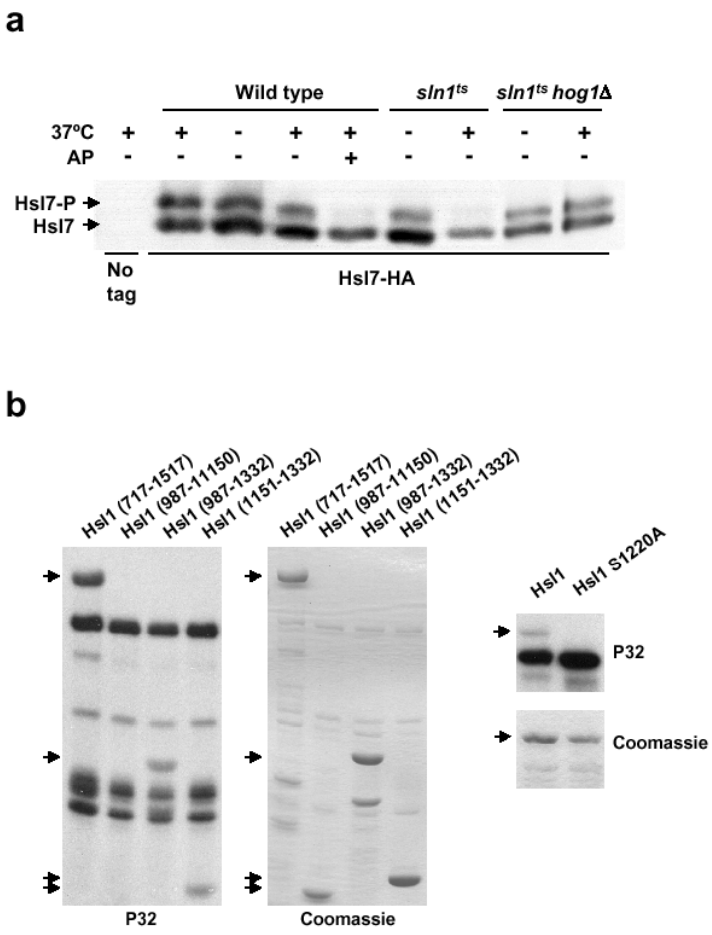

Discussion

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

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Discussion

In response to high osmolarity, the presence of the MAPK Hog1 is crucial for cell survival, because this SAPK triggers the program for cell adaptation. Activation of SAPKs results in the generation of a set of adaptive responses that leads to the modulation of several aspects of cell biology essential for cell survival, such as, gene expression, translation and/or morphogenesis (Hohmann 2002). The presence of stress critically affects progression through the cell cycle. In essence, cells need to modulate the different stages of the cell cycle, so as to allow for proper cellular adaptation in the presence of stress. This is the case when yeast cells are exposed to osmostress. Osmostress induce the activation of the Hog1 SAPK and consequently a transient cell cycle arrest required for proper cell adaptation. Although there are some reports suggesting a possible role of Hog1 within cell cycle regulation, the molecular basis for this regulation is not completely understood (Alexander et al 2001)),(Yaakov et al 2003), (de Nadal et al 2002)). In order to discern the role of the MAPK Hog1 in the cell cycle regulation, we analysed the cell growth arrest caused as a consequence of an osmotic stress or by a sustained activation of the HOG pathway using several alleles able to activate the MAPK in absence of osmotress. From those studies, we propose that the mechanisms which involve the cell cycle control in response to osmostress are of a great complexity and that they could be conserved among eukaryotic cells (from yeast to mammals).

After an osmotic shock, activation of the Hog1 MAPK is essential stages in the generation of adaptive responses which allow survive the cells in these new conditions. This work describes how the MAPK Hog1 is able to regulate cell cycle transition between G1 to S phase by a dual mechanism that involves as a Cdc28-Cln1/Cln2 activity decrease, through a downregulation of G1 cyclin expression, as a Sic1 accumulation through a direct targeting of the CDK-inhibitor protein Sic1.

Cells arrest at G1 phase in response to Sln1 inactivation. Identification of Sic1 as a cell cycle Regulator under osmotic shock

Activation of the HOG pathway produce an accumulation of cells at G1 and, this accumulation was completely prevented in *hog1* deficient cells. This effect was seen either by osmostress or by sustained activation of the MAPK, by the use of the $Sln1^{ts}$ allell.

In yeast, cell cycle transition from G1 to S is driven by the activity of the Cdc28 kinase associated with G1 cyclins (Cln1, Cln2 and Cln3). The G1 arrest mediated by Hog1 was similar to the arrest at G1 achieved by activation in yeast of another MAPK pathway, the mating pathway, which is caused by accumulation of the G1 CDK inhibitor, Far1. We tested whether osmostress produced a Far1-mediated G1 arrest, because there was the possibility of crosstalk between different pathways (the mating pathway and the HOG pathway) due to they are sharing some components (such as Cdc24, Cdc42, Ste11, Ste50 or Ste20). This was not the case, because in *far1* depleted cells, Hog1 activation induced a G1 arrest like the observed in wild type cells. Thus, Far1 does not play a significant role in the G1 arrest imposed by Hog1.

Sic1 is a CDK inhibitor that prevents S phase entry by inhibition of the Cdc28 Clb5/Clb6 complexes. Once the G1 CDK is activated (Cdc28-Cln), this kinase is able to target Sic1 for its degradation. This fact occurs basically through several phosphorylations at the amino terminal region of Sic1. These phosphorylations have a key role for its degradation through ubiquitination, because the phosphorylations are necessary for the recognition of Sic1 by the E3 ubiquitin ligase SCF-Cdc4 (Schwob et al 1994). Moreover, the degradation of Sic1 is a key step to progress into S phase from G1, because Sic1 is necessary to enter into S phase.

The lack of Sic1 degradation results in a delay to enter into S phase, and therefore a G1 arrest. Two mechanisms can promote Sic1 stabilisation; a drop on Cdc28-G1 cyclin activity or regulation of Sic1 protein levels.

To determine whether Sic1 was implicated in the Hog1-mediated G1- phase cell cycle arrest, we studied the activation of the pathway in cells defective in this gene. As we have previously mentioned, an elevated fraction of the cells remained in G1 phase when Hog1 is activated, and only a small proportion of control, *hog1* deleted or *sic1* deleted cells remained blocked in G1 phase upon Hog1 activation. Hence, deletion of *SIC1* eliminates the G1-phase cell-cycle arrest in response to Hog1 activation.

Activation of Hog1 results in the increased concentration of the CDK-inhibitor Sic1.

Sic1 protein levels control progression from G1 to S phase, therefore, in order to enter properly into S phase elimination of the Sic1 protein is required. Therefore, we investigated whether Sic1 protein levels were modified as a result of Hog1 activation. Sic1 protein strongly accumulated as a result of the activation of Hog1. As mentioned previously, this effect could be caused by a reduction in the levels of G1 cyclins, because this causes a decrease on Cdc28-Cln1/Cln2 activity. It was observed a decrease in the levels of RNA corresponding to G1 cyclins (Cln1 and Cln2), but not *SIC*1 mRNA when the pathway was activated, and, this phenomenon was completely dependent on Hog1. The downregulation of cyclin expression need to be explored more profoundly. A possibility to investigate could be whether some transcription factor activities involved in the expression control of G1 cyclins (for instance SBF or MBF) are affected by Hog1 activation.

. Nevertheless, *CLN1* and *CLN2* low levels can not exclusively explain the G1-phase cell cycle arrest mediated by Hog1, because activation of Hog1 in cells with sustained Cln2 levels (achieved by expression of Cln2 under a heterologous promoter), does not avoid Sic1 protein accumulation caused by Hog1 activation. Although, in cells with sustained Cln2 levels, this arrest at G1 (as a consequence of Hog1 activation) was less manifest than that observed in wild type cells with endogenous levels of G1 cyclins. Therefore, we predicted that an additional mechanism, apart from the reduction of Cln mRNA levels, must exist to explain the G1-phase cell cycle arrest mediated by Hog1.

Sic1 is a Direct Substrate of the Hog1 MAPK

Hog1 interacted with Sic1, as seen in a two-hybrid assay and co-immunoprecipitation experiments. Moreover, *in vitro* binding experiments using purified proteins confirmed that Sic1 binds directly to Hog1.
In the protein sequence of Sic1, there are nine serines or treonines putative to be phosphorylated by the MAP kinases. The Cdc28-G1 cyclin kinase complex directly phosphorylates several of these serines or threonines at the N-terminus of Sic1, setting a threshold for its subsequent ubiquitination and degradation triggered by the Cdc4 E3 ligase of the SCF complex. In yeast cells, Hog1 activation induced phosphorylation of Sic1. Interestingly, Sic1 phosphorylation by osmotic stress was completely avoided in *hog1* depleted cells.
We then tested whether Sic1 was able to be phosphorylated directly by Hog1. Certainly, Sic1 was directly phosphorylated by the MAPK Hog1 at the threonine 173. A mutant protein containing threonine 173 mutated was not phosphorylated by the MAPK. Furthermore, under osmotic shock, the phosphorylation observed in Sic1 was absolutely abolished by mutation at the threonine 173. Our results showed that the T173 of Sic1 is the unique site directly phosphorylated by the Hog1 MAPK.

Sic1 Phosphorylation by the Hog1 MAPK is Important for its Biological Function

To consider the relevance of Sic1 phosphorylation by Hog1, we analysed cell cycle progression in cells expressing *Sic1*, mutant non- phosphorylatables by Hog1 or a mutant that mimics Hog1 phosphorylation (S173 to E173). Interestingly, cells that only contain the non-phosphorytable allele of Sic1 were unable to arrest the cell cycle at G1 phase upon activation of the HOG pathway. On the other hand, as a consequence of the Hog1 activation, yeast cells carrying the allele that mimics the Hog1 phosphorylation arrested in G1 phase in a similar manner than yeast cells containing wild-type Sic1. Thus, phosphorylation of Sic1 threonine 173 by Hog1 is an important factor for the arrest of cell-cycle progression at G1 phase in response to osmostress.

It has been described that consecutive phosphorylations over Sic1 protein by Cdc28-Cln produce a threshold for the onset of Sic1 degradation (Nash et al 2001). Hence, we studied whether Hog1 phosphorylation the on carboxi-terminal region of Sic1 modified Sic1 protein degradation rates. We followed Sic1 protein levels of wild type or the Hog1-non-phosphorylatable mutant allele, upon induction of the HOG pathway. We found that Sic1 protein degradation rate was affected as a consequence of Hog1 activation, increasing Sic1 protein half-life. On the other hand, cells that expressed the non-phosphorylatable allele of Sic1, the degradation rate of Sic1 was not affected by activation of the HOG pathway. In addition, the mutant who mimics a constant phosphorylation of Sic1 was stable even in the absence of Hog1 activation. Therefore, introduction of a single phosphorylation at the serine 173 (this residue has not been described as one of the main residues necessary for Sic1 destruction), provoques a clear stabilisation of the inhibitor Sic1. This fact correlates with the arrest at G1 as a consequence of Hog1 activation.

In stress conditions, Sic1 stabilization is crucial to obtain a proper cell cycle progression. For that reason, we checked what happens in yeast cells that can not stabilise Sic1 under Hog1 activation in high osmolarity medium. It has been described that *sic1* depleted cells showed a significant rate of chromossomal instability as compared to wild type cells. This fact is consequence of a premature S-phase onset in the mutant cells. Thus, we checked the chromosomal instability in *sic1* deleted cells, in the presence of a high osmolarity medium. *sic1*Δ cells exhibited chromosomal instability in response to osmostress. A strain containing the Hog1-non-phosphorylatable allele of Sic1 showed a significant percentage genomic instability only when they were submitted to an osmotic shock. Moreover, at high osmolarity conditions, *sic1*Δ cells or cells carrying the Hog1-non-phosphorylatable allele were osmosensitive as compared to wild type cells. Thus, in response to osmotic stress, cells carrying mutations in *SIC1* are not competent to impose a delay at G1 phase appropriately, enter into S phase in inappropriate conditions, which causes genomic instability, this results in cells osmosensitive.

Finally, the last question that we wanted to discern was how the stabilisation of Sic1 was achieved by Hog1 phosphorylation. There were several possibilities; first, Hog1 phosphorylation could be affecting the ability of Cdc28-G1 cyclin to phosphorylate directly Sic1 and, this fact together with the decrease of G1 cyclin expression, produce Sic1the accumulation. Sic1 T173E mutant which mimics the phosphorylation of Hog1 was phosphorylated by Cdc28-Cln2 similarly to a wild type Sic1. Therefore, the phosphorylation on Sic1 T173 does not modify the CDK ability to phosphorylate Sic1 in others sites, which are a necessary step to destruction.

The second possibility was that the Hog1 phosphorylation over Sic1 was affecting the binding of Sic1 to SCF-Cdc4. Sic1T173E showed a reducted affinity to Cdc4 when compared to wild type. Thus, phosphorylation of Sic1 by Hog1 affects binding of Sic1 to the Cdc4 E3 ligase

The MAPK Hog1 interacts physically with Sic1, and phosphorylates a single residue at the carboxyl terminal region of Sic1, which, in combination with the downregulation of the G1 cyclin expression, results in Sic1 stabilization and inhibition of cell-cycle progression at G1 phase. The Cdc28-G1 cyclin kinase complex directly phosphorylates several sites at the N-terminus of Sic1, setting a threshold for its subsequent ubiquitination and degradation, triggered by the Cdc4 E3 ligase of the SCF complex. The MAPK Hog1 seems to be controlling Sic1 stability by down-regulation of Cln1 and Cln2 expression, which diminishes N-terminal Sic1 phosphorylation, (because there are less activity of G1 CDK) and it can explain at least in part the reduction in Sic1 degradation.

On the other hand, the direct phosphorylation of Sic1 at an unique specific threonine at the C-terminus region of Sic1 (Thr173), interferes with its binding to the ubiquitin ligase SCF-Cdc4. Combination of both mechanisms (the downregulation of cyclins and the direct phosphorylation) results in Sic1 stabilization and prevents entry prematurely into S phase.

Which is the advantage of a redundant control over Sic1 to control cell cycle progression? If the direct phosphorylation of Sic1 by the MAPK Hog1 is to permanently stabilize Sic1, there seems no necessity of an extra decrease in Cln1/Cln2-Cdc28 activity to further stabilize Sic1. However, neither the decrease on G1 cyclins levels nor the solely phosphorylation of Sic1 by the MAPK can totally account by the G1 arrest observed upon Hog1 activation, which suggests that

there must be a selective advantage of maintaining such a complex regulatory mechanism.

An obvious advantage of the coordinated effect over Sic1 could be the increase of the efficiency of the G1 arrest by creates two additive systems over Sic1, none of them too stringent to avoid interferences with normal cell cycle progression without an osmotic stress. Otherwise, this system could be envisioned as a two step mechanism. An initial step to cause immediate arrest by acting directly over the CDK inhibitor (Sic1 phosphorylation) and then a decrease on G1 cyclin mRNA would be essential to maintain the arrest for a prolonged time. This scenario could also satisfy the critical step of preventing entry into S phase for different type of cells. G1 phase is an extended phase, and probably is not the same to submit the cells to an osmotic stress in early G1 or in late G1. Thus, cells at early G1 could be easily arrested by the simple decrease on G1 cyclin levels, whereas cells in advanced G1, close to START, would require a more direct effect over the Sic1 inhibitor to restrict their entry into S phase.

Multiple checkpoint activation by the Hog1 MAPK is required for cell survival upon osmostress

(Manuscript under submission)

Discussion

Activation of stress-activated protein kinases (SAPKs) is a vital step for a correct cell adaptation to extracellular stimuli. When yeast cells are exposed to an osmotic stress or mutations that lead to activation of the HOG MAPK pathway results in arrest of cell cycle progression. Once the MAPK Hog1 is activated after an osmotic shock, Hog1 is able to lead to G1 arrest by a dual targeting of the CDK-inhibitor Sic1 (Escote et al 2004). In this work, we demonstrated that survival to osmostress not only requires regulation of G1 but also the regulation of G2 progression. Interestingly, like in G1, activation of the HOG pathway regulates G2. This arrest is mediated by the direct control of the CDK activity, and like in G1, by a dual mechanism which involves the CDK inhibitor Swe1 and the decrease of G2 cyclin transcription. Again, the coordinated action of the MAPK Hog1 over various components of the cell cycle apparatus, mediates cell cycle progression. Thus, Hog1 activity prevents a premature entry into mitosis, when cells are not adapted to the new extracellular conditions.

Activation of the HOG pathway results in G1 and G2 cell cycle arrest.

In response to osmotic stress, the Hog1 SAPK mediates a transient cell cycle arrest, which is a vital step for the proper generation of the cell adaptation responses. The Hog1 MAPK controls cell cycle progression at the G1 phase by a dual mechanism which involves as a down-regulation of cyclin expression as a direct phosphorylation of the CDK-inhibitor protein Sic1. This double action results in Sic1

protein accumulation and inhibition of cell cycle progression between G1 and S phase (Escote et al 2004).

Previous reports have suggested that activation of Hog1 also results in a delay at G2 progression (Alexander et al 2001), (de Nadal et al 2002),(de Nadal et al 2003).

In order to understand the role of the MAPK Hog1 in G2 progression, we analysed cell cycle progression in response to sustained activation of the HOG pathway (Posas et al 2000), (Wurgler-Murphy et al. 1997), (Maeda et al 1995), (Posas et al 1997)and osmostress.

We initially investigated the effect of Hog1 activation on cell cycle progression in cells. After release from alfa factor, and shift to non permissive temperature when cells were in G2, an elevated fraction of cells continued in G2 phase upon Hog1 activation, whereas only a little percentage of non activated cells remained in G2. Hence, when yeast cells are submitted to an osmotic shock, they arrest temporally at G2 in a Hog1 dependent manner. Thus, activation of Hog1 results in a G1 arrest as well as in a G2 delay.

Swe1 mediates the G2 arrest caused by Hog1 activation. Activation of Hog1 causes a decrease on Clb2-Cdc28 activity.

At G2, a prolongated activation of the Hog1 MAPK produced an accumulation of cells with an elongated bud, which was suggestive of down regulation of Clb2-Cdc28 activity. The CDK activity at G2 can be regulated by phosphorylation state and by the levels of the G2 cyclins, such as Clb2. These two elements, the CDK-Clb2 associated activity and the Clb2 protein levels were reduced upon activation of the Hog1 MAPK.

The phosphorylation state of Cdc28 and thus, its regulation at G2, is a consequence of the balance between the activities of the Swe1 kinase and the Mih1 phosphatase. In Mih1 depleted cells, effects of Hog1 activation were more pronounced than a wild type cells. However, in Swe1 depleted cells, the bud enlargement provoked by Hog1 activation was prevented, and in addition, these cells partially bypassed the G2 delay induced by the MAPK. Therefore, Swe1 mediates the Hog1 induced G2 cell cycle arrest.

Stability of Swe1 is critical to regulate its activity towards Cdc28-Clb2 (Sia et al 1996), (Sia et al 1998). Swe1 degradation is triggered by phosphorylation by a subset of protein kinases (McMillan et al 2002). Then, we analyzed whether the Swe1 patern of phosphorylation changed in the presence or absence of stress. Interestingly, osmostress induced a delay in Swe1 phosphorylation, which could suggest that Swe1 stability was affected as a consequence of Hog1 activation. We then analysed whether Swe1 accumulates in response to Hog1 activation, and found that upon Hog1 activation there was a strong accumulation of Swe1 inhibitor. Therefore, activation of Hog1 results in accumulation of the Swe1 CDK-regulator as a consequence of the inhibition of its degradation.

Thus, as in G1, where the MAPK mediated cell cycle arrest by a dual mechanism which involves a down regulation of cyclin expression and control of Sic1 protein stability (Escote et al 2004); in G2 phase, the MAPK Hog1 also triggers a dual mechanism to control cell cycle progression, which involves the down regulation of Clb2 levels and the stabilization of the cell cycle regulator Swe1.

This dual mechanism of control at G2 could be a robust mechasnim to avoid transition to M phase without proper adaptation upon osmotic stress down regulation of G2 cyclins. This is down regulation of the CDK activity, but still medium/long term effect, because the cyclin protein present in the cell could associate to Cdc28. To have a short term effect, the stabilization of Swe1 inhibitor could be important; this would lead to rapid down regulation of the G2-CDK activity, and avoid transition to M phase. Stabilization of Swe1 is accomplished at a specific place of the cell, the bud neck. The delocalization of Hsl7 from the bud, leads a rapid accumulation of Swe1 in this area, which results in a fast decrease of Cdc28 activity in this zone. It has been described that in the mitotic apparatus, tubuline cytoskeleton is critical to obtain a mother cell with a nucleus and the daughter cell with the other nucleus. There are some proteins of the spindle that interact with proteins at the neck to orientate the spindle in the mother-bud axes. Actually, Hog1 activation in a *swe1*Δ strain, in which Hsl7 is delocalized from the bud neck, the spindle results in inapropiate spindle orientation which results in binucleated cells.

A possible explanation of this dual mechanism control at G2 could be a synergic method to avoid a transition to M phase in non proper conditions. Upon osmotic stress, cells activate the HOG pathway and this leads to an arrest at G2 phase by a down regulation of G2 cyclins. This fact implies a general down regulation of the activity CDK at G2, but in a medium-long term, because the cyclin protein synthesized could associate to Cdc28 and rest active. In a short term, there is the stabilization of Swe1 inhibitor, which implies a rapid down regulation of the G2-CDK activity, to avoid the transition to M phase. This stabilization is caused in a specific place of the cell, the bud neck. The delocalization of Hsl7 from the bud, leads a rapid accumulation of Swe1 in this area, which means a fast fall of Cdc28 activity in this zone. It has been described that the mitotic apparatus is based tubuline spindle cytoskeleton to obtain a mother cell with a nucleus and the bud-daughter cell with the other nucleus. There are some proteins of the spindle that interacts with proteins at the neck to orientate the spindle in the axes mother-bud. Therefore, upon Hog1 activation in a *swe1*Δ strain, the complex Hsl1-Hsl7 is delocated and the spindle is not able to orientate properly, obtaining as a result binucleated cells, because the cells enter prematurely in mitosis even the down regulation of the cyclins.

*sic1*Δ cells can not arrest at G1 upon Hog1 activation, this causes premature entry into S phase, and consequently, these cells become osmosensitive (Escote et al 2004). Consistent with these observations, *swe1* deleted cells do not properly arrest at G2 upon stress and also become osmosensitive. Actually, these cells enter prematurely into mitosis without proper adaptation, and these results in high percentage of cells with two nucleus. Synthetic *sic1* and *swe1* mutations results in cells even more osmosensitive than the single mutations alone, osmosensitivity comparable to the osmosensitivity observed *hog1*Δ cells. For that reason, modulation of cell cycle progression by the MAPK Hog1 through diverse checkpoints is a necessary step for the existence of the cells upon stress.

Modulation of G1-S transition and the G2-M is important to essential events in cell cycle, such as replication of DNA and chromosome distribution. These events are critical because improper DNA replication leads to an introduction of errors during duplication, which can lead to in mutations. On the other hand, chromosome distribution is critical for the cell viability. Therefore, upon stress the role of Hog1

MAPK in cell cycle is essential to prevent mutations or altered chromosome segregation.

Its worth to mention that the Hog1 MAPK is implicated at least two checkpoints, the G1-S transition and also the G2-M, which regulate important events in cell cycle, such as the replication of DNA and the chromosome distribution. These events are critical in the offspring, because an improper DNA replication leads to an introduction of mistakes in the duplication, which can translate in mutations. On the other hand, a chromosome distribution in non-better conditions is critical for the viability of the cell. Therefore, upon stress the role of Hog1 MAPK in cell cycle is essential to avoid mutations or cell death.

Localisation of Hsl7 is affected by activation of the HOG pathway

Swe1 stability is regulated by localisation in the bud neck of the septin cytoskeleton, the activity of the checkpoint protein kinase Hsl1 and its interacting protein Hsl7. Actually, Hsl7 interacts directly with both Hsl1 and Swe1 and mutations that impair either of these interactions, or change the localisation of Hsl1 or Hsl7, stabilize Swe1 protein (Lew 2003), (Theesfeld et al 2003) (Hanrahan et al 2003). For that reason, we monitored localisation of septins at the bud neck upon activation of HOG pathway (for instance Cdc11 and Cdc12). After osmostress, septins did not modify their localisation from the bud neck. Therefore, the G2 arrest caused by Hog1 is not caused by the modification of septin localisation. Then we checked whether the localisation at the bud neck of Hsl1 kinase was affected as a consequence of Hog1 activation. Like the septins, Hsl1 did not change its localisation upon osmostress.

Finally, we monitored the Hsl7 localisation at the bud neck in absece or presence of osmostress. Hsl7 localisation noticeably changed upon Hog1 activation. Cells exposed to osmostress showed a delocalised Hsl7 from the bud neck, specially forming patches at the membrane surface. Moreover, the absence of Hsl7 at the bud neck was transient and correlated with Hog1 activation. It was reported that when Hsl7 is delocalised from the bud neck, Hsl7 is rapidly dephosphorylated (Sakchaisri et al 2004). Correspondingly, Hog1 activation results in rapid Hsl7 dephosphorylation. Therefore, activation of the HOG MAPK pathway causes a delocalisation of Hsl7 from the bud neck, which induces Swe1 protein stabilisation.

Hog1 phosphorylates the Hsl7 binding site in Hsl1. Phosphorylation of Hsl1 Ser1220 by Hog1 determines Hsl7 localisation.

We studied whether the MAPK Hog1, in order to regulate Hsl7 localisation to the bud neck, was able to interact with Hsl1 or Hsl7. Co-precipitation assays demonstrated that the MAPK is not able to interact with Hsl7, but interacts with Hsl1 kinase. Therefore upon osmostress the MAPK Hog1 is able to interact with Hsl1 kinase.

Phosphorylation assays also showed that whereas the Hog1 MAPK was not able to phosphorylate Hsl7 or Swe1, Hsl1 was phosphorylated in its C-terminal domain by the MAPK. Furthermore, when yeast cells were treated with an osmotic shock, Hsl1 was phosphorylated, dependent on the presence the Hog1. Therefore, Hog1 MAPK is not only able to interact with the Hsl1 kinase, but also specifically phosphorylates Hsl1 protein in its C-terminal region.

It has been reported that Hsl7 recruitment to the bud neck requires the binding of Hsl7 to the Hsl1, at the C-terminal domain of Hsl1**.** Several kinase assays indicated that Hog1 phosphorylated the Serine 1220 in the C-terminal region of Hsl1. Interestingly, Serine 1220 lays in the middle of the Hsl7-binding domain in Hsl1 (Cid et al 2001). Mutation of Serine 1220 to Alanine completely eliminated the Hog1 phosphorylation of Hsl1. Therefore, Hog1 directly phosphorylates Hsl1, and this phosphorylation is located in the middle of the binding domain to Hsl7.

The relevance of the Hog1 phosphorylation site was monitored by the analysis of the localisation of Hsl7 in cells containing Hsl1 or $Hsl1^{SA}$, (which contains a mutation in the Hog1 phosphorylation site). In cells containing Hsl1, the localisation of Hsl7 was altered dramatically in response to activation of the HOG pathway; on the other hand the localisation of Hsl7 did not change in cells containing the $Hsl1^{SA}$ protein. Hence, the phosphorylation of Hsl1 at the serine 1220 by the Hog1 MAPK is a critical step for the proper localisation of Hsl7 upon an osmotic stress.

Moreover, it is important to mention that an elevated number of cells expressing the Hsl1^{SE} (which can not be phosphorylated by Hog1, but the glutamic mimics the phoshorylated state of this site) did not suitably localised Hsl7 even in absence of stress and, showed an elongated bud morphology reminiscent to *hsl1*Δ cells, which correlates with an accumulation of the Swe1 inhibitor.

Taken together, Hog1 phosphorylation of Hsl1 in the serine 1220 is a critical step for Hsl7 localisation in response to high osmolarity, in order to adapt the cells to the new extracelular conditions delaying the transition between G2 and M phase. Correspondingly, cells containing the Hog1- unphosphorytable allele Hsl1^{SA}, in response to Hog1 activation, were unable to arrest properly at G2 and these cells were more sensitive to osmostress than cells carrying wild type Hsl1.

Exposure of mammalian cells to osmotic imbalances results in the activation of the p38 SAPKs. As observed in yeast, mammalian cells also respond to high osmolarity by modulating cell cycle progression. Essentially, different reports indicate that different type of mammalian cells arrest at several stages of the cell cycle (G1-S, G2 and mitosis) upon osmostress. 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. 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. From the yeast studies we propose 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 cell cycle arrest at

several steps of cell cycle to allow for proper cellular adaptation to extracellular stimuli.

Conclusions

- Osmostress induces a transient cell cycle arrest.

- Upon osmotic stress, the Hog1 MAPK is able to control cell cycle progression at G1 and G2 phases. Cells defiencient in these controls become osmosensitives.

- At G1, Hog1 controls the transition to S phase by a dual mechanism down-regulate Cdc28 activity, which involves G1 cyclins and the Sic1 inhibitor.

- Down-regulation of the expression of G1 cyclins leads to a decrease in the activity of Cdc28 associated to Cln1 and Cln2.

- Upon osmotic stress, Hog1 is able to stabilize the Sic1 inhibitor by a direct phosphorylation on the T173, which reduces the binding of the E3 ligase Cdc4 to Sic1. This results in Sic1 stabilisation.

- At G2, Hog1 controls the transition to M phase by a dual mechanism to down regulate the Cdc28 activity, a similar scenario than in G1, but involving G2 cyclins and the Swe1 inhibitor.

- Down-regulation of G2 cyclins expression leads to a decrease in the activity of Cdc28 associated to Clb2.

- Upon osmotic stress, Hog1 is able to accumulate the Swe1 kinase by an indirect effect. Hog1 phosphorylates Hsl1 kinase at Hsl7-binding domain, at the S1220, which leads to Swe1 accumulation.

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