QUALITATIVE AND QUANTITATIVE STUDY OF THE EFFECT OF OSMOSTRESS ON THE CELL CYCLE OF *Saccharomyces cerevisiae*

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SUMMARY

Control of cell cycle by Stress Activated Protein Kinases (SAPKs) is an essential aspect for adaptation to extracellular stimuli. In *Saccharomyces cerevisiae*, the activation of the Hog1 SAPK, results in a delayed transcription of the G_1 cyclins *CLN1,2* and the stabilization of the B-type cyclin inhibitor *SIC1*, therefore postponing entry into S phase. The results displayed here, show, by a combination of mathematical modelling and quantitative *in vivo* experiments, that, before Start, the control of G_1-S transition is mainly exerted by inhibiting expression of cyclins, both G_1 (*CLN1,2*) and S phase (*CLB5,6*) cyclins. On the other hand, after Start, it is the phosphorylation and stabilization of Sic1 by Hog1 that becomes imperative to prevent inadequate firing of replication before adaptation. Therefore, we found that there is a distinct temporal role for Sic1 and cyclins on the G_1 regulation by a SAPK in response to stress. We have also found that Hog1 induces a G_2 delay, by down-regulating *CLB2* transcription and phosphorylating Hsl1 to promote Hsl7 delocalization and subsequent accumulation of Swe1, an inhibitor of Clb1,2-Cdc28, and thus postponing anaphase. Altogether, we demonstrate novel Systems Biology approaches are useful to better understand how an intracellular signalling pathway incises on cell cycle control, beyond a mechanistic description, as well as showing how a single MAPK modulates different cell cycle checkpoints to improve cell survival upon stress.

El control del cicle cel·lular per Proteïna Cinases Activades per Estrès (SAPKs) es un aspecte essencial per a l'adaptació als estímuls extracel·lulars. A *Saccharomyces cerevisiae*, l'activació de la SAPK Hog1, resulta en un retardament de la transcripció de les ciclines de G_1 (*CLN1,2*) i l'estabilització del inhibidor de les ciclines del tipus B, *SIC1*, i per tant posposa l'entrada en fase S. Els resultats que aquí s'exposen, mostren, mitjançant la combinació de modelatge matemàtic i experiments quantitatius *in vivo*, que, abans d'*Start*, el control de la transició es duu a terme principalment inhibint l'expressió de les ciclines, tant les de G1 (*CLN1,2*) com les de la fase S (*CLB5,6*). Per altra banda, després d'*Start*, la fosforilació i estabilització de Sic1 per part de Hog1 esdevé un fet necessari per a prevenir la iniciació de la replicació abans d'adaptar-se. Per tant, hem descobert aquí que la regulació de Sic1 i les ciclines juguen un paper diferent segons el moment en que apareix l'estrès. Hem descrit també, que Hog1 produeix una parada a G_2 a través de la inhibició de la transcripció de *CLB2* i la fosforilació d'Hsl1, la qual promou la deslocalització d'Hsl7 i la subsegüent estabilització de Swe1, un inhibidor específic de Clb1,2-Cdc28, i d'aquesta manera es posposa l'entrada en Anafase. Tot plegat, demostra que l'ús d'aproximacions pròpies de la Biologia de Sistemes és útil per a entendre de quina forma una via de senyalització intracel·lular incideix sobre el control del cicle cel·lular, mes enllà de la pura descripció de la mecànica del sistema. D'aquesta forma, proposem que una sola MAPK modula distints punts de control del cicle cel·lular per millorar la probabilitat de supervivència en front de l'estrès osmòtic.

PREFACE

The accumulation of multiple efforts and landmark advances along years, make possible the fulfilling of any current scientific work. The dissertation hereafter expounded, could be considered hire of both lineages of investigation: the study of cell cycle control and cell signalling. Starting from the first genetic descriptions of cell cycle, forty years ago, and after discovery of all the different regulators, scientists have nowadays a quite complete picture of the biochemical mechanism of cell cycle control in budding yeast. This knowledge constitutes, at the present time, the basis for further understanding of cell cycle in higher eukaryotes. Similarly, the current understanding of signal transduction processes is the result of numerous contributions made to the field over many years by different research groups all over the world. After the finding of the structure of these signalling pathways by means of genetic tests of epistasis, the cloning and identification of their components, functional and biochemical characterization, the field is now moving on the road to a different era: the understanding of their regulation as a whole.

This work is at the boundary between classical cell biology studies and the upcoming Systems Biology approaches, being a good example of the expansion of research in the field towards quantitative biology and its interplay with mathematical modelling. Besides describing a new mechanism of cell cycle control and how it relates to a pre-existing checkpoint, this work is an endeavour to find out the reason why such a mechanism to be the way it is, rather than just making a mere description of the system.

My personal feeling, as a young scientist, is quantitative biology, as well as yeast as model organism, will be widely used in fundamental biomedical research.

And the future is jet to be written.

The author

TABLE OF CONTENTS

SUMMARY.. 1

PREFACE.. 3

INTRODUCTION.. 9

1. OSMOTIC STRESS.. 9

1.1. Fluctuations in osmolarity…………………………………… 9

1.2. Signalling pathways involved in osmoadaptation........... 11

2. MAPK PATHWAYS…………………………………………… 12

2.1. Architecture of the central core………………………….. 13

2.2. Signalling specificity……………………………………… 14

2.3. MAPK pathways in *Saccharomyces cerevisiae*……….. 15

3. THE HOG PATHWAY.. 17

3.1. Osmosensors of the HOG pathway.............................. 18

3.2. Signal transduction and feedback regulation................ 20

4. PHYSIOLOGICAL ROLES OF THE HOG PATHWAY........ 22

4.1. Metabolic adaptation.. 22

4.2. Regulation of protein synthesis.................................... 23

4.3. Gene expression.. 24

5. REGULATION OF CELL CYCLE IN *Saccharomyces cerevisiae*.. 25

5.1. The cell cycle in *S. cerevisiae*...................................... 25

5.2. Cell cycle regulation in *S. cerevisiae*............................ 26

5.2.1. The Cyclin Dependent Kinase Cdc28................. 26

5.2.2. The cyclins.. 28

5.2.3. Cdc28 phosphorylation and cell-cycle-dependent inhibitors; Sic1 and Swe1.................. 30

5.2.4. Cell cycle transcriptional regulation.................... 31

5.2.5. Regulation of cell cycle by proteolysis................ 33

5.3. Cell cycle checkpoints.. 37

5.3.1. The Intra S Phase DNA Damage Checkpoint...... 37

5.3.2. Spindle Checkpoints.. 38

5.3.3. The Morphogenesis Checkpoint......................... 39

6. CELL CYCLE CONTROL BY THE HOG1 MAPK............... 41

6.1. Implications of SAPKs on the control of cell cycle......... 41

6.2. Hog1-mediated arrest at G1.. 42

6.2. Hog1-mediated arrest at G2-M.. 43

7. MATHEMATICAL MODELING OF SIGNALLING PATHWAYS AND CELL CYCLE... 45

OBJECTIVES.. 47

RESULTS AND DISCUSSION.................................. 49

1. A quantitative approach reveals that the Hog1 SAPK regulates G1-S progression upon stress by independently controlling Clns, Sic1 and Clb5... 49

2. Phosphorylation of Hsl1 by Hog1 leads to a G2 arrest essential for cell survival at high osmolarity…………………....... 107

GLOBAL DISCUSSION.. 129

CONCLUSIONS... 133

REFERENCES.. 135

INTRODUCTION

1. OSMOTIC STRESS

Single cell organisms living freely in nature are exposed to highly variable environmental conditions that may threaten their survival or reduce their fitness. These environmental threats are commonly termed cell stresses. Cellular stresses may be of a physical nature; changes in temperature, pressure or presence of radiation. Or of chemical nature; nutrient and oxygen availability, pH or concentration of solutes (Hohmann, 2002). In response to these stresses, cells must be able to coordinate a range of biological activities in order to adapt to these conditions and improve chances of survival and proliferation.

Osmotic stress is the condition in which cells encounter difficulties maintaining proper and steady intracellular water activity (a_W). We refer to water activity as the chemical potential of free water in solution. As in mild environments, a_W depends mostly on the concentration of osmotically active solutes (osmolarity), it is often measured as the inverse value of the osmotic pressure. Osmotic stress may be caused by sudden changes in water activity due to variations in the osmolarity of the surrounding solution, or by constantly low water activity in the medium due to external osmolyte accumulation.

It is important for cell survival that intracellular osmolarity keeps a_W inside the cell lower than the one in the surrounding medium, allowing for retention of enough water to support biochemical reactions and generate turgor pressure.

1.1. Fluctuations in osmolarity

In a free environment, two different situations might alter water activity; a decrease in osmolarity (hypoosmotic shock), or an increase osmolarity (hyperosmotic shock). During a hypoosmotic shock, environmental a_W rises up, water flows into cells, increasing turgor pressure and inducing swelling.

When cells experience a hyperosmotic shock, in which a_W of the environment drops, water rapidly outflows cells, as water follows its concentration gradient by passive diffusion (Hohmann, 2002). This water outflow drops turgor pressure and cells shrink. The resulting phenomenon is an increased concentration of biomolecules and ions inside the cell that compromise several aspects of cellular activity; an imbalance of the membrane potential and thereby the activity of transmembrane transporters is compromised (Norbeck and Blomberg, 1998); disruption of ion homeostasis and intracellular pH equilibrium (Vindelov and Arneborg, 2002) result in protein synthesis impairment and generation of reactive oxygen species (ROS) (Koziol *et al.*, 2005; Norbeck and Blomberg, 1998).

In the natural environment of budding yeast, *Saccharomyces cerevisiae*, osmolarity may widely and rapidly fluctuate. Hence, yeast cells have developed mechanisms to cope with constant changes in osmotic pressure. Since passive flow of water occurs very fast, this mechanisms evolved to be rapidly activated after sudden osmotic variations (Blomberg and Adler, 1992; Brown, 1976). The adaptation to increased osmolarity comprises three active essential processes: sensing external osmolarity, intracellular signalling and effective response to restore optimal cellular activity. In *S. cerevisiae*, the production and accumulation of chemically inert osmolytes, mainly glycerol, allows cells to increase the internal osmolarity, recovering the osmotic balance and playing a central role in the process of osmoadaptation (de Nadal *et al.*, 2002; Gustin *et al.*, 1998; Hohmann, 2002). This allows yeast cells to be metabolically active and proliferate over a wide range of external water activities. The time course of events upon osmotic shock depends on its severity and the ability of cells to respond to it.

1.2. Signalling pathways involved in osmoadaptation

Several MAPK cascades with functional preservation from yeast to mammals have been identified to respond to hypertonicity, and have been studied at the molecular level (Sheikh-Hamad and Gustin, 2004). In fission yeast, *Schizosaccyaromyces pombe*, the SAPK Sty1 responds to a whole range of stress conditions (Hohmann, 2002). Among the triggers of the Sty1 response there is the osmotic stress, as deletion of Sty1 or its MAPKK, Wis1 renders osmosensitive cells, among other effects (Millar *et al*., 1995). In mammalian cells, several are the signalling pathways activated by osmotic stress; the three major MAPK subfamilies p38, ERK and JNK, although p38 is the major coordinator of the response to hyperosmolarity; the non-receptor tyrosine kinases Fyn and Syk, PKC, PKA, PAK2 and the DNA damage-inducible kinase (Ferraris *et al*., 2002; Irarrazabal *et al*., 2004; Jiang *et al*., 1996; Miah *et al*., 2004; Sheikh-Hamad and Gustin, 2004).

A number of signalling pathways are activated upon osmotic stress in *S. cerevisiae*: the Protein Kinase A pathway, the phosphatidylinositol-3,5-bisphosphate pathway and the best characterized system, the <u>H</u>igh <u>O</u>smolarity <u>G</u>lycerol response pathway (HOG). The protein kinase A (cyclic AMP cAMP-dependent protein kinase) pathway affects expression of genes upon hyperosmotic shock (Norbeck and Blomberg, 2000). Moreover, this pathway mediates a general stress response observed under essentially all stress conditions, such as heat shock, nutrient starvation, high ethanol concentration, oxidative stress and osmotic stress (Marchler *et al*., 1993). For this reason, protein kinase A most probably does not respond directly to osmotic changes. In fact, it is not clear how the activity of protein kinase A is regulated by stress. It has also been observed that an osmotic stress stimulates production of phosphatidylinositol-3,5-bisphosphate, a molecule that might be a new type of phosphoinositide second messenger in an osmotic signalling

system (Dove *et al*., 1997). The HOG pathway is a Mitogen-Activated Protein Kinase (MAPK) cascade. It consists of a conserved eukaryotic signal transduction module, and its involvement in the process of osmoadaptation has been clearly established (de Nadal *et al*., 2002; Gustin *et al*., 1998; Hohmann, 2002). Many basic principles of osmoadaptation are conserved across eukaryotes, and therefore the HOG pathway in *S. cerevisiae* is an ideal model system for the study of these processes. The HOG pathway is the best-understood osmoresponsive system in eukaryotes. It is activated within less than one minute by a hyperosmotic shock and cells defective for this pathway, or unable to activate it, can not survive in high-osmolarity medium (Brewster *et al*., 1993). Thus, the role of HOG is to orchestrate a significant part of the response of yeast cells to high osmolarity.

2. MAPK PATHWAYS

Eukaryotic cells have highly complex signalling pathways; each of them is preferentially activated by diverse stimuli, thereby allowing cells to quickly adapt to changing environments. Amongst signalling pathways, the Mitogen-Activated Protein Kinase (MAPK) signal transduction pathways, originally identified as transducers of growth and differentiation promoting signals, are found both in higher and unicellular eukaryotic cells and show a high level of conservation between organisms.

The ERK, JNK and p38 families are amongst the mammalian MAPK pathways and can be activated by a wide variety of different stimuli like hormones, growth factors and cytokines to coordinate cell growth, proliferation and survival (Chen *et al*., 2001). But it is now known that this MAPKs respond to environmental stresses, as well, such as osmotic shock, ionizing radiation, heat stress, and ischemic injury (Kyriakis and Avruch, 2001). These pathways even respond to some intracellular signals like DNA damage or protein synthesis impairment (Benhar *et al*., 2001; Kyriakis *et al*., 1994). It

is because of this kind of triggering stimuli that these pathways are commonly termed Stress-Responsive MAPKs (SAPKs).

2.1. Architecture of the central core

MAPK cascades convey intracellular signals in the form of sequential phosphorylation events. MAPKs are phosphorylated by MAP kinase kinases (MAPKKs), which in turn are phosphorylated by MAP Kinase Kinase Kinases (MAPKKKs), see **Figure 1**.

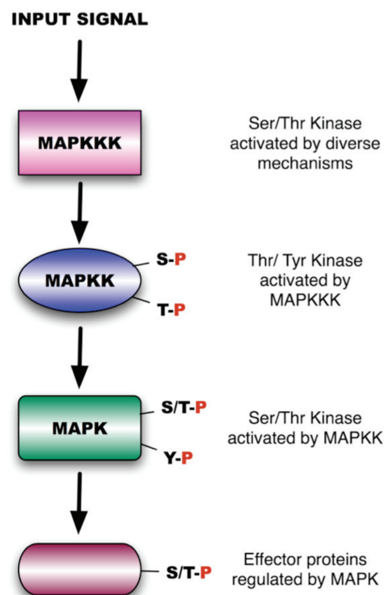

Figure 1. Schematic diagram of a MAPK core module. The central core of a MAPK pathway is composed of three protein kinases, a MAPK kinase kinase, a MAPK kinase and the MAPK that are sequentially activated by specific phosphorylations.

MAPKKKs consist of a regulatory N-terminal domain and a C-terminal catalytic kinase domain. The regulatory domain keeps the C-terminal kinase domain locked until the kinase domain is unleashed by phosphorylation through upstream protein kinases or by interaction with other proteins as small G-proteins. Once MAPKKKs become active, they phosphorylate MAPKKs on serine and threonine within a conserved part at the N-terminal lobe of the

kinase domain. Subsequently, MAPKKs phosphorylate a MAPK on a threonine or serine and a tyrosine residue separated by a single amino acid (Thr/Ser-X-Tyr). These phosphorylation sites are located in the activation loop of the catalytic domain and are essential for the activation of the MAPK.

The activation of MAPKs often stimulates its nuclear accumulation, where it phosphorylates target proteins on serine/threonine residues followed by a proline. However, some MAPK protein remains in the cytoplasm, indicating that it can mediate other events than those in the nucleus (Reiser *et al*., 1999). Most of the already defined substrates for MAPKs are transcription factors. However, MAPKs have the ability to phosphorylate many other substrates including other protein kinases, phospholipases, cytoskeleton-associated proteins and ionic transporters (Chen *et al*., 2001).

2.2. Signalling specificity

Different MAPK pathways may interact through crosstalk and even some individual upstream elements can be found participating in different pathways, as they are often subjected to regulation by multiple inputs. Given the complexity and diversity of MAPK regulation and function, it is critical for cells to preserve an appropriate regulation and selectivity of each MAPK pathway. For this reason, pathway wiring and specificity of signal transduction are controlled by means of scaffolding proteins. Scaffold proteins bind and sequester selected MAPK pathway components, maintaining the architectural integrity and allowing a coordinated and selective activation of the intermediate proteins in response to specific types of stimuli (Pawson and Scott, 1997). In some MAPK pathways, the signalling components themselves possess intrinsic scaffolding properties, such as the yeast MAPKK Pbs2.

Alternatively; accessory scaffolding elements bind and segregate the activation of groups of proteins, therefore intervening in the regulation of signalling components. This is the case of the *S.*

cerevisiae Ste5 protein that allosterically modulates the signal output (Bhattacharyya *et al*., 2006). More over, it has been recently shown that the regulation of scaffolding proteins is an important modulator of the signal flow through MAPK pathways (Garrenton *et al*., 2008).

Despite the important role of scaffolding proteins in maintaining and modulating signalling specificity, there are other mechanisms to ensure the integrity of the signalling transduction, such as the MAPK substrate specificity. Although MAPKs are proline-directed kinases, MAPK achieve substrate selectivity by recognizing specific docking sites on physiological substrates. These docking sites are often at a considerable distance from the phosphorylation site in the primary sequence, but not in the tertiary structure. It is this docking specificity of elected MAPKs on target substrates that excludes the interaction with other MAPKs, avoiding inappropriate phosphorylations (Kallunki *et al*., 1996; Tanoue and Nishida, 2002). On the other hand, MAPK pathways are negatively controlled by protein phosphatases acting on both the MAPKK and the MAPK (serine-threonine phosphatases) or only on the MAPK (tyrosine phosphatases) (Keyse, 2000). The regulation by phosphatases widens out of individual MAPK pathways, proven to be also a mechanism of crosstalk (Junttila *et al*., 2008; Martin *et al*., 2005).

2.3. MAPK pathways in *Saccharomyces cerevisiae*

The understanding of the *S. cerevisiae* MAPK pathways is more complete than MAPK pathways in other organisms. For this reason, budding yeast is nowadays an ideal model to study these pathways. Extensive genetic and biochemical analysis revealed that *S. cerevisiae* contains five MAPKs conforming five functionally distinct cascades (Gustin *et al*., 1998; Hunter and Plowman, 1997). Even though these pathways conform distinct cascades they share upstream regulators, giving rise of crosstalk events, see **Figure 2** (Chen and Thorner, 2007). Four of these pathways, the mating, the

filamentation-invasion, the cell integrity and the high osmolarity pathways, are present in growing cells. The Smk1 MAPK, part of the spore wall assembly pathway, appears during sporulation and is the master regulator of this developmental process (Krisak *et al.*, 1994).

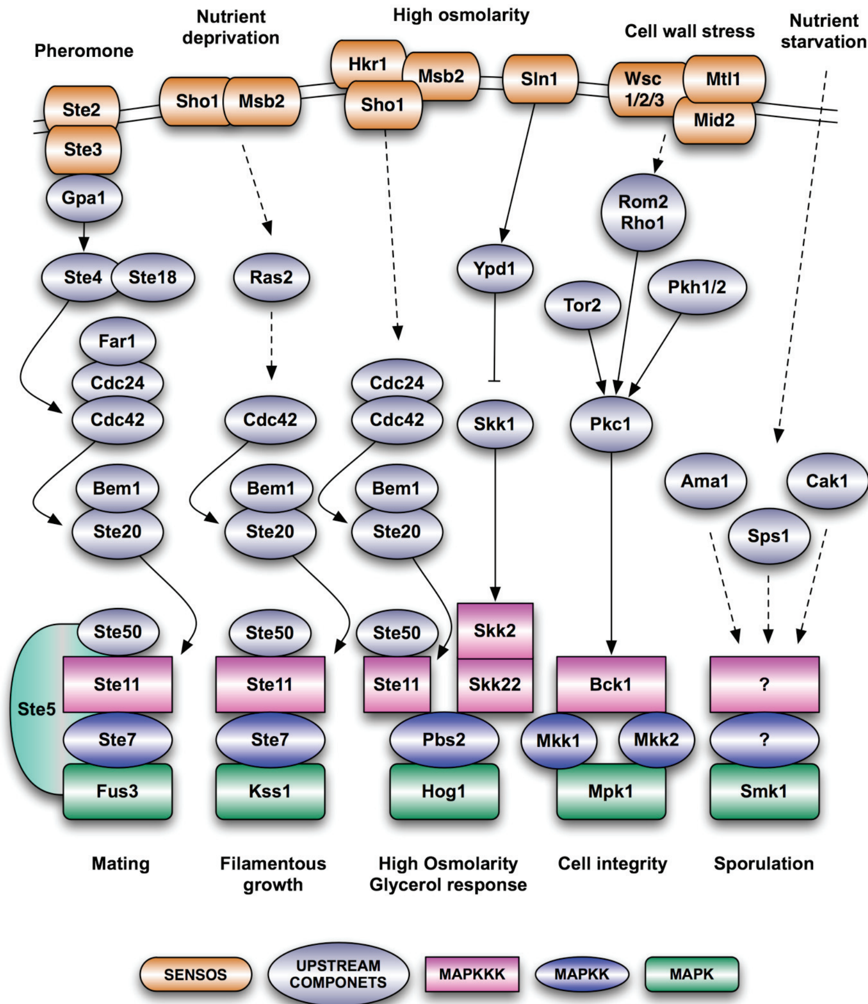

Figure 2. Scheme of MAPK pathways in *S. cerevisiae*. Five MAPK pathways described in budding yeast; mating response, filamentation, osmoadaptation, cell wall integrity and sporulation. All pathways show the MAPK core structure and some share upstream components.

3. THE HOG PATHWAY

The HOG pathway is the best-characterized osmoresponsive system in eukaryotes and hence serves as a prototype for the study of MAPK pathways. The HOG pathway specifically responds to increased extracellular osmolarity and is required for cell survival under these conditions (de Nadal *et al*., 2002; Hohmann, 2002). The activation of this pathway results in the initiation of a set of osmoadaptive responses, which includes metabolic regulation, delay in cell cycle and gene expression regulation. As many other yeast signalling pathways, the HOG pathway has its equivalent system in mammalian cells, the p38 and the c-Jun N-terminal kinase (JNK) pathways. The high homology between the Hog1 and the JNK and p38 SAPKs became relevant by the functional replacement of Hog1 by these two mammalian SAPKs in yeast (Galcheva-Gargova *et al*., 1994; Han *et al*., 1994).

The Hog1 MAPK cascade consists of five protein kinases; three MAPKKKs, Ssk2, Ssk22 (Maeda *et al*., 1995) and Ste11 (Posas and Saito, 1997) activate a single downstream MAPKK, Pbs2, that in turn activates a single MAPK, Hog1 (Brewster *et al*., 1993; Maeda *et al*., 1994), see **Figure 3**.

The pathway is activated by two upstream independent mechanisms that converge on the MAPKK, commonly termed “branches”. The first branch involves a two-component osmosensor constituted by the Sln1-Ypd-Ssk1 proteins and the MAPKKKs Ssk2 and Ssk22. The second mechanism involves the transmembrane protein Sho1 and the mucins Hrk1 and Msb2, which most probably correspond to the osmosensor system (Tatebayashi *et al*., 2007), and the MAPKKK Ste11, accompanied by the Ste11-binding proteins Ste50, Ste20 (p21-activated kinase, PAK), and the small GTP-ase Cdc42 (Hohmann, 2002; Posas *et al*., 1998).

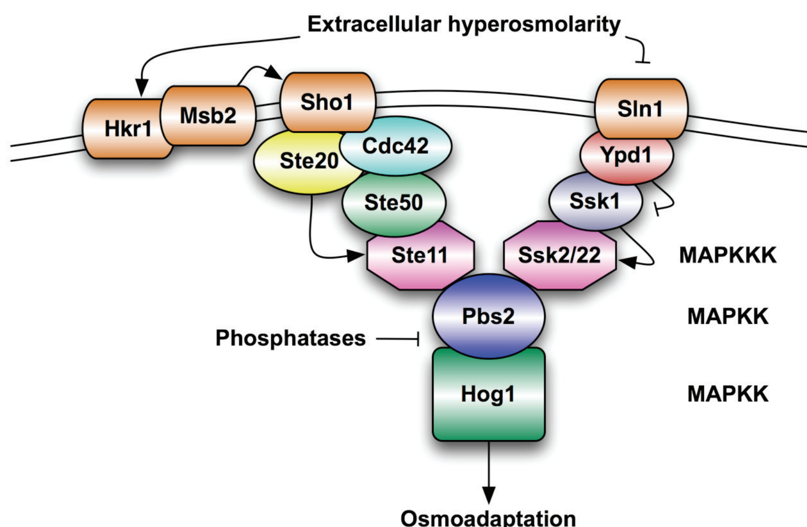

Figure 3. Simplified schematic representation of the HOG pathway. Two independent osmosensing mechanisms activate their corresponding subset of MAPKKKs that converge on the activation of a single MAPKK, Pbs2. Upon increased external osmolarity, Pbs2 activates the MAPK, Hog1, which is the responsible for triggering the osmoadaptive response.

3.1. Osmosensors of the HOG pathway

The HOG pathway accounts with two independent mechanisms upstream of the MAPKK Pbs2; namely the Sln1 and the Sho1 branches, according to the first described most upstream molecule, Sln1p and Sho1p respectively. Genetic evidences suggest that the upstream branches of the HOG pathway operate independently of each other; blocking one branch of the pathway still allows for Hog1 phosphorylation upon an osmotic shock, and such cells are apparently fully resistant to high osmolarity. Although these observations suggest redundant functions, it has been proposed that different sensitivities of the two branches may allow the cell to respond over a wide range of osmolarity changes by being differentially sensitive to changes in osmolarity, on top of having completely different signalling properties (Maeda et al., 1995).

The Sln1 branch involves a “two-component” osmosensor composed of a sensor molecule, Sln1 (Maeda *et al*., 1994; Ota and Varshavsky, 1993); and a response-regulator, the complex Ypd1-Ssk1. The Sln1-Ypd1-Ssk1 complex constitutes a phosphorelay system able to detect changes in turgor pressure and transmit the signal through the HOG pathway (Posas *et al*., 1996; Reiser *et al*., 2003). Typically, the sensor protein has an extracellular receptor domain and a cytoplasmatic histidine kinase domain. The response-regulator is cytosolic and contains a phosphate-receiver domain and a DNA binding domain. At normal osmolarity, the osmosensor Sln1 constantly autophosphorylates itself and the phosphate is then sequentially transferred to its receiver domain, then to a specific histidine on Ypd1p and finally, to an aspartic residue on Ssk1, which, in turn, represses the activity of two redundant MAPKKs: Ssk2 and Ssk22. At high osmolarity, however, the Sln1 histidine kinase activity is inhibited, resulting in an accumulation of unphosphorylated Ssk1, which turns into an activator of both Ssk2/Ssk22 redundant MAPKKKs and activates the signal cascade (Posas and Saito, 1998). Genetic disruption of the *SLN1* gene is lethal, due to the resulting constitutively activation of the HOG pathway (Maeda *et al*., 1994).

The Sho1 branch has been more elusive, for it engages many more proteins with diverse functions (Chen and Thorner, 2007). Up to date, the actual osmosensing mechanism is still unknown, despite the highly glycosylated mucin family proteins Hkr1 and Msb2 have been identified as potential osmosensors of the branch with most likelihood (Tatebayashi *et al*., 2007). These two transmembrane proteins have been proven to be the most upstream activators of Sho1 and therefore, the idea of Sho1 being the actual osmosensor molecule seems declinable (Raitt *et al*., 2000; Tatebayashi *et al*., 2007). Sho1 is a transmembrane protein with a cytoplasmic Src-homology 3 (SH3) domain (Maeda *et al*., 1995). The activation of the Sho1 branch entails a rapid recruitment of proteins to the cell surface (Raitt *et al*., 2000).

In high osmolarity conditions, both Hrk1 and Msb2 activate Sho1 (Tatebayashi *et al*., 2007). Once activated, Sho1 recruits Pbs2 to the plasma membrane by interacting via its SH3 domain with the proline-rich N-terminal domain on the MAPKK (Maeda *et al*., 1995; Posas and Saito, 1997). Then the rho-like G protein Cdc42 is recruited (Raitt *et al*., 2000). On one hand, active Cdc42 binds and activates the PAK-like kinase Ste20 and the kinase Cla4, on the other hand, Cdc42 binds the Ste11-Ste50 complex through a conserved C-terminal RAS-association (RA) domain of Ste50 (Truckses *et al*., 2006). Thus Cdc42 serves as scaffold to enclose active Ste20 and Cla4 their substrate Ste11 (Tatebayashi *et al*., 2006). Concurrently, active Ste11 and its substrate, Pbs2, are brought together by Sho1, as the Ste11-Ste50 complex and Pbs2 bind to the same cytoplasmic domain of Sho1 (Zarrinpar *et al*., 2004). At the final course of events, Ste11 activates Pbs2, which in turn, activates Hog1 (Posas and Saito, 1997). Opy2, a type 1 transmembrane protein, has also been implicated in this branch, as the mutations *opy2Δ ssk1Δ* are synthetically osmosensitive (Wu *et al*., 2006), but its role in signalling seems to be the targeting of Ste50 to the plasma membrane, more than intervening in the process of sensing (Tatebayashi *et al*., 2007; Wu *et al*., 2006). Besides the mechanism described above, Msb2 is able to sense osmostress and trigger the HOG pathway signalling though a Sho1-independent mechanism but that implies the whole branch downstream of Sho1 (O'Rourke and Herskowitz, 2002; Tatebayashi *et al*., 2007). Thus, upon osmotic stress, Hrk1 and Msb2 induce the formation of a complex that includes Cdc42, Ste50, Sho1 and Opy2, which acts as adaptor to control the flow of the osmostress signal from Ste20 to Ste11 and then to Pbs2.

3.2. Signal transduction and feedback regulation

Upon activation of the sensor systems, the MAPKK Pbs2 becomes activated through phosphorylation on Ser514 and Thr518 by any of the three MAPKKKs; Ssk2, Ssk22 or Ste11. A dual Pbs2-mediated phosphorylation on the conserved residues Thr174 and Tyr176 activates the MAPK Hog1 (Brewster *et al*., 1993). This

phosphorylation induces a fast conspicuous accumulation of Hog1 in the nucleus, although under normal conditions, Hog1 appears to be distributed between the cytosol and the nucleus (Ferrigno *et al*., 1998; Reiser *et al*., 1999). Both Hog1 phosphorylation and nuclear localization are transient events (Ferrigno *et al*., 1998). The timing of activation depends on the severity of the osmotic shock: lasting for about 30 minutes under mild osmotic stress (0.4M NaCl), up to few hours under severe osmotic shock (1.4M NaCl) (Van Wuytswinkel *et al*., 2000). Correspondingly, this delay correlates with a delay in stress-responsive gene expression (Zapater *et al*., 2007).

The nuclear accumulation of Hog1 upon its activation, suggests a significant part of Hog1 activity takes place in the nucleus. However, a portion of active Hog1 protein remains in the cytosol, where it mediates other regulatory effects. Amongst the best documented cytosolic effects of Hog1 there is the activation of the protein kinase Rck2 (Bilsland-Marchesan *et al*., 2000), which controls translation efficiency (Teige *et al*., 2001). Another example of cytosolic activity of Hog1 is the phosphorylation of ion transporters (Proft and Struhl, 2004).

The observed transient Hog1 phosphorylation and activation hints the pathway might be controlled by some kind of feedback mechanisms. If truth be told, a downstream mechanism has been described, which involves several phosphatases. Two phosphotyrosine phosphatases, Ptp2 and Ptp3, as well as three phosphoserine/threonine phosphatases, Ptc1 to Ptc3, are known to genetically interact with HOG. Over-expression of any of these phosphatases suppresses the lethality caused by permanent activation of the pathway (Jacoby *et al*., 1997; Maeda *et al*., 1994; Mattison and Ota, 2000; Ota and Varshavsky, 1992; Warmka *et al*., 2001; Wurgler-Murphy *et al*., 1997).

There is an obvious and direct negative feedback loop between Hog1 and Ptp2/Ptp3, as Hog1 activity itself enhances Ptp2 activity (Wurgler-Murphy *et al*., 1997) and Ptp3 expression (Jacoby *et al*.,

1997). This feedback regulation has been proposed to play a critical role in the dynamic activation and inactivation of Hog1, given that Pbs2 and Ptp2 interact with Hog1 through adjacent docking sites (Murakami *et al*., 2008). Since, even in the *ptp2*Δ *ptp3*Δ double mutant, the levels of tyrosine-phosphorylated Hog1 are still reactive to osmotic shock, it seems that additional dephosphorylation mechanisms must exist (Jacoby *et al*., 1997; Wurgler-Murphy *et al*., 1997). Among the serine/threonine phosphatases, Ptc1 seems to be the one that truly functions in the deactivation of the HOG pathway, by directly dephosphorylating Hog1 (Warmka *et al*., 2001).

4. PHYIOLOGICAL ROLES OF THE HOG1 PATHWAY

Once activated, Hog1 elicits the program for cell adaptation to osmotic stress, which includes modulation of several aspects of cell biology essential for cell survival, such as gene expression, cell cycle progression, protein synthesis and metabolic adaptation (Hohmann, 2002), see **Figure 4**. Moreover, it is likely that still unknown effects like chromatin remodelling or modulation of protein degradation are also mediated by this MAPK.

4.1. Metabolic adaptation

Hog1 modulates metabolism through induction of gene expression and posttranslational modification of some enzymes. Among the Hog1 targets one can find the relevant stress responsive transcription factors Hot1, Sko1 and Msn2,4 (Rep et al., 2000); (Proft et al., 2001); (Hohmann, 2002); (Alepuz et al., 2003). These transcription factors induce the expression of osmolyte-synthesizing genes, e.g. *GPD1* (encoding glycerophosphate dehydrogenase 1) (Albertyn *et al*., 1994) and *TPS2* (encoding trehalose phosphate phosphatase) (Gounalaki and Thireos, 1994). The induction of these genes increases the levels of the compatible osmolytes glycerol and trehalose and restores the osmotic gradient

through the plasmamembrane after a hyperosmotic shock. On the other side, Hog1 phosphorylates and activates the 6-phosphofructo-2-kinase (Pfk26) (Dihazi *et al*., 2004). The activation of Pfk2 stimulates the upstream glycolisis pathway, improving substrate availability for glycerol synthesis and allowing for up to three times more glycerol accumulation, which is necessary to generate osmoresistance (Dihazi *et al*., 2004).

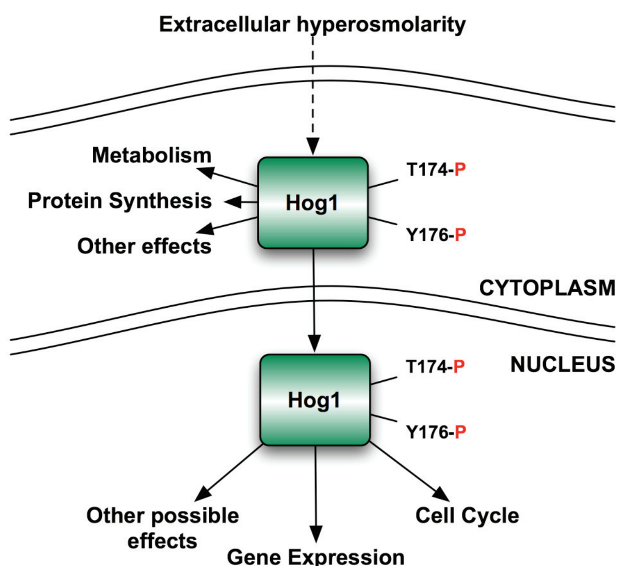

Figure 4. Downstream effector mechanisms of the MAPK Hog1. Upon activation by phosphorylation, Hog1 controls several functions both in the cytoplasm and in the nucleus.

4.2. Regulation of protein synthesis

There is evidence that under osmotic stress protein translation is transiently diminished (Norbeck and Blomberg, 1998). Rck2 is a member of the calmodulin protein kinase family that phosphorylates and thereby inhibits the translation elongation factor EF-2. Rck2 is a direct target of Hog1 (Bilsland-Marchesan *et al*., 2000) and both Hog1 and Rck2 are needed for osmostress-induced inhibition of protein translation (Teige *et al*., 2001).

It has been known for a long time that there is an overall reduction of protein synthesis upon osmotic stress (Varela *et al*., 1992). It was much later, though, that direct regulation over translation was acknowledged and it has even proposed that stress granules and P-bodies are implicated in yeast (Buchan *et al*., 2008; Uesono and Toh, 2002), as well as global changes in ribosomal association (Melamed *et al*., 2008). However, there is expression of genes that are important for stress adaptation (Posas *et al*., 2000) and, evidently, their translation must be ensured (Uesono and Toh, 2002).

Gradually more evidences of a preferential translation of subsets of mRNAs under certain conditions have arisen. For instance, Hog1 increases the levels and stabilizes stress-responsive transcripts, being this effect quite widespread among these transcripts. It has been shown that cells destabilize stress-induced mRNAs as a mean to recover the initial transcriptome in preparation for the subsequent recovery during a transient osmostress (Molin et al., 2009), and also that differentially regulates stress-responsive mRNAs import and export from P-Bodies (Romero-Santacreu et al., 2009).

4.3. Gene expression

Approximately the 7% of all genes in *Saccharomyces cerevisiae* show significant transient changes in their expression levels following osmotic shock (Causton *et al*., 2001). These osmostress-regulated genes are implicated in carbohydrate metabolism, general stress protection, protein biosynthesis and signal transduction (Hohmann, 2002), and are, in a large part, dependent on the Hog1 MAPK (Posas *et al*., 2000).

Hog1 directly regulates gene expression under osmostress conditions through at least five known transcription factors. That is; the zinc finger proteins Msn2 and Msn4 (Rep *et al*., 2000), Hot1 (Rep *et al*., 1999), the MADS box protein Smp1 (de Nadal *et al*.,

2003) and the bZIP protein Sko1 (Proft *et al*., 2001). Extensive studies of global gene expression have allocated different subsets of genes to ach of these transcription factors, although only a subset of the Hog1-induced genes are dependent on them, indicating that others may also intervene in the transcriptional response to osmotic stress.

Besides the regulation over transcription factors, the MAPK Hog1 is found in the promoters and ORFs directly intervening in transcription initiation and elongation (Alepuz *et al*., 2001; Proft *et al*., 2006). Hog1 drives chromatin remodelling of stress responsive genes, via the recruitment of the Chromatin Structure Remodelling (RSC) Complex (Mas *et al*., 2009). Moreover, Hog1 is involved in the recruitment of various complexes that facilitate the transcription. These include the RNA polymerase II complex, the Histone Deacetylase Rpd3-Sin3 complex, the mediator and SAGA complexes and the nucleosome remodelling SWI/SNF complex (Alepuz *et al*., 2003; De Nadal *et al*., 2004; Zapater *et al*., 2007). Perturbation of the function of any of these complexes impairs the adequate transcriptional induction of Hog1 target genes.

5. REGULATION OF CELL CYCLE IN *Saccharomyces cerevisiae*

5.1. The cell cycle in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a single-celled fungus from the phylum Ascomicota that experiences asexual and sexual reproductive cycles. However, the most common mode of vegetative growth in yeast is asexual reproduction by budding (Balasubramanian *et al*., 2004). Under certain conditions diploid cells undergo pseudohyphal growth or sporulation by entering into sexual reproduction, or meiosis. The resulting haploid spores can enter into a vegetative mitotic haploid cell cycle or go on to mate, reconstituting a new diploid cell (Neiman, 2005).

The mitotic haploid cycle shows sexual dimorphism, determined by two alleles of the *MAT* locus; *MATa* and *MATα*. It divides in four prototypical phases: G1, when growth mostly occurs; S-phase, when DNA is replicated, bud emerges and the spindle pole body is duplicated; G2, when the spindle alignment occurs; and M-phase, or mitosis, when DNA segregates and cytokinesis takes place. Analogous to the mammalian restriction point, Start is a point nearby the G1-S transition where cells become committed to irreversibly enter the mitotic cycle and assures coordination of division and growth (Hartwell *et al*., 1974), see **Figure 5**.

5.2. Cell cycle regulation in *S. cerevisiae*

All cell cycle events in *S. cerevisiae* are biochemically coordinated by a single cyclin-dependent kinase (CDK), Cdc28 in association with phase-specific activator proteins, the cyclins, see **Figure 5**. These complexes constitute the core machinery of cell cycle control.

Conceptually, cell cycle control in *S. cerevisiae* can be interpreted as a multilayer regulatory program, consisting of distinct coupled mechanisms that ensure cell cycle progression in an orderly fashion. A first layer is the time-wise election of CDK specificity by binding to cyclins, being these latter ones timely controlled by transcriptional, posttranslational modification, inhibition and proteolysis events. A second layer is the control of CDK activity through phosphorylation and phase-specific inhibitors, and a third layer, is an intricate transcriptional program, which is at the same time, regulating and regulated by cyclins.

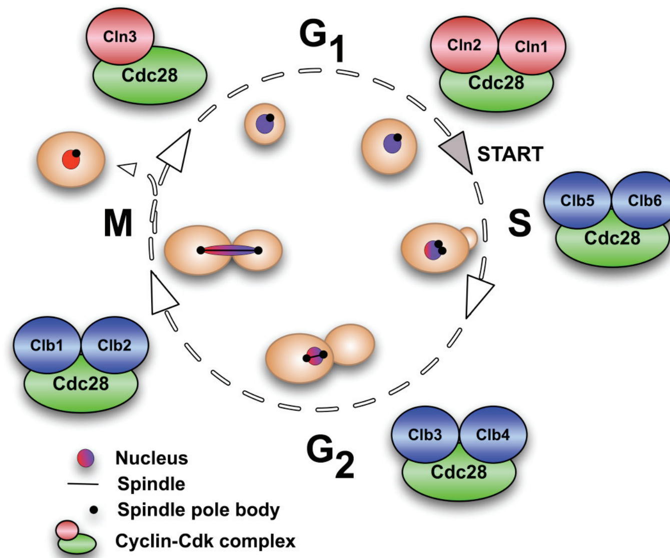

Figure 5. The haploid cell cycle of budding yeast. The CDK Cdc28 associates to phase-specific cyclins (represented in the outer part of the circle) to regulate the timing of the events along cell cycle such as bud emergence, nuclear migration and DNA duplication and distribution among daughter cells (represented in the inner part of the circle).

5.2.1. The Cyclin Dependent Kinase Cdc28

Although other cycle-dependent kinases exist in *S. cerevisiae*, there is a single prototypical CDK, Cdc28 or Cdk1, encoded by the essential gene *CDC28*. It was originally described as a *ts* mutation that blocked cell cycle at Start and it was quickly recognized as the main coordinator of budding yeast cell division cycle (Hartwell *et al*., 1973).

Cdc28 is a highly conserved proline-directed serine/threonine kinase, although, like CDKs from other organisms, individual Cdc28-cyclin complexes have more stringent specificities. The Cdc28-cyclins complexes constitute the central core of the cell cycle control machinery. Unlike other proteins, its protein levels are not regulated, for they are virtually steady throughout the cell cycle and proven to naturally occur in excess (Mendenhall *et al*., 1987; Wittenberg *et al*., 1990). Cdc28 activity and specificity are,

otherwise, timely and tightly controlled through the regulation of different cyclins, phase-specific phosphorylations and inhibitors, see **Subheadings 5.2.2 to 5.2.5**.

5.2.2. The Cyclins

Cyclins were first discovered as proteins that appear and disappear in synchrony during cell cycle. They were soon defined by their ability to bind and activate a CDK and are often recognized by the presence of a conserved domain, the *cyclin box*, which is a region required for binding and activation of CDKs (Evans *et al*., 1983; Kobayashi *et al*., 1992; Mendenhall and Hodge, 1998).

In *S. cerevisiae*, Cdc28 cyclins have historically been classified into two broad groups; G1 cyclins (Cln1-3), that regulate events during the interval between mitosis and DNA replication; and B-type cyclins (Clb1-6), needed for replication, G2 progression and passage through Mitosis (Mendenhall and Hodge, 1998). All cyclins, but Cln3, experience waves of production and destruction in pairs, see **Figure 6** and **Subheadings 5.2.4 and 5.2.5**.

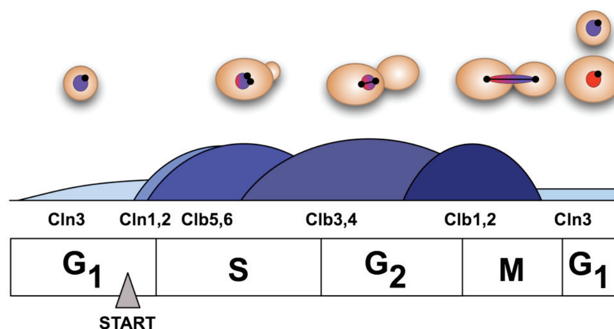

Figure 6. Waves of cyclins along cell cycle in *S. cerevisiae*. Quantities of the distinct Cdc28 cyclins are represented as overlapping domes. The different phases of cell cycle are represented in the lower rectangle. And morphological changes along cell cycle on top.

G1 cyclins split into two functional groups, although being genetically redundant, on one hand Cln3, which levels are rather low and steady along cell cycle, is regulated by relocalization and is the primary initiator of events at Start (Tyers *et al*., 1993; Verges *et*

al., 2007). Amongst Start-specific events there is the transcription of the highly redundant Cln1,2, which mediate bud emergence, spindle pole body (SPB) duplication, degradation of the S-phase inhibitor Sic1, and the expression of a whole transcriptional regulone that encompasses the subsequent S-phase cyclins and Cln1,2 themselves (Breeden, 1996; Cross and Tinkelenberg, 1991; Koch *et al*., 1993; Nash *et al*., 2001; Nasmyth and Dirick, 1991; Spellman *et al*., 1998).

B-type cyclins form a family of six proteins generated by several rounds of gene duplication (Archambault *et al*., 2005; Archambault *et al*., 2004). B-type cyclins are commonly subdivided into three pairs, based on their homology and transcriptional pattern. The first pair being transcribed along cell cycle is Clb5,6, they are produced at Start and their primary role is to initiate S-phase in a timely fashion (Schwob and Nasmyth, 1993). Clb5,6-Cdc28 complexes phosphorylate components of the pre-replication complexes to fire replication and also to prevent reinitiation of the already fired replication origins (Dahmann *et al*., 1995; Masumoto *et al*., 2002; Tanaka *et al*., 2007). The second pair of cyclins is formed by Clb3,4, it appears in mid S-phase and contributes in DNA replication and drive spindle assembly (Richardson *et al*., 1992). The last wave of B-type cyclins appears before anaphase, it generates two highly homologous cyclins, Clb1,2, that promote isopicnic bud growth, chromosome separation and inhibit G1-specicic events (Amon *et al*., 1993; Fitch *et al*., 1992; Lew and Reed, 1993).

Although cyclins show high level of functional redundancy, specificity of each cyclin is assured by a variety of mechanisms (Bloom and Cross, 2007; Haase and Reed, 1999). Cyclins are disparately sensitive to cell-cycle-regulated inhibitors, differentially restricted to subcellular locations, they are timely controlled by specific transcriptional activators, degraded by phase-specific events, bind only to specific targets and differentially allow for Cdc28 inhibitory phosphorylations when bound to it. Thus, rather than a single regulator, the combination of multiple oscillatory

mechanisms that collaborate in providing alternate periods of low and high levels of the different cyclins-CDK activities ensures the orderly progression through cell cycle (Cross, 2003; Morgan and Roberts, 2002).

5.2.3. Cdc28 phosphorylation and Cell-Cycle-Dependent inhibitors; Sic1 and Swe1

Besides binding to cyclins, all eukaryotic CDKs are also regulated by phosphorylation and cycle specific inhibitors. A required step in the activation of all eukaryotic CDKs, is the phosphorylation of a conserved threonine residue within the activation domain, the *T-loop* (Morgan, 1995). In budding yeast, the activating phosphorylation of Cdc28 at Thr169 by a single CDK-activating kinase, Civ1 (also named Cak1), is essential for cell cycle progression (Thuret *et al*., 1996). A second reversible phosphorylation on tyrosine 19 of Cdc28, results in the inhibition of its CDK activity. It is mediated by a kinase expressed in S-phase and G2, Swe1 (Booher *et al*., 1993; Lim *et al*., 1996). Swe1 specifically recognizes Clb1,2-assotiated Cdc28 molecules, therefore phosphorylation on Cdc28 by Swe1, selectively restricts Clb1,2-Cdc28 activity, for it only affects to a minor extent Clb3,4-Cdc28 and Clb5,6-Cdc28 show to be insensitive to this phosphorylation (Hu and Aparicio, 2005). Therefore, Swe1 primarily restricts Clb1,2 activity and is the major mechanism by which cells delay entry into mitosis until critical cell size has been reached (Kellogg, 2003; Rupes, 2002) or bud morphogenesis has been successfully completed (Cid *et al*., 2002; Lew, 2003). Swe1 phosphorylation is opposed at mitosis by the Mih1 phosphatase (Russell *et al*., 1989).

On top of phosphorylation, stoichiometric CDK inhibitors (Oehlen *et al*.) inactivate different cyclin-Cdc28 complexes at certain times of the cycle. Far1 is a Cln-Cdc28 specific inhibitor that acts upon activation of the pheromone pathway, see **Figure 2**. The activation of Far1 represses Start-specific transcription, causing in Cln-Cdc28

activity total depletion and resulting in a G1 arrest in preparation for mating (Peter *et al*., 1993; Peter and Herskowitz, 1994). Sic1 is a potent Clb-Cdc28 specific inhibitor that excludes substrates from the active site of Clb-bound Cdc28. Sic1 plays a role in the timing and robustness of regulation of DNA replication by holding under inhibition Clb5,6-Cdc28 complexes and setting a threshold for Clb-Cdc28 activation (Cross *et al*., 2007). The levels of Sic1 are maximal at early G1, but at late G1, a rise in Cln1,2-Cdc28 activity results in the phosphorylation of Sic1, which targets it for ubiquitination and degradation. Adequate progression into S phase requires degradation of Sic1 or an overcoming activity of cyclin B-associated CDK activity, see **Subheading 5.2.5** (Schwob *et al*., 1994; Verma *et al*., 1997). Sic1 has also an important role in down-regulating Clb2 to allow for spindle degradation and exit from mitosis.

5.2.4. Cell cycle transcriptional regulation

In *S. cerevisiae*, a significant fraction of genes (>10%) are transcribed with cell cycle periodicity (Spellman *et al*., 1998). These genes can be organized into clusters exhibiting similar patterns of periodic transcription, which are achieved via both repressive and activating mechanisms, which are ruled by the CDK and a network of transcription factors that has an oscillatory property by itself (Orlando *et al*., 2008; Wittenberg and Reed, 2005). Roughly, these clusters are the G1, the S-phase, the Clb2 and the M-G1 clusters.

The G1-specific gene cluster is triggered at Start. It is targeted by two heterodimeric transcription factors that share the same transactivating protein (Swi6) and two differing DNA binding proteins (Swi4 and Mbp1). The first is the Swi4 Cell cycle Box Binding Factor (SBF) and the second is the Mlu Cell cycle Box Binding Factor (MBF). SBF typically targets genes involved in cell morphogenesis and the cyclins Cln1,2, meanwhile MBF target genes are those necessary for DNA replication and the cyclins Clb5,6 (Breeden, 1996; Koch *et al*., 1993; Nasmyth and Dirick, 1991). Under physiological conditions, Cln3-Cdc28 is the primary

activator of these transcription factors (Tyers *et al*., 1993). Upon Cln3 activation, the CDK phosphorylates and inactivates the G1 specific inhibitor Whi5, analogous to the retinoblastoma (Rb) in mammals, and releasing both SBF and MBF dependent transcription. When cells reach Start, a burst of Cln1,2-Cdc28 activity further activates SBF and MBF through a positive feedback loop that involves phosphorylation of Whi5 (Cross and Tinkelenberg, 1991; Dirick *et al*., 1995; Skotheim *et al*., 2008).

During S-phase the histone cluster shows to be under the control of SBF/MBF but its timing of expression is shifted by two co-repressors Hir1 and Hir2 (Sherwood *et al*., 1993). It has been recently described that Hcm1, a transcription factor from the Forkhead family, is responsible for the correct timing of transcription of a number of genes implicated in chromosome separation, and that belong to this S-phase cluster (Pramila *et al*., 2006).

The Clb2 cluster encloses 35 genes that are transcribed from the end of S-phase until nuclear division. Several genes important for progression through mitosis are included in this cluster; the mitotic cyclins *CLB1,2*; *CDC5*, the yeast polo-like kinase homolog; *CDC20*, a mitotic specificity factor for the APC protein–ubiquitin ligase; and *SWI5* and *ACE2*, transcription factors required for late M/early G1-specific gene expression (Cho *et al*., 1998; Ghiara *et al*., 1991; Spellman *et al*., 1998). The promoters of these genes are permanently bound by the transcription factors Fkh1,2 and Mcm1 and the key to cell cycle regulation is the co-activator Ndd1, expressed periodically during S-phase and turned off during mitosis (Koranda *et al*., 2000; Loy *et al*., 1999). Ndd1 activation is mediated by CDK-dependent phosphorylation, being the primary activator Clb2 itself, and therefore the regulation of this cluster accounts with a positive loop, similarly to the G1 cluster (Reynolds *et al*., 2003).

The M and early G1 cluster is formed by genes required for G1 functions, like the MCM complex (component of the prereplication

complex), transcription factors required for G1 gene expression and proteins involved in the yeast mating response, which occurs during G1 (Cho *et al*., 1998; Spellman *et al*., 1998). A large number of these genes require Mcm1 and the repressors Yox1 and Yhp1, which bind to the 'early cell cycle box' (ECB) for their correct timing of expression (McInerny *et al*., 1997; Pramila *et al*., 2002). Other genes, like *SIC1* depend on Ace2 and Swi5, which in turn depend on Fkh1 and Fkh2, and therefore their regulation depends on the regulation of the Clb2 cluster as well (Nasmyth and Shore, 1987; Zhu *et al*., 2000). A third group of genes is formed by those normally induced by mating pheromone and depend on the transcription factor Ste12 and two co-repressors, Dig1 and Dig2, that are targeted by Fus3 upon pheromone sensing (Breitkreutz *et al*., 2003; Kusari *et al*., 2004; Oehlen *et al*., 1996; Spellman *et al*., 1998).

Recent studies point that not only CDK activity but also a set of regulatory interactions occurring between cell cycle-specific gene clusters, are critical for maintaining the organization of cell cycle events (Futcher, 2002; Kato *et al*., 2004; Lee *et al*., 2002; Orlando *et al*., 2008). These interactions seem to be via three general mechanisms: (i) a transcription factor can be regulated by the preceding cluster regulation (McInerny *et al*., 1997); (ii) an enzymatic activity is required for regulation of transcription of another gene cluster (Amon *et al*., 1993); (iii) a transcriptional repressor is expressed as a member of another gene cluster (Spellman *et al*., 1998).

5.2.5. Regulation of cell cycle by proteolysis

Besides transcriptional control, cell cycle regulators are also timely regulated by ubiquitin-proteasome-mediated proteolysis to promote cell cycle irreversibility (Hershko and Ciechanover, 1998). Two important complexes of ubiquitin ligases intervening in cell cycle regulation; Skp1/Cullin/F-box (SCF) and the Anaphase Promoting Complex (APC), also termed the cyclosome.

The SCF system is built of an E2 ligase (Cdc34), a scaffold protein (Cdc53 or cullin), an F-box binding protein (Skp1) and an interchangeable F-box protein, which is responsible for substrate recognition and jointly with Cdc53 and Skp1 constitutes the E3 complex, see **Figure 7**. (Willems *et al*., 1996). It catalyzes ubiquitination of phosphorylation-targeted cell-cycle-regulating proteins, including the G1 cyclins, CDK inhibitors (Sic1 and Far1), and proteins implicated in DNA replication, like Cdc6 (Barral *et al*., 1995; Henchoz *et al*., 1997; Schwob *et al*., 1994; Tyers and Jorgensen, 2000; Willems *et al*., 1996).

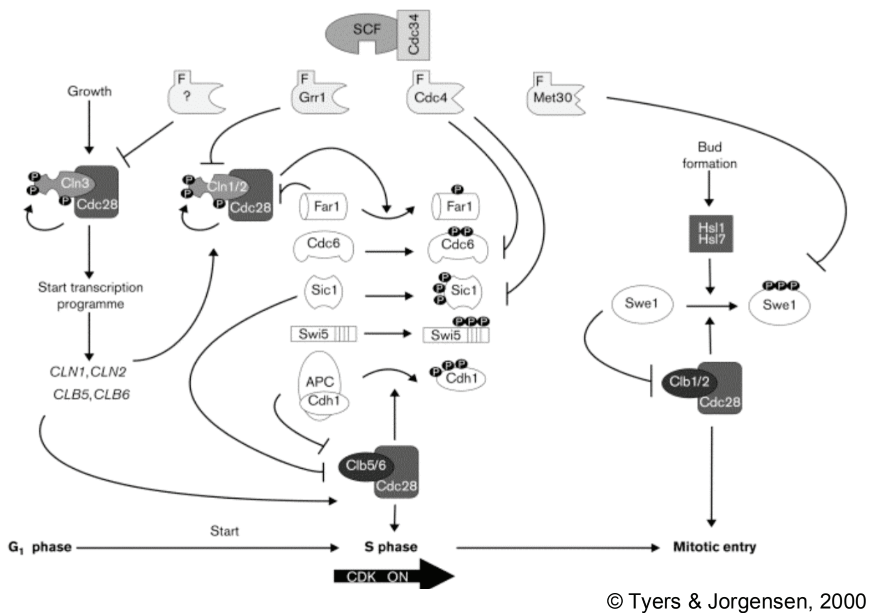

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Figure 7. SCF-dependent proteolysis and CDK activity that control the budding yeast cell cycle. At late G1, Cln–Cdc28 phosphorylates Sic1 to target it for ubiquitination by Cdc4-SCF, liberating the Clb5,6–Cdc28 kinases. Grr1-SCF, targets phospho-Cln1,2 for degradation After bud emergence is complete, Met30-SCF eliminates the CDK-inhibitory kinase Swe1, which allows Clb1,2–Cdc28 to initiate the events of mitosis.

SCF is active throughout the cell cycle and the degradation of its substrates is controlled at the level of phosphorylation, which is in many cases mediated by the CDK activity (Lanker *et al*., 1996). A classical example of an SCF-regulated event of cell cycle is the degradation of Sic1 and Far1 at the G1/S transition. It is triggered

by Cln1,2–Cdc28-dependent phosphorylation (Nash *et al*., 2001). Phospho-Sic1 and phospho-Far1 are both bound by the F-box protein Cdc4 and are ubiquitinated by the E2 enzyme Cdc34. Once Sic1 is eliminated, Clb5,6–Cdc28 activity is liberated form inhibition by Sic1, and free to initiate DNA replication (Schwob *et al*., 1994). Once Far1 is degraded cells become insensitive to the arrest imposed by mating pheromone (Henchoz *et al*., 1997).

The autophosphorylation of Cln–Cdc28 complexes on the PEST domain of these cyclins tethers them to Grr1, a different F-box protein, for ubiquitination and degradation, thus, active Cln1,2–Cdc28 complexes are intrinsically labile and highly responsive to changes in rates of transcription brought on by different environmental conditions (Schneider *et al*., 1998). The F-box protein Met30 mediates another cell-cycle role for the SCF. It targets the kinase Swe1 for degradation in the G2/M phase and thereby restricts the window in which Swe1 can inhibit Cdc28 (Kaiser *et al*., 1998). Swe1 degradation depends both on Clb–Cdc28 activity and a conserved upstream kinase, Hsl1, in association with the adaptor protein Hsl7, which together target Swe1 for degradation once the bud site is properly assembled (Asano *et al*., 2005; McMillan *et al*., 1999; Tyers and Jorgensen, 2000).

The APC core complex is necessary for progression through anaphase, exit from mitosis and maintenance of G1 phase, see **Figure 8** (Zachariae and Nasmyth, 1999).

The regulation of the APC is hierarchically achieved by association with two conserved accessory factors, Cdc20 and Cdh1, which serve as substrate adaptors for the APC complex. Cdc20 activates the APC at the transition from metaphase to anaphase. Its abundance is cell-cycle-regulated, accumulating in S-phase, peaking in mitosis, and dropping in G1 due to transcriptional up-regulation by Clb-Cdc28 in mitosis and protein degradation by Cdh1-APC in G1 (Pesin and Orr-Weaver, 2008; Prinz *et al*., 1998). Its activity requires the phosphorylation of APC core subunits by

the polo-like kinase (Cdc5) and Cdc20 by Clb-Cdc28 (Nigg, 1998; Rudner and Murray, 2000). Clb3, Clb5 and the anaphase inhibitor Pds1 (Securin) are the only essential substrates of Cdc20-APC (Thornton and Toczyski, 2003). Anaphase is initiated upon Clb2-Cdc28-mediated activation of Cdc20-APC. Then active Cdc20-APC eliminates the anaphase inhibitor Pds1, which holds Esp1 inactive. Esp1 cleaves the cohesin protein Scc1 to allow for sister chromatid separation (Uhlmann *et al.*, 1999).

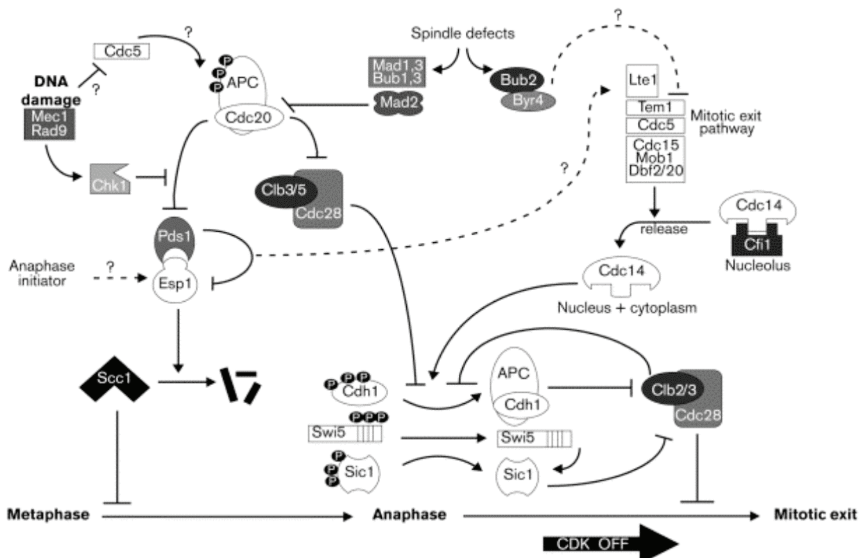

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Figure 8. APC-dependent proteolysis and CDK activity that control the budding yeast cell cycle. Cdc20 and Cdh1 activate at metaphase and anaphase the APC, respectively. Cdc20-APC eliminates Pds1, freeing Esp1 to promote cleavage of Scc1. Cdh1-APC depends on Clb5-Cdc28 activity and dephosphorylation by Cdc14, which also dephosphorylates Sic1 and Swi5. Cdh1-APC and Sic1 collaborate to collapse Clb2,3–Cdc28 kinase activity and allow exit from mitosis. Checkpoint pathways that impinge on anaphase and mitotic exit, like spindle defect and DNA damage checkpoints converge on the regulation of the APC complex.

Complementarily to Cdc20, Cdh1 mediates APC activation from the end of anaphase until late G1. It has other targets from M throughout G1, including Clb1,2, factors that regulate spindle function, sister chromatid cohesins, and even Cdc20 (Zachariae

and Nasmyth, 1999). In contrast to Cdc20, Cdh1 is expressed throughout the cell cycle and is held inactive by Clb-Cdc28-dependent phosphorylation until the end of mitosis, when it is dephosphorylated by Cdc14, upon activation of the Mitotic Exit Network (MEN) (Jaspersen *et al*., 1999; Zachariae and Nasmyth, 1999).

5.3. Cell Cycle Checkpoints

Cell cycle progression is surveyed at any time by checkpoint mechanisms that postpone critical steps to allow error-free completion of such processes (Hartwell and Weinert, 1989). In *S. cerevisiae* several checkpoints have been extensively studied; the DNA damage checkpoint, DNA replication, kinetochore attachment to the mitotic spindle, spindle assembly and dynamics, and bud morphogenesis (Amon, 1999; Lew and Reed, 1995; Rudner and Murray, 1996; Weinert and Hartwell, 1989; Weinert *et al*., 1994). These checkpoints delay cell cycle by inferring either on CDK, CDK inhibitors or APC activity and show to be responsive to both internal and external stimuli.

5.3.1. The Intra S phase DNA Damage Checkpoint

DNA damage or impairment of replication may result in checkpoint-mediated arrest at S-phase or M. These blockades save daughter cells from inheriting lesions on the DNA (Longhese *et al*., 2003). When replication is underway, elongating replication forks may stall if the pool of nucleotides is depleted (this may occur if genotoxic drugs like Hydroxyurea are present), or when replication forks stumble upon a lesion in the DNA strand of any kind. When replication forks stall, it is crucial for maintaining DNA integrity that they are stabilized in order to preserve their ability to proper resume replication (Lopes *et al*., 2001; Tercero *et al*., 2003). Besides stabilizing undergoing replication forks, cells have also means to delay initiation of the origins still to be fired (Santocanale and Diffley, 1998). Stalled replication forks are themselves the

main sensors of the DNA damage checkpoint pathway. Stalled forks are sensed by the generation of single stranded DNA (Zou and Elledge, 2003). Two kinases from the ATR/ATM family are recruited to stalled forks, Mec1 and Tel1, and are the most upstream components of the DNA checkpoint mechanism (Osborn and Elledge, 2003; Zou and Elledge, 2003). Once recruited to stalled forks, Mec1 and Tel1 initiate the massive autophosphorylation of the essential Rad53 effector kinase. This event occurs in coordination with the adaptor proteins Rad9 and Mrc1, which physically recruit Rad53, allowing its full activation. Once active, Rad53 mediates both the essential stabilisation of forks and inhibition of initiation of late origins (Alcasabas *et al*., 2001; Branzei and Foiani, 2006; Gilbert *et al*., 2001; Sweeney *et al*., 2005). Among the targets of Rad53 there is the DDK complex, and this has been proposed to be the mechanism through which Rad53 inhibit firing of late origins, slowing down cell cycle in S phase (Dohrmann *et al*., 1999; Duncker *et al*., 2002; Weinreich and Stillman, 1999). Rad53, in combination with another kinase, Chk1, also induce an arrest in metaphase upon DNA damage. Rad53 ultimately delays the activation of the APC complex via Cdc20 and thus postpones entry into anaphase. Chk1 directly inhibits Pds1 and thus stabilizes the cohesins that hold sister chromatids together, see **Subheading 5.2.5** and **Figure 8** (Sanchez *et al*., 1999; Tinker-Kulberg and Morgan, 1999).

5.3.2. Spindle Checkpoints

In budding yeast the boundary between mother and daughter cell resides at the bud neck, where cytokinesis takes place at the end of the cell cycle. Since budding and bud neck formation occur much earlier than spindle formation, spindle positioning is a finely regulated process, so that proper heritage of divided nuclei at the end of cell cycle is accomplished. A surveillance mechanism called the Spindle Position Checkpoint (SPOC) delays mitotic exit and cytokinesis until the spindle is properly oriented, thus ensuring adequate karyogamy. As long as the spindle is not properly placed, the SPOC holds inhibited the Mitotic Exit Network (MEN)

transduction cascade. The MEN involves several factors, including the polo kinase (Cdc5), that ultimately promote the activation of a key phosphatase Cdc14. Cdc14 is in turn essential for mitotic exit and cytokinesis through inactivation of mitotic CDKs and dephosphorylation of their targets, see **Figure 9** (Fraschini *et al*., 2008; Sullivan and Morgan, 2007). Thus, the SPOC mediates a cell cycle arrest at mitosis by maintaining high Clb-Cdc28 activity until proper spindle alignment.

The Spindle Assembly Checkpoint Pathway monitors proper microtubule-to-kinetochore attachments, that is, each chromatid of each pair must attach to microtubules from opposite poles. In response to a single unattached kinetochore, this checkpoint arrests cell cycle at metaphase by inhibiting Cdc20-APC through a complex containing the proteins Mad1-3 and Bub1-3, see **Figure 8** (Brady and Hardwick, 2000; Chen *et al*., 1999; Shah and Cleveland, 2000).

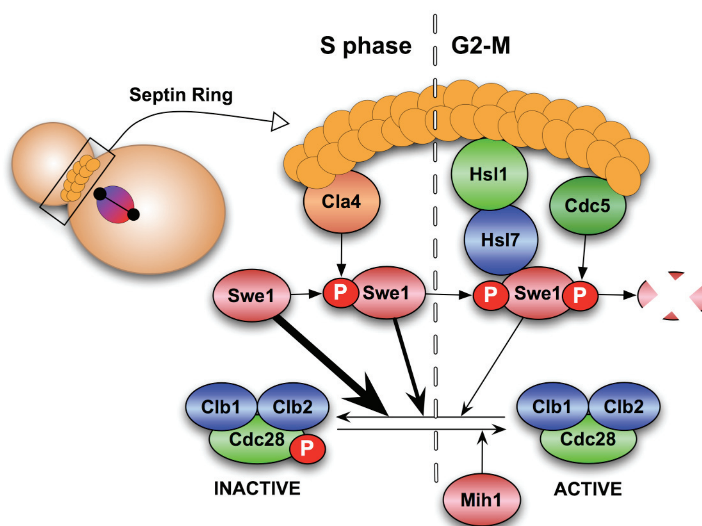
During anaphase, the spindle pulls the sister kinetochores apart until the sister chromatids are fully separated from each other to form two nucleated cells once cytokinesis occurs. Recent studies indicate that, at least in budding yeast, a checkpoint called NoCut prevents abscission when spindle elongation is impaired, and might delay cytokinesis until all chromosomes are pulled out of the cleavage plane (Mendoza and Barral, 2008).

5.3.3. The Morphogenesis checkpoint

Phosphorylation of Cdc28 on tyrosine 19 by Swe1 is maximal between S and G2, therefore, during these phases, Clb2-Cdc28 is held inactive to prevent premature entry into mitosis, see **Subheading 5.2.3** (Amon *et al*., 1992). In a normal cell cycle, Swe1 is degraded in G2 and the opposing activity of Mih1 dephosphorylates Cdc28, which becomes competent to initiate entry into mitosis, see **Subheading 5.2.5**. Swe1 degradation prior Clb2-Cdc28 activation is a necessary step to enter into mitosis,

thus any delay of this process, results in a pre-mitotic cell cycle arrest.

Swe1-mediated phosphorylation on Cdc28 is an essential effector of the still controversial checkpoint, called the Morphogenesis Checkpoint, which monitors actin cytoskeleton and septins structures and delays entry into metaphase until bud neck formation is complete, arresting cells with short mitotic spindles (Barral *et al.*, 1999; Booher *et al.*, 1993; Cid *et al.*, 2002; Lew, 2003; Lew and Reed, 1995; McMillan *et al.*, 1999). Septins form filaments shaping a double-ring structure at the bud neck (Byers and Goetsch, 1976). Two kinases, Cla4 and Cdc5 are targeted sequentially to the bud neck in a septin-dependent manner; Cla4 in S phase and Cdc5 in G2, and are responsible for the stepwise phosphorylation and down-regulation of Swe1, see **Figure 9** (Sakchaisri *et al.*, 2004)..



Adapted from Versele and Thorner, 2005

Figure 9. The Morphogenesis Checkpoint. Swe1 phosphorylation in S phase by Cla4 is not sufficient to trigger its degradation but it reduces its activity. In G2, Swe1 tethering to the Septin Ring by Hsl1-Hsl7, allows the polo-like Kinase (Cdc5) to phosphorylate it, which labels Swe1 for degradation, relieving Clb2-Cdc28 inhibition. Thus, this mechanism links proper assembly of septin filaments with efficient passage through G2 into mitosis.

In G2, the Septin Dependent Kinase (SDK) Hsl1 directly associates with the septin filaments and recruits Hsl7, which in turn binds Swe1 (Barral *et al*., 1999; Shulewitz *et al*., 1999; Versele and Thorner, 2005). Upon completion of bud formation, tethering of Swe1 to the bud neck by these two proteins, allows Cdc5 to phosphorylate Swe1, which leads to its ubiquitination and degradation, see **Subheading 5.2.5** (Cid *et al*., 2001; Ma *et al*., 1996; McMillan *et al*., 1999; Sakchaisri *et al*., 2004)

Stresses like changes in temperature or osmolarity in the medium disrupt actin polarity impairing bud formation. If morphogenesis defects may occur, Hsl1 and Hsl7 do not target Swe1 to the bud neck, resulting in its stabilization and subsequent delay of karyokinesis until a proper bud emerges (Lee and Amon, 2003; Lew, 2003; Neef *et al*., 2003)

6. CELL CYCLE CONTROL BY THE Hog1 MAPK

6.1. Implications of SAPKs on the control of cell cycle

The role of SAPKs in cell cycle control was first proposed in *Schizosaccharomyces pombe* for the Sty1 MAPK pathway, linking the control of cell cycle to environmental responses (Shiozaki and Russell, 1995). Soon after these findings, the mammalian SAPKs ERK1, ERK5, and JNK were also shown to affect cell cycle at different stages (Kamakura *et al*., 1999; Sewing *et al*., 1997; Wang *et al*., 1999). More recently, a role for p38 MAPK pathway in cell cycle progression was reported. In fact, different types of mammalian cells arrest at several stages along the cell cycle (G1-S, G2 and mitosis) upon osmostress (Dmitrieva *et al*., 2002; Dmitrieva *et al*., 2001; Kishi *et al*., 2001).

Diverse 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 (Ambrosino and Nebreda, 2001; Goloudina *et al*., 2003; Pearce and Humphrey, 2001; Todd *et al*., 2004; Wilkinson and Millar, 2000). It is still not clear, though, whether specific mechanisms are used to respond to different stimuli or if different cell types use different mechanisms to cope with stressful situations. The involvement of Hog1 in the progression of cell cycle under osmotic stress conditions has recently been established to be a central component of the adaptive response. Cells unable to properly regulate cell cycle under osmostress conditions are rendered osmosensitive (Belli *et al*., 2001; Clotet *et al*., 2006; Escote *et al*., 2004). At the current moment our group has described some of the mechanisms elected by the SAPK Hog1 to control cell cycle progression in *S. cerevisiae* in G1 and in the G2-M transition.

6.2. Hog1-mediated arrest at G1

Hog1 activity induces a transient arrest in G1, at least, via a dual mechanism that involves the direct phosphorylation of Sic1 on Thr173 at the carboxyl terminus of Sic1, which interferes with its degradation, and down-regulation of transcription of both G1 cyclins (*CLN1* and *CLN2*) and the S-phase *CLB5* (Clotet and Posas, 2007; Escote et al., 2004). Thus, although the molecular mechanism through which Hog1 down-regulates the mRNAs of these cyclins is still to be elucidated, it results in both the delay of Start-specific transcription and Clb-associated Cdc28 activity onset. As Sic1 phosphorylation by Cln1,2-Cdc28 on multiple residues targets it for degradation, see **Subheading 5.2.3,** and the single phosphorylation on Thr173 of Sic1 by Hog1, results its stabilization, for it interferes Sic1 binding to Cdc4 and thus reduces its degradation, it could be considered that Hog1 targets Sic1 degradation via both the down-regulation of *CLN1,2* and the direct phosphorylation on Thr173, see **Figure 10**. This Hog1-mediated

G1 arrest certainly plays an important role in osmoadaptation, as cells lacking Sic1 or that carry a mutant form of Sic1 that is not able to be phosphorylated on Thr173, are unable to grow in high osmolarity conditions (Escote *et al*., 2004).

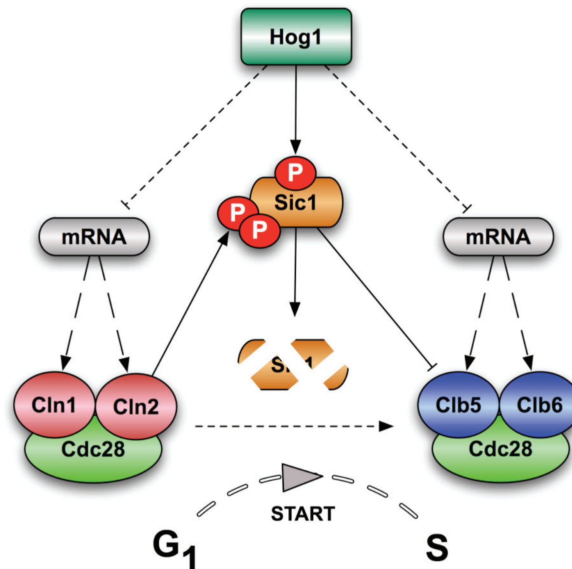


Figure 10. Control of Start by Hog1. Hog1 delays the onset of the activity of Clb-Cdc28 through a double mechanism; the down-regulation of mRNA of G1 and G2 cyclins and direct phosphorylation (and stabilization) of Sic1. Short dashed lines represent known, but probably indirect, effects. Long dashed lines represent intermediate processes not pictured in the figure

6.2. Hog1-mediated arrest at G2-M

Previous reports suggested that osmostress induces a delay at G2 (Alexander *et al*., 2001; de Nadal *et al*., 2002). As mentioned above, entry into mitosis is controlled by the activity of the Clb2-Cdc28 complex, which is held in check by the protein kinase Swe1. As in G1, Hog1 also controls progression through G2 under hyperosmotic conditions, in a very similar way. Namely, it involves the combined prolonged half-life of a cell cycle inhibitor and the down-regulation of cyclins. That is to say, Hog1 stabilizes Swe1 and therefore it induces a transient arrest in G2 by decreasing

Clb2-Cdc28 activity via its inhibitory phosphorylation, as well by the down-regulation of *CLB2* transcript levels (Alexander *et al*., 2001; Clotet *et al*., 2006). Similarly to G1, the mechanism elected by Hog1 to control *CLB2* mRNA levels remains still unclear. By contrast to Sic1 in G1, Swe1 is not directly phosphorylated by Hog1 during the G2 arrest, but is rather stabilized by a Hog1-mediated phosphorylation on Hsl1. As stated before, the SDK Hsl1 is part of the Morphogenesis Checkpoint, which mediates Swe1 degradation, see **Subheading 5.3.3.** Hog1 interacts with and directly phosphorylates Hsl1 on Serine 1220 within its Hsl7-docking site. This phosphorylation leads to the delocalization of Hsl7 from the neck that results in the lack of recruitment of Swe1 nearby Cdc5 and therefore it is not phosphorylated to be targeted for ubiquitination and degradation, see **Figures 9 and 11** (Clotet *et al*., 2006). This model is supported by the fact that cells harbouring point-mutated Hsl1 that cannot be phosphorylated by Hog1 neither accumulate Swe1 nor arrest in G2 and become osmosensitive, as wel

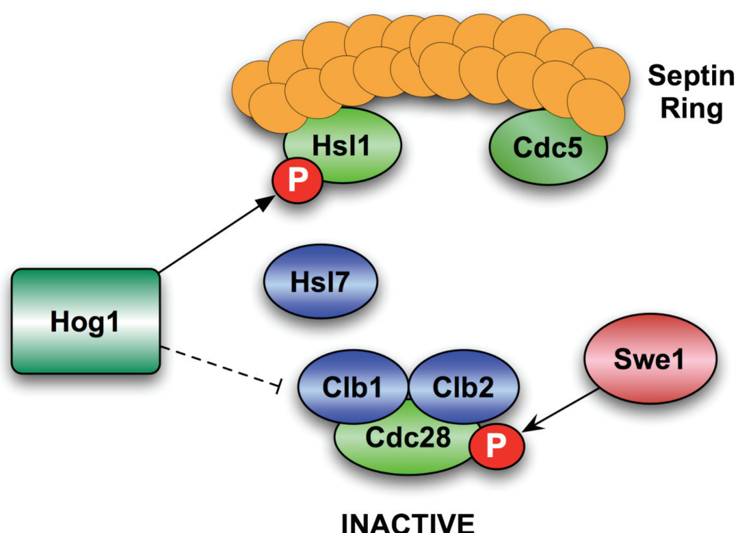


Figure 11. Control of the G2-M transition by Hog1. The direct phosphorylation of Hls1 by Hog1 interferes in Swe1 degradation, taking profit of the machinery of the Morphogenesis Checkpoint. Hog1 also down-regulates transcription of Clb1 and Clb2, contributing of inhbition of anaphase onset.

As a matter of fact, the combined deletion of *SIC1* and *SWE1* results in a synergistic osmosensitivity, pinpointing the relevance of proper cell cycle progression under osmostress at different stages of cell cycle (Clotet *et al*., 2006).

7. MATHEMATICAL MODELLING OF SIGNALLING PATHWAYS AND CELL CYCLE

Up to date, a large number of quantitative computational simulations have proven to support understanding of regulatory systems. These approaches have been useful for characterizing threshold properties, bistability, timings, crosstalk events or feedback effects of a wide variety of biological systems. MAPK pathways have now been investigated for over ten years through mathematical modelling (Bluthgen and Legewie, 2008). These pathways have been extensively studied and subjected to many quantitative systems biology approaches that give rise to abstract models that emphasise some key features of the signalling process, but also detailed models that describe the dynamics of specific pathways (Heinrich *et al*., 2002; Schoeberl *et al*., 2002; Swameye *et al*., 2003). Some of these models have even been experimentally tested (Klipp *et al*., 2005; Papin *et al*., 2005).

One of these efforts was performed on the Hog1 pathway some years ago in order to investigate the dynamics and logic of the response of yeast cells to osmotic shock (Klipp *et al*., 2005). This model comprised receptor stimulation, the HOG signaling pathway, activation of gene expression, adaptation of cellular metabolism, glycerol accumulation and a thermodynamic description of the control of volume and osmotic pressure. It helped in the understanding of the activation and downregulation of the signaling pathway, finding underlying feedback control of the HOG pathway. But, although being a comprehensive model of the pathway this model did not picture the interaction of Hog1 with the cell cycle control machinery. Recently, an abstract model of the activation of

the signalling process through the HOG pathway has helped in explaining the advantage of having a basal signal input for the kinetics of the pathway activation (Macia *et al*., 2009).

Already existing mathematical models of cell cycle in *S. cerevisiae* have been based on both Ordinary Differential Equations (ODEs) and Boolean networks. In rather comprehensive cell cycle models, based on ODEs, modellers have incorporated detailed molecular concentrations and reactions for each mRNA or protein, extracted from a significant amount of wild type and mutant data (Cross *et al*., 2002; Chen *et al*., 2000; Novak *et al*., 2007). More detailed models of specific processes have also been produced. Rather than being holistic approaches to cell cycle modelling, these models focus on a single regulatory program, like the transition from G1 to S phase or mitotic exit (Barberis *et al*., 2007; Queralt *et al*., 2006; Toth *et al*., 2007). ODE models, however, need detailed analysis of a large number of parameters and a not small number of unknown parameters need to be estimated. Therefore, simpler models that allow the main qualitative features of the system to be analysed more easily, are also useful. This is the case of Boolean Network Models, where each gene/protein is represented as an ON or OFF kind of interaction. These models need less computational effort for analysing the logical structure of the system. Boolean models that capture cell cycle dynamics and show remarkable robustness have been previously proposed (Braunewell and Bornholdt, 2007; Li *et al*., 2004). However, these approaches show remarkable limitations. To account for ‘noise’ in the yeast cell cycle, stochastic models have also been produced, where the adaptation of ODE models give stochastic Petri net models or Langevin-type equations (Mura and Csikasz-Nagy, 2008; Steuer, 2004). A recent boolean network model for the S. cerevisiae cell cycle, which incorporates knowledge from existing models and current literature, shows to be consistent with wild type and mutant phenotypes, capture the essential features of the system, but, as the rest of these models shows no plasticity to be used as tool to investigate the effects of *in silico* deformations of the modelled cell cycle (Irons, 2009).

OBJECTIVES

Main objective

To better understand how the Stress-Activated-Protein Kinase Hog1 regulates cell cycle of *Saccharomyces cerevisiae*

Specific objectives

Quantitatively describe the impact of Hog1 activation on the arrest of cell cycle at Start

Elucidate the role of the different regulators controlled by Hog1 in the osmostress-imposed arrest at Start

Describe the molecular mechanism underlying the Hog1-induced arrest in G2-M.

RESULTS AND DISCUSSION

A quantitative approach reveals that the Hog1 SAPK regulates G1-S progression upon stress by independently controlling Clns, Sic1 and Clb5

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A quantitative approach reveals that the Hog1 SAPK regulates G_1-S progression upon stress by independently controlling Clns, Sic1 and Clb5

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Key words: Cell cycle/Cyclins/Osmostress/Mathematical modelling/SAPK/Sic1

Abstract

Control of cell cycle progression by stress-activated protein kinases (SAPKs) is essential for cell adaptation to extracellular stimuli. Exposure of yeast to osmostress activates the Hog1 SAPK, which modulates cell cycle progression at G_1 by direct phosphorylation of Sic1 and delay of the onset of Cln1,2. We took profit of mathematical modelling, along with quantitative *in vivo* experiments, to define the role and the direct contribution of the individual mechanisms that regulate G_1 downstream of Hog1. Our results show that the length of the arrest depends on the degree of stress and the proximity to Start. More importantly, we found that, rather than Cln1,2 regulation, Clb5 regulation by Hog1 is critical to modulate entry into S phase. Interestingly, the effect of Hog1 on Clb5 is exerted independently of its effect on Clns. Therefore, the control of different cyclins is determinant for a proper arrest at G_1, whereas the control of Sic1 degradation by Hog1 is only important to regulate Clb5-Cdc28 activity at late G_1, thus defining a distinct temporal role for Sic1 and cyclins on G_1 upon stress.

Introduction

Stress-activated protein kinases (SAPKs) are essential for proper cell adaptation to extracellular stimuli (Kyriakis and Avruch, 2001). In budding yeast, the increased extracellular osmolarity results in the activation of the p38-related stress-activated Hog1 kinase, which elicits an extensive program required for cell adaptation, implicating regulation of gene expression, translation and cell cycle progression (Hohmann, 2002;de Nadal *et al.*, 2002). Diverse stresses, such as heat stress, extracellular hyperosmolarity and DNA damage, critically affect progression through the cell cycle (Flattery-O'Brien and Dawes, 1998;Li and Cai, 1999;Wang *et al.*, 2000;Alexander *et al.*, 2001). When yeast cells are exposed to osmostress, the activation of the Hog1 SAPK mediates a transient cell cycle arrest both at G_1 and G_2 phases (Alexander *et al.*, 2001;Yaakov *et al.*, 2003;Escote *et al.*, 2004;Clotet *et al.*, 2006;Clotet and Posas, 2007).

In budding yeast, control of G_1-S transition is exerted at Start by an extensive transcriptional program under the control of the SBF and MBF transcription factors that mediate transcription of the G_1 cyclins, Cln1 and Cln2, and the S phase cyclins, Clb5 and Clb6, respectively (Nasmyth and Dirick, 1991;Koch *et al.*, 1993;Breeden, 1996). Under physiological conditions, Cln3-associated CDK (Cln3-Cdc28) is the primary activator of these transcription factors (Tyers *et al.*, 1993). When cells reach Start, the burst of Cln1,2-Cdc28 activity, in addition to initiate budding, also triggers transcription of *CLN1,2* and *CLB5,6* though a positive feedback loop that involves the SBF/MBF inhibitor Whi5 (Cross and Tinkelenberg, 1991;Dirick *et al.*, 1995;Skotheim *et al.*, 2008). Moreover, Cln1,2-Cdc28 phosphorylates Sic1 at several sites of its N-terminal domain, setting a threshold for its ubiquitination and subsequent degradation (Nash *et al.*, 2001). DNA replication is initiated when Clb5,6-Cdc28 phosphorylates components of the pre-replication complex (Masumoto *et al.*, 2002;Tanaka *et al.*, 2007). At the end of G_1, the net activity of newly formed Clb5,6-Cdc28 depends on the levels of Clb5,6 cyclins and the levels of the CDK-inhibitor Sic1 (Schwob *et al.*, 1994). Sic1 associates and inactivates Clb5,6-Cdc28 complex and thus, adequate

progression into S phase requires degradation of Sic1 or an overcoming activity of cyclin B-associated CDK activity (Verma *et al.*, 1997;Cross *et al.*, 2007).

Previous reports showed that cell cycle delay at G_1 imposed by Hog1 entailed the down-regulation of *CLN1* and *CLN2* expression and the direct phosphorylation by Hog1 of Sic1 at Thr173, which interfered with its ubiquitination (Escote *et al.*, 2004). Actually, cells unable to postpone onset of Cln1,2, lacking Sic1 or containing a Sic1 allele mutated on the Hog1 phosphorylation site were unable to properly arrest at G_1 upon osmotic stress, entering prematurely into S phase and became partially sensitive to osmostress (Escote *et al.*, 2004;Zapater *et al.*, 2005). The existence of the dual mechanism of *CLN1,2* down-regulation and Sic1 stabilization by Hog1 posed the question of the biological significance of such a complex regulatory mechanism. Nevertheless, neither the delay of *CLN1,2* transcription alone, nor the solely phosphorylation of Sic1 by the SAPK can totally account for the whole G_1 arrest observed upon osmostress (Escote *et al.*, 2004). These findings suggest a selective advantage of maintaining such a dual control mechanism.

The use of Sic1 mutants and alteration of Cln1,2 regulation have been useful to elucidate mechanistic properties of the regulatory machinery at the G_1–S transition, albeit showing to affect normal cell cycle progression (e.g., Nash *et al.*, 2001;Cross *et al.*, 2007). Therefore, although useful, these techniques may need the help of computational analysis to fully understand the regulation of G_1 transition by Hog1. Previous qualitative modeling approaches have proven to be a powerful tool to elucidate regulatory principles on cell cycle progression (Thornton *et al.*, 2004;Barberis and Klipp, 2007;Novak *et al.*, 2007) as well as MAPK signaling (Schoeberl *et al.*, 2002;Klipp *et al.*, 2005). Here, we combined quantitative *in vivo* experiments and mathematical modelling with parameters constrained by the quantitative experimental data to analyse the impact of Hog1 on the regulation of G_1-S upon stress. We show here, that before Start the control of G_1-S transition is exerted mainly by delaying the expression of G_1 cyclins (Cln1 and Cln2) and more importantly the S phase cyclin Clb5, while the phosphorylation of Sic1 by Hog1 seems not to play a critical role. It is worth noting that the regulation of Clb5 is independent of the regulation of Clns by the SAPK. At Start, a different scenario is established. When Clb5 is induced to some extent which can not be completely blocked by Hog1, then the stabilization of Sic1 by Hog1 is important to prevent inadequate firing of replication before adaptation. Therefore, our data show that there is a distinct temporal role for Sic1 and cyclins on the G_1 regulation by a SAPK in response to stress.

Results

A mathematical model to study the effect of Hog1 on cell cycle modulation at G_1

As a first attempt to understand the biological relevance of each regulatory element downstream of Hog1 in G_1, we took advantage of mathematical modelling and created a qualitative model that illustrated the interaction of Hog1 with the basic cell cycle machinery that governs the G_1-S transition, onset of Cln1,2 transcription and Sic1 stabilization (Supplementary Fig. 1). The choice of parameters for this initial model was completely arbitrary, at maximum a sensible guess, since we aimed to study the qualitative behaviour of the network and not its precise dynamical properties. We considered progression into S phase to be indicated in the model by the time point when Clb5-Cdc28 levels overcame Sic1 levels, referred to here as ”cross point”. The simulations yielded two main predictions with respect to the effect of the timing and strength of osmotic stress on cell cycle progression. First, cells similarly arrested for a given Hog1 activation, independently of the stage of G_1 in which Hog1 was activated. However, when cells overcame a critical point, they were unable to arrest in G_1. Thus, cells at any stage of G_1 are capable to delay progression in response to stress (Supplementary Fig. 2a-f). Second, an increase on Hog1 activation lead to a longer cell cycle delay (Supplementary Fig. 2g-l). Therefore, the length of the activation of Hog1 is critical for cell cycle progression.

Based on the initial simulations we quantified the impact of Hog1 on the cell cycle arrest. Yeast cells were synchronized using a-factor, released into fresh media in the absence or presence of different concentrations of NaCl (0.2-0.8 M NaCl). As shown in Figures 1a and 1b, exposure of cells to increasing amounts of NaCl resulted in longer Hog1 phosphorylation, which extended cell cycle arrest at G_1 (shown by DNA content analyses). Correspondingly, bud formation was also delayed upon osmostress (Supplementary Fig. 3). It is worth noting that there is a strong correlation between the period Hog1 remains active and the time of

arrest at G_1 (Fig. 1c). Thus, as suggested by the simulations, the length of cell cycle arrest at G_1 depends on the strength of the extracellular osmolarity and correlates with the timing of Hog1 phosphorylation.
We then tested whether the cell cycle arrest depended on the stage of G_1 in which cells were exposed to stress. Cells were stressed (0.4 M NaCl) at different times after release from pheromone and cell cycle progression was monitored. As shown in Figure 1d, the length of the arrest was constant when cells were stressed before 20 minutes after pheromone release but the arrest dramatically shortened after this time. Similar results were observed when cells were subjected to higher osmolarity (not shown). Therefore, cells retain the ability to arrest for a specific period when stressed at different stages of G_1 until they reach a point when the ability to arrest is strongly compromised.
Taken together, the predictions of the initial model were validated *in vivo* and suggested that further efforts with mathematical modelling could be interesting to understand how the Hog1 MAPK controls G_1-S transition.

Cln1,2 and Sic1 play different roles on the Hog1-mediated cell cycle arrest at G_1

To create a more comprehensive and quantitative mathematical model, we collected time course data sets by stressing cells at different times after release from pheromone and in response to different strengths of osmostress. Wild type cells bearing HA-tagged Cln2 and Myc-tagged Sic1 on their genomic loci were synchronized using a-factor, released into fresh media and then subjected to osmostress (0.4 M NaCl) at different times (0-30 min) after release. Time course series with quantification of total Cln2 and Sic1, together with measurements of DNA content and budding index are shown in Figure 2. In the absence of stress, cells reached Start 20 minutes after release, as indicated by the onset of Cln2 production and accelerated Sic1 degradation (Fig 2a). Under all the conditions tested, Sic1 degradation was initiated before Cln2 was detectable and this degradation was not mediated by Cln3 (data not shown). Interestingly, passage through Start coincided with the time that cells loose the ability to arrest at G_1 upon osmostress (Fig. 1d). It is worth noting that, whereas under non-stress conditions there was a tight relationship between low Sic1 levels, peak of Cln2 protein, DNA replication and budding (Fig. 2a), when cells were stressed, this relationship was partially lost, for Sic1 levels seemed to correlate better with the onset of DNA replication than the Cln2 peak (Fig. 2b-c). Surprisingly, when cells were stressed at Start, although they were able to arrest, both regulators lost their time relationship with entry into S phase, for DNA replication and budding occurred before the total degradation of Sic1 and the Cln1,2 peak (Fig 2d).
It is worth noting that in the absence of stress, DNA replication and bud formation are induced almost simultaneously. When cells were stressed after release (t=0) the concomitancy of both processes was kept (Fig. 2b). However, when cells were stressed 30 minutes after release, there was no delay on DNA replication but bud formation was still delayed (Fig. 2e). Correspondingly, Cln2 levels notably decreased after an osmostress at 30 minutes whereas a second peak appeared at 80 minutes as when cells were stressed at earlier times (Fig. 2b-e). Thus, although both processes are regulated simultaneously under normal conditions, our data suggest that they might be independently regulated by the SAPK in response to osmostress.

Neither Sic1 stabilization nor Cln1,2 regulation by Hog1 can solely account for the G_1 delay upon osmostress

To dissect the individual contribution of the Hog1-mediated down-regulation of Cln1,2 and stabilization of Sic1 to control G_1 we analysed G_1-S progression in *sic1D* cells or cells expressing *CLN2* under the *GAL1* heterologous promoter in response to osmostress. Wild type and *sic1D* cells were synchronized using a-factor, released into fresh media and then subjected to osmostress (0.4 M NaCl). Strikingly, under the conditions tested, *sic1D* cells showed almost the same ability to delay DNA replication just like wild type (Fig. 3a). Then, we tested whether over-expression of *CLN2* could prevent cell cycle arrest upon osmostress. Wild type cells bearing an empty plasmid or a plasmid expressing *CLN2* under the *GAL1* promoter (pYes2-*CLN2-HA*) were synchronized and subjected to stress as before. Cells ectopically expressing Cln2 also were able to delay entry into replication in response to osmostress even having high Cln2 levels and promoting bud formation (Fig. 3b and Supplementary Fig. 4c). These data are consistent with previous results (Escote *et al*., 2004) and suggest that neither the sole down-regulation of *CLN1,2* expression nor Sic1 phosphorylation are sufficient to explain

the cell cycle arrest mediated by Hog1 upon osmostress. There could be the possibility that the stabilization of Sic1 and down-regulation of *CLN2* expression had a combined effect. Thus, we tested whether the deletion of *SIC1* together with the simultaneous over-expression of *CLN2* was able to suppress the cell cycle arrest upon osmostress. *sic1Δ*cells bearing a plasmid expressing *CLN2* under the *GAL1* promoter (pYes2-*CLN2-HA*) were synchronized and subjected to stress as before. Although these cells have a weaker arrest and enter S phase without the same synchrony as wild type cells, they are still able to arrest upon osmostress (Fig. 3c). All together seems to indicate that an additional component of the cell cycle machinery might be regulated by Hog1 to delay cell cycle upon osmostress.

Clb5 is differentially down-regulated depending on the stage of G_1 in which cells are subjected to osmostress

Previous data showed that hyper-activation of Hog1 resulted in a down-regulation of *CLB5* gene expression, although it was not clear whether this was a consequence of Cln2 down-regulation and whether this effect was also observed upon osmostress (Escote *et al.*, 2004). Thus, first we analysed the levels of Clb5 in response to osmostress. Cells carrying endogenously TAP-tagged Clb5, together with Myc-Sic1 and HA-Cln2 were stressed with 0.4 M NaCl after release from pheromone (t0) or 20 minutes later (t20). Quantitative western blot was used to follow Clb5 protein levels (Fig. 4a) as well as in parallel Sic1 and Cln2 (not shown). DNA content was determined by flow cytometry (Fig. 4b). Interestingly, Clb5 accumulation was delayed similarly to Cln2 upon osmostress, indicating that not only Cln2 is down-regulated but also Clb5. It is worth noting that whereas Clb5 levels are undetectable when stress is applied just after release indicating a tight regulation of Clb5 production, a leaky production of Clb5 that reached around 20% of the maximum level was observed when cells were stressed at Start. Strikingly, this amount of Clb5 was not sufficient to induce DNA replication (Fig. 4b), suggesting the existence of an additional control over Clb5-Cdc28 activity under these conditions. Thus, Clb5 is tightly down-regulated by osmostress during G_1 and displays a loose control when cells are subjected to stress at Start, although this does not represent a loss in arrest capability.

Sic1 restricts the activity of Clb5 when cells are stressed at Start.

The observation that a leaky production of Clb5 at Start upon osmotic stress does not generate a loose timing of replication onset, led us to monitor Clb5-assotiated activity. The activity of Clb5-Cdc28 and entry into S phase can be precisely monitored *in vivo* by the analysis of the phosphorylation of some of the components of the replication machinery (e.g., Sld2) known to be targeted by the kinase complex (Masumoto *et al.*, 2002;Tanaka *et al.*, 2007). Thus, we monitored Sld2 phosphorylation in response to stress at different stages of G_1 and its dependency on Sic1.

Mutant *sic1Δ* cells containing endogenously TAP-tagged Sld2 and Clb5 were transformed either wild type *SIC1* or the *SIC1* mutant allele encoding Sic1^{T173A}, which cannot be phosphorylated by Hog1 (Escote *et al.*, 2004). Cells were released from pheromone in the presence of 0.4 M NaCl and phosphorylation of Sld2 was followed by western blot. In the absence of stress, phosphorylation of Sld2 was concomitant to Clb5 induction (Figures 4a and 5a). Correspondingly, when cells were subjected to osmostress just after release from pheromone, Clb5 production was delayed as well as Sld2 phosphorylation (Figures 4a and 5b). It is worth noting that a similar pattern of Sld2 phosphorylation was observed in *sic1* cells or cells containing either wild type or the mutant non-phosphorylatable Sic1 (Fig. 5b). Thus, the presence of Sic1 is not critical to delay Sld2 phosphorylation when cells are subjected to stress before Start.

However, in clear contrast, when these cells were subjected to osmostress at Start (20 minutes after pheromone release) the scenario was completely different. Whereas wild type cells were able to arrest efficiently and display strong synchrony in S phase entry (sharp increase in Sld2 phosphorylation), *sic1D* cells or cells containing the mutant *SIC1^{T173A}* allele showed partial and progressive phosphorylation of Sld2 earlier than wild type cells (Fig. 5c). Correspondingly, DNA content analyses showed that *sic1D* cells or containing the *sic1^{T173A}* mutation, although progressing as wild type cells under normal conditions (Fig. 5d), arrested similarly to wild type cells when stressed before Start, but were not able to properly arrest when stressed at Start (Fig. 5e-f). Therefore, the role of Sic1 upon osmostress is to restrict the activity of Clb5 at Start, in a moment when the regulation by the

SAPK Hog1 over the production of cyclins is not sufficiently efficient.

The regulation of Clb5 protein production is most critical to arrest at G_1

The observation that Clb5 activity is tightly regulated by Hog1 during G_1, lead us to test whether the over-expression of Clb5 could abolish the cell cycle arrest at G_1 imposed by osmostress. Cells bearing an ectopic copy of *CLB5* under the *GAL1* promoter (*GAL::CLB5*) or an empty vector (vector) were released from pheromone in the presence of 0.4 M NaCl. Clb5 production was induced by galactose addition only 20 minutes before release from pheromone. Samples of the indicated times were taken to score DNA content by FACS analyses. In clear contrast with cells over-expressing *CLN2*, cells over-expressing Clb5 were not able to delay replication when subjected to stress, although showing a clear delay in bud formation (Fig. 6b and Supplementary Fig. 4b). Therefore, the presence of Clb5 is sufficient to prevent cell cycle delay upon osmostress.

The inactivation of the thermosensitive mutant of Sln1 (*sln1^{ts4}*) leads to the sustained activation of Hog1 and a subsequent block of cell cycle at the G_1-S transition (Escote *et al*., 2004). This is an ideal model to study the cell cycle arrest by Hog1 without the presence of stress. Thus, we analysed whether over-expression of *CLB5* or *CLN2* were able to prevent cell cycle arrest mediated by hyperactivation of Hog1. *sln1^{ts4}* cells carrying an empty vector or a vector expressing either *GAL1-CLB5* or *GAL1-CLN2* were synchronized with pheromone, incubated only for 20 minutes in the presence of galactose, released at the non-permissive temperature and then DNA content was analyzed by FACS. As shown in Supplementary Figure 4d, over-expression of *CLB5* totally abolished the Hog1-imposed arrest at G_1, whereas over-expression of *CLN2* only had a minor effect. Taken together, our data indicate that Hog1-mediated *CLB5* control upon stress is a key determinant to mediate cell cycle delay whereas down-regulation of *CLN2* under stress seems to be mainly important to coordinate bud formation with exit from G_1.

The delay on Clb5 is independent of the positive loop of the G_1 cyclins

It has been proposed that expression of Clb5 can be under the control of a positive feed-back loop exerted by G_1 cyclins (Skotheim *et al.*, 2008). To test whether the effect of Hog1 was direct on Clb5 rather than an indirect effect of Hog1 on Clns, we tested whether Cln2 was controlling Clb5 expression upon stress. Cells carrying TAP-tagged Clb5, together with Myc-Sic1 and HA-Cln2 were transformed either with empty plasmid or a plasmid expressing HA-tagged *CLN2* under the *GAL1* promoter (pYES2-Cln2-HA), and arrested in G_1 with pheromone, galactose was added 20 minutes before release and then, released in the presence of 0.4 M NaCl. Samples of the indicated times were taken to score DNA content by FACS analyses and total Sic1 and Clb5 protein amounts by quantitative western blot. Albeit cells expressing *CLN2* entered into S phase faster than wild type, in response to stress they delayed Clb5 production similarly to cells bearing empty plasmid (Fig. 7a and 7b).

Pervious works indicate that the positive loop of Cln1,2 on the transcription of the SBF/MBF-regulated genes involves the transcriptional repressor Whi5 (Skotheim *et al.*, 2008). Thus, we tested whether onset of Clb5 was still delayed in the absence of *WHI5*. Wild type and *whi5D* cells bearing TAP-tagged Clb5 at the endogenous locus were synchronized at G_1 with pheromone and stressed with 0.4 M NaCl after release. In response to osmostress, *whi5D* cells were able to delay Clb5 production just like wild type cells (Fig. 7c and 7d). Taken together our results suggest that the effect of Hog1 in the regulation of Clb5 upon osmotic stress is independent of Cln2 and the G_1 feedback loop.

A quantitative mathematical model predicts and quantifies the differential role for Hog1 targets on the cell cycle machinery at different stages of G_1.

To quantitatively assess the impact of Hog1 over the different components that regulate G_1-S progression, we created a new version of the qualitative model, which included all quantitative data. We refined and carefully parameterized the components to make it useful for studying its regulatory properties. Briefly, we created an ordinary differential equation model describing the temporal changes of the amounts of the components and complexes involved in the regulation of the G_1-S transition. Figure 8a shows a schematic representation of this model. A full description of the model, black boxes and parameter values is provided in the supplementary information. In-sample data

fittings (data used for parameter estimation) showed that the model simulations quantitatively reproduce the experimental data under different scenarios (in-sample fit, Supplementary Fig. 7). We next validated the model with additional experimental data sets that were not used in parameter estimation (out-sample fit, Supplementary Fig. 8). The quantitative model was able to predict different lengths of the arrest at G_1, the delay of the appearance of Cln1,2 and Clb5 as well as Sic1 degradation depending on the stress and Hog1 phosphorylation (Supplementary Fig. 9). The model was also able to predict the response curves for the cases of cells being stressed at different stages of G_1 (Supplementary Fig. 10). Then, the model was used to perform a quantitative study of the relevance of the regulation of each element downstream of Hog1 by analysing different *in silico* "mutants" deficient for Hog1-mediated *CLN2* or *CLB5* down-regulation or for Sic1 stabilization (Sic1^{T173A} mutant). Initially, we simulated with the three *in silico* mutants and a stress of 0.4 M NaCl applied immediately after release from pheromone (Fig. 8b-d) or at Start, 20 minutes after release (Fig. 8e-g). Results from the simulations indicate that a mutant deficient on the stabilization of Sic1 mediated by Hog1 phosphorylation only displayed a minor defect on the arrest caused by osmostress when cells were stressed after release. However, when stressed at Start, the ability of the cells to arrest upon stress was seriously compromised and only retained about a 40% of their ability to arrest (Fig. 8b and 8e; Supplementary Fig. 11q-r). It is worth noting that cells deficient on the down-regulation of *CLN2* displayed a similar lack of arrest when stressed after release and only partially maintained their ability to arrest when stressed at Start (Fig. 8c and 8f; Supplementary Fig. 12q-r). Thus, both the down-regulation of *CLN2* and stabilization of Sic1 only displayed a defect on cell cycle arrest when cells were stressed at Start.

In clear contrast, when simulations were carried out with a mutant deficient in the down-regulation of the *CLB5* cyclin in response to stress, showed almost a total inability to arrest both at the beginning of G_1 and at Start, only retaining 12% and 5% of its ability to arrest respectively (Fig. 8d and 8g; Supplementary 13q-r). Thus, the model predicted that the down-regulation of *CLB5* by Hog1 is critical for the arrest at any stage of G_1.

Taken together the model allowed to quantify the different contribution of the elements downstream of Hog1 in G_1 arrest and showed that whereas the down-regulation of *CLN2* and the stabilisation of Sic1 are important to prevent S phase entry in response to stress only at Start, the down-regulation of *CLB5* is critical at any stage of G_1.

Finally, we used the model to analyse the results from the ectopic expression of *CLN2* and *CLB5*. The simulations of cell cycle progression were carried out for a stress of 0.4 M NaCl applied immediately after release from pheromone or 20 minutes later. The simulations showed that, on one hand, the over-production of *CLN2* did not remarkably affect cell cycle arrest in response to stress (Supplementary Fig. 14q-r, Supplementary Fig. 16a and c), and on the other hand, that the ectopic expression of *CLB5* precipitated entry into S phase in the presence of stress and its effect was similar at any stage of cell cycle (Supplementary Fig. 15q-r, Supplementary Fig. 16b and d). According to the *in vivo* results, only the over-production of *CLB5*, but not *CLN2*, abolished the delay on cell cycle in response to osmostress. Therefore, the model supports the observation that Hog1 directly regulates *CLB5* independently of *CLN2* and, that this direct regulation might be the most significant for the regulation of Start upon osmostress.

Discussion

Regulation of cell cycle progression by external stimuli requires complex regulatory mechanisms. For instance, regulation of G_1 progression in response to osmostress was shown to be mediated, at least, by the down-regulation of *CLN1,2* and the direct phosphorylation of Sic1 by the Hog1 MAPK (Escote *et al.*, 2004;Zapater *et al.*, 2005). Although the use of mutants and over-expression experiments has proven to be useful to elucidate mechanistic properties of cell cycle regulation, these approaches may alter the normal cell cycle and make it difficult to assess specific dynamic properties, differential roles of the cell cycle regulators and their real *in vivo* contribution on the control of G_1 upon stress. On the other hand, computational analyses have shown to be very useful to elucidate regulatory principles of cell cycle progression (Thornton *et al.*, 2004;Chen *et al.*, 2004;Barberis *et al.*, 2007;Novak *et al.*, 2007) as well as MAPK signalling (Schoeberl *et al.*, 2002;Klipp *et al.*, 2005). To analyze the contribution of the different cell cycle regulators at the G_1-S transition, we have performed *in vivo* quantitative experiments complemented with a mathematical model, created through extensive parameter estimation from

experimental data. Thus, we generated, as far as we know, the first model that defines the impact of a MAPK signalling pathway on the cell cycle machinery.

Our analyses show that upon increasing osmolarities, Hog1 is phosphorylated for longer periods and this correlates with the duration of cell cycle arrest. Interestingly, when cells are subjected to stress at different times between release from pheromone and Start, the length of the arrest is identical. Thus, to a given stress, the arrest time required for adaptation is always similar.

Our experimental data, together with the model analyses, showed that neither down-regulation of *CLN2* nor stabilisation of Sic1 account for the cell cycle arrest mediated by Hog1. Actually, cells over-expressing *CLN2* in a *sic1* background were still able to arrest in response to osmostress, which suggested that besides Sic1 stabilization and down-regulation of *CLN2,* there should be an additional mechanism to delay cell cycle upon Hog1 activation. We show here that the regulation of Clb5, the major S phase promoting cyclin, is critically required for G_1 arrest upon stress. Indeed, Clb5 production is strongly delayed in response to stress. Correspondingly, previous results showed that *CLB5* transcription was down-regulated upon Hog1 hyperactivation and heat shock (Li *et al.*, 1999;Escote *et al.*, 2004). Thus, we show that previously described components play restricted roles and that cooperate with a new regulatory element of the G_1 arrest in response to osmotic stress.

Mathematical modelling profoundly contributed to the understanding of the systems properties. For example, our initial model could not satisfactorily fitted to data for Cln2, Sic1 and G_1-S-transition. However, once we included Clb5 production inhibition by Hog1, the fitting results were significantly improved which lead us to *in vivo* measurements of Clb5. Moreover, the final model based on data shown in Supplementary Figure 7 made and interesting prediction for different roles of Cln2 and Clb5 for regulating G_1-S transition (Fig. 8). This was then confirmed by *in vivo* experiments (Figs. 3 and 4). On the other hand, the model also reproduced experimental results not considered in its construction, for example the effect of Sic1^{T173A} mutant (Figs. 5 and 8).

In addition, mathematical modelling combined with experimental data, permits a quantitative assessment of the relevance of each component in the G_1 arrest through the use of *in silico* knockout of each specific component involved in the G_1 regulation (Cln2, Clb5 or Hog1-specific Sic1 phosphorylation; see Figure 8 for a summary of the predictions). These analyses, together with the *in vivo* data, have been instrumental to quantify and clarify the role of the different components regulated by Hog1. The phosphorylation of Sic1 by Hog1 has been shown to increase the stability of Sic1 (Escote *et al.*, 2004). The model predicts the role of Sic1 stabilization is to modulate cell cycle only at Start, for it also predicts that before Start, Clb5 production is tightly down-regulated and therefore the presence of its inhibitor, Sic1, is irrelevant. Correspondingly, when cells are stressed at Start, they show a deficient arrest if containing non-phosphorylatable Sic1 or lacking Sic1, whereas wild type cells show to be competent to arrest in this scenario, as monitored by Sld2 phosphorylation and DNA content analyses. Thus, Sic1 phosphorylation plays a key role in preventing the slow increase of Clb5 activity. Otherwise, cells progressively initiate replication without proper adaptation, since Clb5 levels are not as tightly regulated by Hog1 at this stage It is worth noting that a deficient Cln2 down-regulation results in the same G_1-deficient arrest that the lack of Sic1 stabilization at Start. Therefore, both *in vivo* and *in silico* data, define a specific temporal role for Sic1 and *CLN2* on the arrest at G_1. In contrast, the *in silico* mutants clearly show that the lack of *CLB5* down-regulation strongly affects the ability of the cells to delay cell cycle progression. Correspondingly, over-expression of *CLB5* prevents cell cycle arrest upon osmostress at any stage of G_1.

Additionally, our results show the main role of the Hog1 regulation on *CLN1,2* is to regulate budding. In a normal cell cycle, budding is simultaneous to replication, but upon stress, budding correlates to *CLN2* expression independently of replication, hinting replication is delayed through a downstream mechanism of Cln1,2-induced Sic1 degradation. Furthermore, the over-expression of *CLB5* promotes replication but not budding formation, upon osmotic stress. In contrast, the over-expression of *CLN2* in the presence of stress promotes budding but not replication. Thus, the main role of the down-regulation of *CLN2* by Hog1 seems to be the coordination of the arrest in replication and cell morphogenesis.

It has been proposed that expression of Clb5 can be under the control of a positive feed-back loop exerted by G_1 cyclins (Skotheim *et al.*, 2008). Here, our results show that induction of *CLB5* expression under the *GAL1* promoter is sufficient to abolish the delay of the replication onset caused by osmostress. In contrast, over-expression of *CLN2*, albeit

resulting in higher protein levels than Clb5, did not abolish the delay of replication onset upon osmostress. In addition, the lack of *WHI5*, a mediator of the positive loop of Cln1,2 on the transcription of the SBF/MBF-regulated genes did not alter the G_1 arrest in response to osmostress. Thus, in response to osmostress, the effect of Hog1 is direct on *CLB5* rather an indirect effect on *CLN*s.
Taken together, modelling and quantitative analyses have allowed us to define Clb5 as a novel key regulator for the arrest at G_1 upon stress, independently of Cln2, and reveal the specific and different temporal roles of Sic1 and *CLN*s for cell adaptation in response to stress.

Material and methods

Yeast strains and plasmids. Strains used: W303 (*MATa his3 leu2 trp1 ura3 ade2 can1*) and its derivatives YAN7 (*CLN2-3HA::KanMx SIC1-9Myc::TRPKl*), YAN32 (*CLN2-3HA::KanMx::Nat SIC1-9Myc::TRPKl CLB5-TAP::KanMx*). BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and its derivatives YAN37 (*CLB5-TAP::HIS3 sic1::KanMx::Nat SLD2-TAP::KanMx*), YAN38 (*cln3::KanMx::Nat SIC1-TAP::KanMx*) and YAN50 (*CLN2-3HA::KanMx::Nat SIC1-9Myc::TRPKl CLB5-TAP::KanMx whi5::LEU2*). TM141 (*MATa his3 leu2 trp1 ura3*) and its derivatives YPC38 (*sln1-ts4*) and YPC29 (*sln1-ts4 hog1::LEU2*). Plasmids used: Full-length wild type Sic1-Myc (pMZ55) and the mutant in the Thr173 to alanine (pMZ57) were cloned into the pRS416 centromeric plasmid and expressed under the native promoter. Wild type Sic1-Myc (pMZ65) and the mutant in the Thr173 to alanine (pMZ62) were cloned into the pRS414, and expressed under the native promoter. Full length HA-Clb5 was cloned into YCpIF16 (pMAD23) under the *GAL1* promoter. Full length HA-Cln2 cloned into pYES2 under the *GAL1* promoter (pCM249) and is a generous gift from Dr. Eloy Garí (UdLl).

Growth conditions, cell synchrony and cytometry analyses. Cells were grown in YPD at 25ºC. Cell synchrony was accomplished by treatment of cells with 40 mg/ml of α̃factor for 3h at 25ºC. In the case of *GAL1::CLB5* and *GAL1::CLN2* over-expression experiments cells were grown overnight in SD with 2 % rafinose, synchronized for 2h in the same medium at 30ºC with pheromone. *GAL1* promoter was induced with 2% galactose, 20 minutes prior release from pheromone. For flow cytometry analyses, cells were fixed in ethanol, treated overnight with RNAse A at 37ºC in 50mM Sodium Citrate, stained with propidium iodide and analysed in a FACScan flow cytometer (Becton Dickinson). A total of 10000 cells were analysed and the population of G_1 quantified for each time point using WinMDI 2.9.

Western-blot and quantification analyses. TCA protein extracts were resolved in SDS-PAGE, total amounts of the indicated proteins were detected by immunobloting and chemoluminescence. Exposed films were scanned in 16bits/channel and quantified using the ODISSEY™ Aplication Software 2.1.

Mathematical modeling. The details of the mathematical model are presented in the Supplementary Methods online. The parameter estimation for the final quantitative model was implemented in SBML-PET tool (Zi and Klipp, 2006). The ordinary differential equations were solved using Mathematica and SBML-SAT tool (Zi *et al.*, 2008) in Matlab.

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Figures

Adrover *et al.*
Figure 1

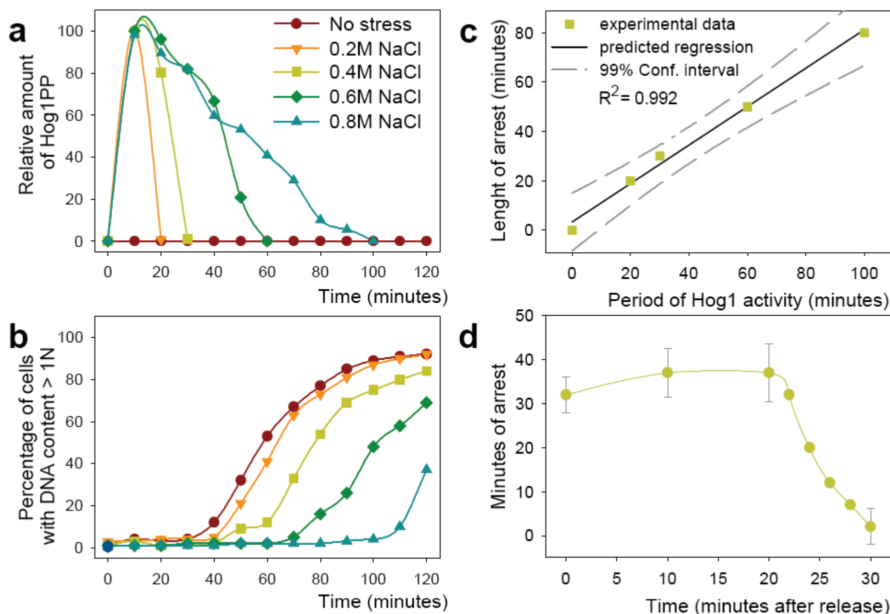


Figure 1. ***In vivo* validation of the predictions from the initial model.**
(**a**) Higher osmolarities induce longer Hog1 activation. W303 cells bearing tagged Cln2-3HA and Sic1-13Myc on their chromosomal locus (YAN7), were synchronized for 3h with α̃factor at 25ºC, shifted to YPD, containing different concentrations of NaCl (0.2 to 0.8 M). Cultures were sampled every 10 minutes and phosphorylated Hog1 (expressed as percentage of maximum level) was analyzed by quantitative western blot. (**b**) Longer activation of Hog1 results in a longer arrest at G_1. YAN7 cells were synchronized, stressed and sampled as in **a**. DNA content was assessed by flow cytometry and 1C and 2C populations were quantified (represented % of cells in S-G_2). **(c)** The length of the arrest linearly correlates with the length of Hog1 activation. Minutes of phosphorylated Hog1 for cells exposed to different NaCl concentrations (0,2-0,8 M) are represented in the x-axis and minutes of the onset of DNA replication is delayed with respect to control cells are represented in the y-axis. The 99% confidence interval is represented by slashed gray lines. Experimental data are shown with green squares and regression line is in black. **(d)** The data shows the relationship between the delay of replication onset (y-axis) and the stress time (x-axis). The arrest at G_1 upon stress is effective along G_1, but vanishes nearby the Start

Adrover *et al*.
Figure 2

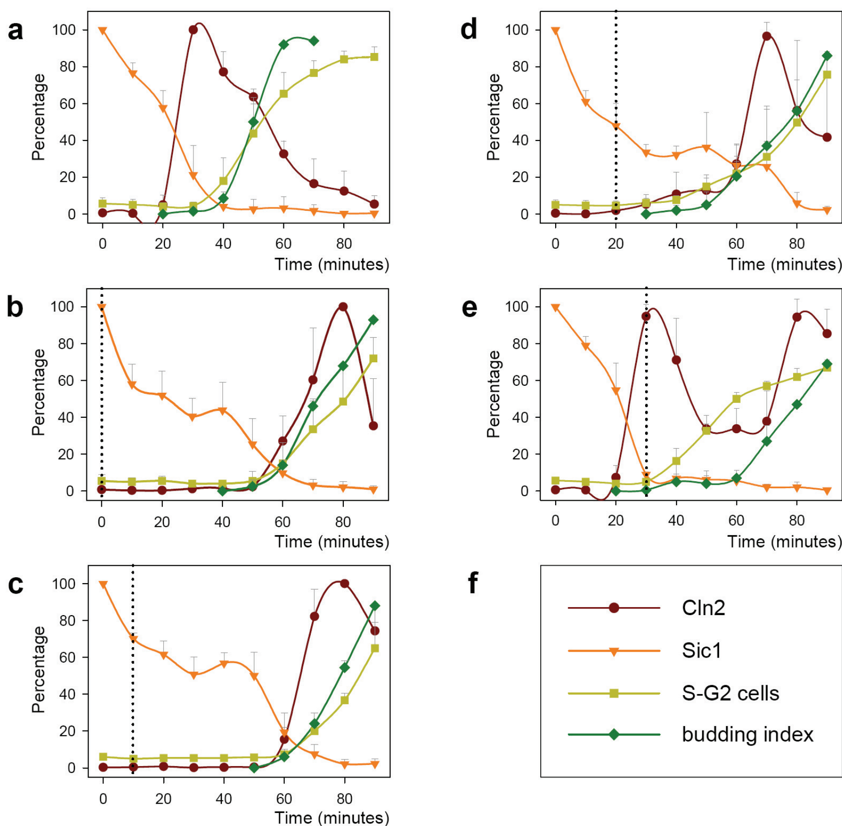


Figure 2. Sic1 degradation time correlates with DNA replication and Cln2 levels upon osmostress.
(**a**) Sic1 is degraded before Cln2 reaches its maximal levels and ten minutes before S phase onset. YAN7 cells were synchronized for 3h with ãfactor at 25ºC, shifted to YPD and sampled every 10 minutes. Sic1 and Cln2 proteins (expressed as percentage of their maximal levels) were determined by quantitative western blot. DNA content was scored by flow cytometry, and budding index determined by microscopy. **(b-d)** Sic1 total degradation, Cln2 production and S phase are delayed in a similar fashion when stress appears at different times along G_1. YAN7 cells were synchronized and released as in **a**, and stressed at 0 (b), 10 (c) or 20 (d) minutes (as indicated by vertical dotted lines) after release in 0.4 M NaCl, and finally analyzed as above. **(e)** Cells loose ability to arrest when stressed nearby the G_1/S phase boundary. YAN7 cells were synchronized and released as in **a,** stressed with 0.4 M NaCl 30 minutes after ãfactor release, and sampled and analyzed as described above. (**f**) Legend.

Adrover *et al.*
Figure 3

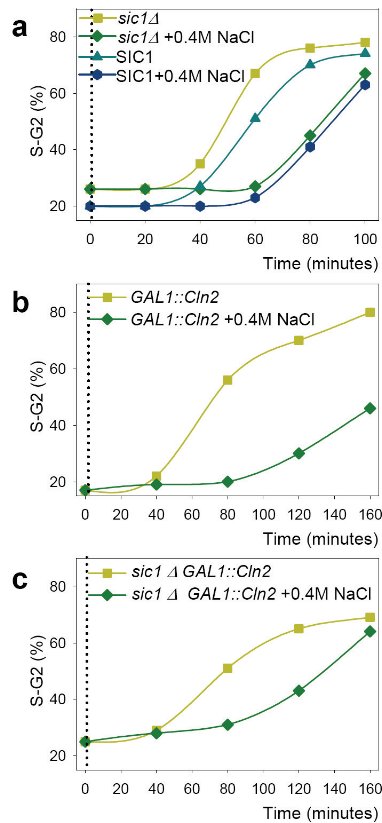


Figure 3. Lost of regulation of Sic1 and/or Cln1,2 by Hog1 is not sufficient to fully abolish the delay in cell cycle upon osmotic stress
(**a**) Sic1 is not essential for cell cycle arrest after pheromone release upon osmostress. W303 and *sic1D* cells were arrested in G_1 with pheromone and released in the absence or presence of 0.4 M NaCl. Cultures were sampled at the indicated times to determine DNA content by flow cytometry. (**b**) The ectopic expression of Cln2 does not prevent the osmostress-imposed delay. W303 cells were transformed with empty plasmid or *GAL1-CLN2-HA* and synchronized with pheromone for 2h at 30ºC in SD plus raffinose. Galactose was added 20 min prior release in absence or presence of 0.4 M NaCl. Cultures were sampled and analysed as in **a**. (**c**) The ectopic expression of Cln2 does not prevent the osmostress-imposed delay in the absence of Sic1. *sic1D* cells were transformed with the same plasmids, treated and sampled as in **b**. In all panels, vertical dotted lines indicate the time point osmostress is applied.

Adrover *et al.*
Figure 4

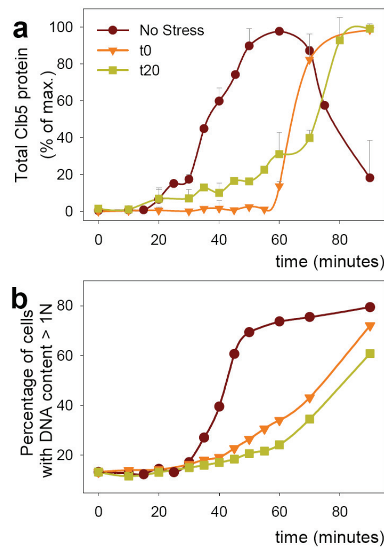


Figure 4. Clb5 protein levels are regulated upon osmostress.
(**a**) Osmostress delays production Clb5 at the protein level. W303 cells bearing epitope-tagged Clb5-TAP, Sic1-13Myc and Cln2-3HA at their respective chromosomal locus (YAN32), were synchronized for 3h with pheromone in YPD at 25ºC, shifted to YPD, divided into aliquots, stressed with 0.4 M NaCl after release (t0) or 20 minutes later (t20), and analyzed by quantitative western blot to determine levels of Clb5. (**b**) DNA replication is postponed even when Clb5 production is not tightly repressed. Samples from the same cultures as in **a** were analyzed by flow citometry to score for DNA content.

Adrover *et al.*
Figure 5

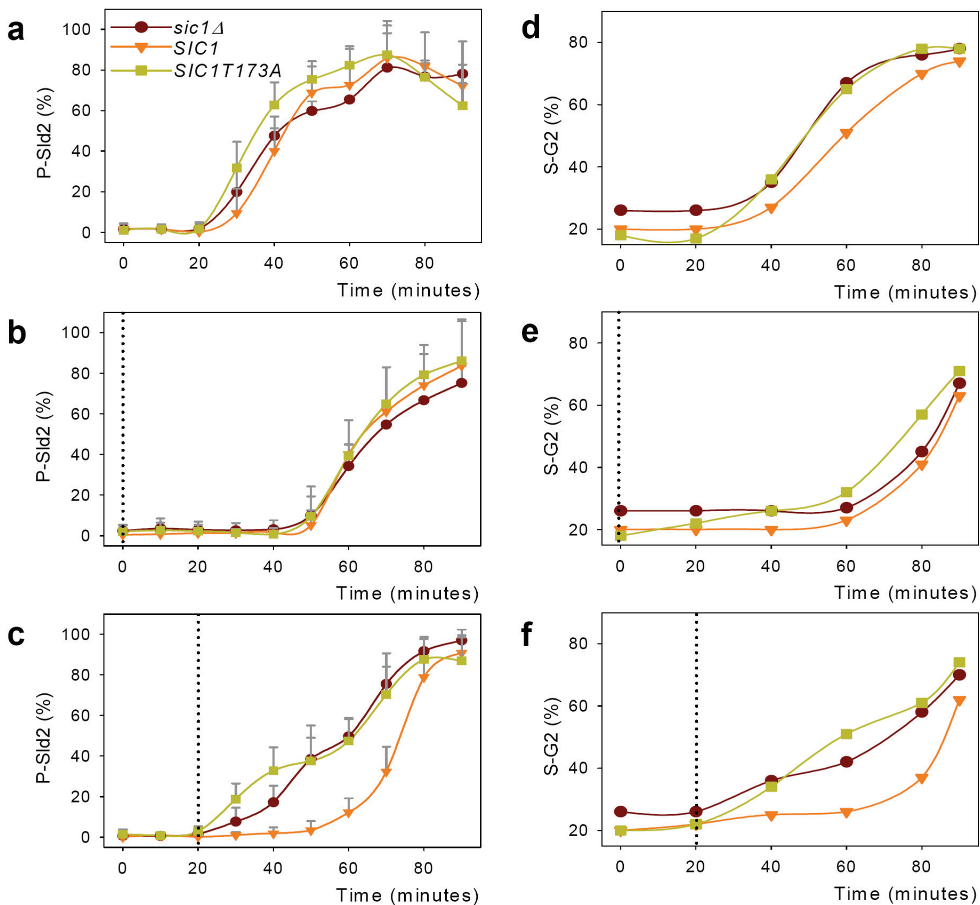


Figure 5. Sic1 stabilization at Start by Hog1 is necessary to properly postpone Sld2 phosphorylation and progression into S phase upon osmostress.
(**a**) Mutation of Sic1 does not greatly alter timing of Sld2 phosphorylation under normal conditions. s*ic1*D cells bearing genomically tagged Clb5-TAP and Sld2-TAP (YAN37) were transformed with centromeric plasmids expressing tagged Sic1-9Myc from its own promoter (pMZ55), its equivalent expressing Sic1T173A (pMZ57) or empty plasmid, synchronized for 3h with ãfactor in YPD at 25ºC, released into YPD and sampled every 10 minutes. TCA protein extracts were resolved in SDS-PAGE and analyzed by quantitative western blot to determine levels of phosphorylated Sld2 (expressed as a percentage of the total amount of Sld2). (**b** and **c**) Sic1 stabilization is a key determinant on Sld2 phosphorylation upon osmostress at Start. YAN37 bearing plasmids pMZ55, pMZ57 or empty plasmid were synchronized as in **a**, released from the arrest and stressed with 0.4 M NaCl after release (b) or 20 minutes later (c), as indicated by vertical dotted lines. Samples were analyzed as above. (**d**) Mutation of Sic1 does not greatly alter timing of progression into S phase under normal conditions. Cells as in a were sampled at the indicated times to determine DNA content by flow cytometry. (**e** and **f**) Sic1 stabilization is a key determinant for cell cycle progression upon osmostress at Start. Cells as in e and f respectively were sampled at the indicated times to determine DNA content by flow cytometry.

Adrover *et al.*
Figure 6

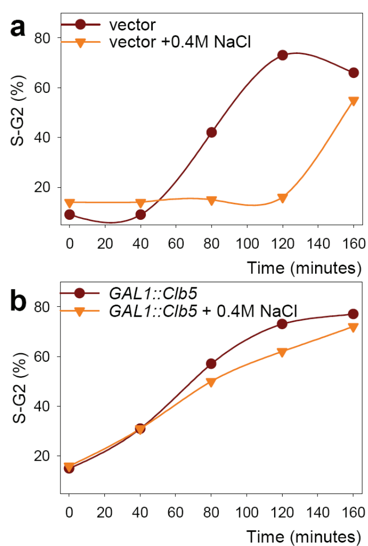


Figure 6. The relevance of Sic1 is restricted to Start and the ectopic expression of Clb5 accelerates the entry into S phase.
The ectopic expression of Clb5 from *GAL1* promoter in G_1 induces an earlier DNA replication and abolishes the delay imposed by osmostress. (**a**) W303 cells were transformed with empty plasmid (vector) and synchronized with pheromone for 2h at 30ºC in raffinose. Galactose was added 20 min prior release in absence or presence of 0.4 M NaCl. Cultures were sampled at the indicated times to determine DNA content by flow cytometry. (**b**) W303 cells containing a plasmid expressing *CLB5* from the *GAL1* promoter (*GAL1::CLB5*) were treated and scored as in a.

Adrover *et al.*
Figure 7

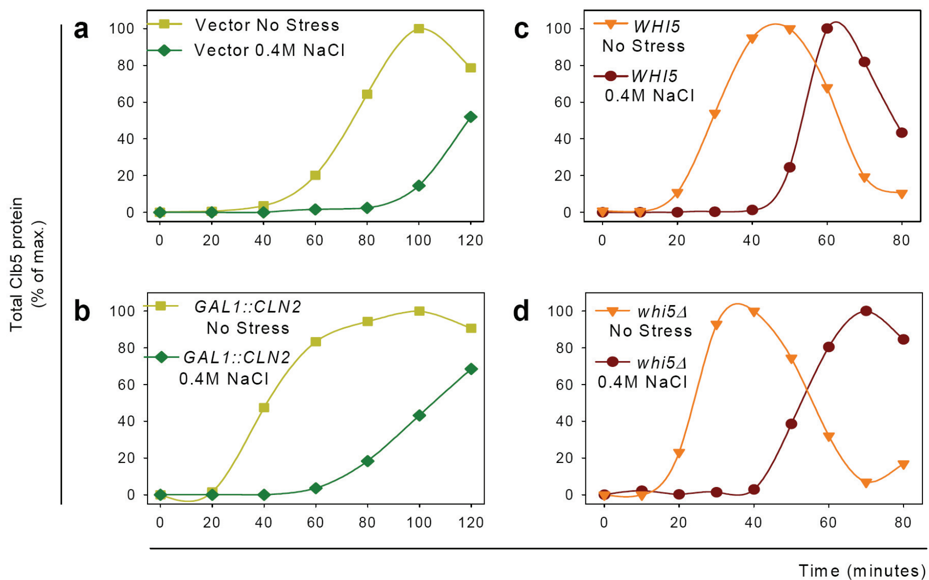
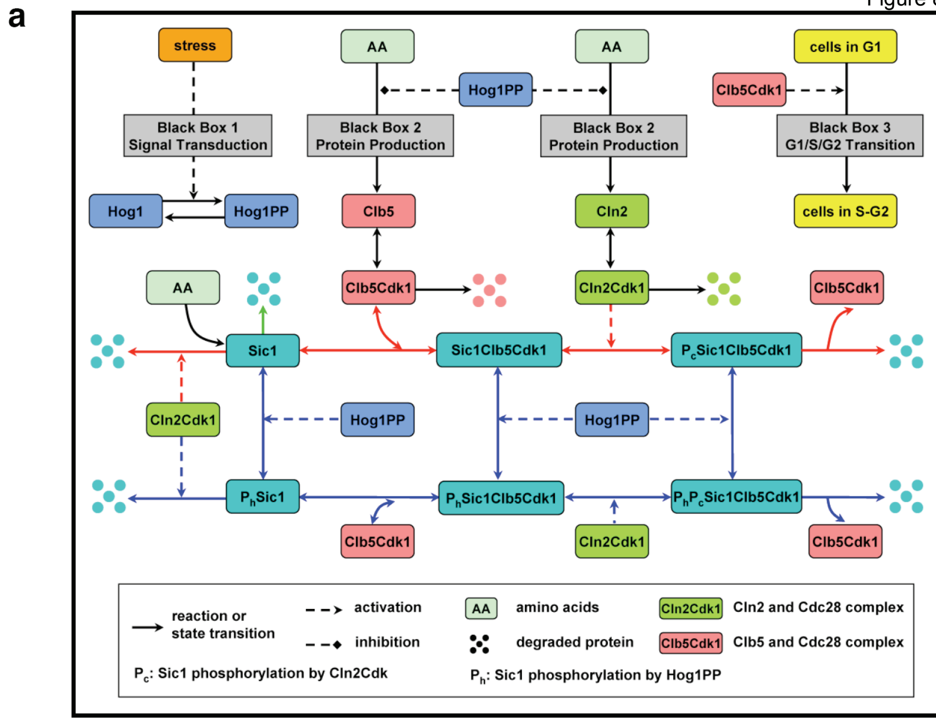


Figure7. The regulation of Clb5 upon osmostress is not through the G_1 cyclins feedback loop.
(**a** and **b**) The ectopic expression of Cln2 does not prevent the osmostress-imposed delay on Clb5 production. YAN32 cells were transformed with empty plasmid (**a**) or pYES2-Cln2-HA (**b**) and synchronized with pheromonefor 2h at 30ºC in raffinose. Galactose was added 20 min prior release in absence or presence of 0.4 M NaCl. Cultures were sampled at the indicated times to determine DNA content by flow cytometry and total Clb5 protein levels by quantitative western blot. (**c** and **d**) The regulation of Clb5 upon osmotic stress does not involve the transcriptional repressor Whi5. W303 (**c**) and YAN50 cells (**d**) were synchronized with pheromone and released in the presence of 0.4 M NaCl, samples of the indicated times were taken to monitor Clb5 levels by quantitative western blot.

Adrover *et al.*
Figure 8



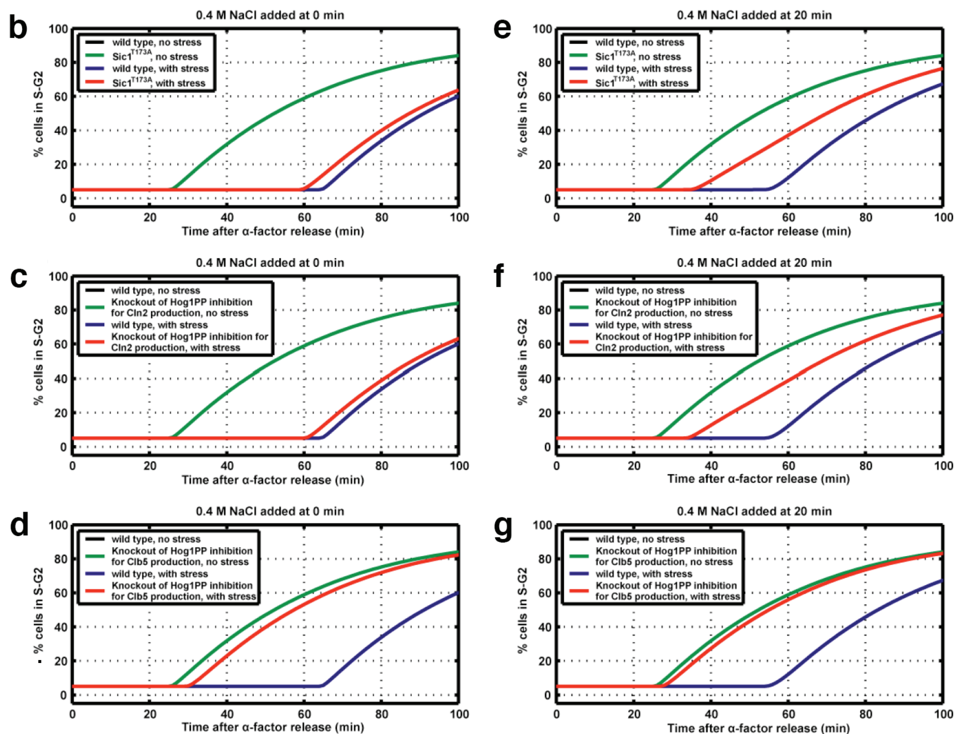


Figure 8. Scheme of the quantitative model and predictions of cell cycle progression for *in silico* mutants on Hog1 targets upon stress.
(**a**) The core module of the model is Sic1, which experiences a constitutive degradation (green arrow) that is independent on Cln2-Cdc28, Clb5-Cdc28 or phosphorylated Hog1 (Cln2Cdk1, Clb5Cdk1 and Hog1PP respectively). Sic1 is also degraded by some Hog1-independent reactions (red arrows), and through some other reactions that involve Hog1 activity (blue arrows). Non central parts of the model are simplified in the mode of black boxes (gray rectangles) Detailed information about the mathematical model is described in the supplementary information. (**b**-**g**) Predictions for time courses of the percentage of cells in S-G_2 obtained with the unperturbed model (wild type) under normal conditions (black), or stressing with 0.4M NaCl (blue) immediately (**b**-**d**) or 20 minutes (**e**-**g**) after release from alpha-factor; and simulations for different perturbations of the model; non-stabilized Sic1 (*Sic1T173A*) (**b** and **e**), non-regulated Cln2 (**c** and **f**), and non-regulated Clb5 (**d** and **g**) under normal conditions (green) or stressing with 0.4M NaCl (red). In all cases, the black curve overlaps with the green curve.

Supplementary Information

Supplementary Information for the Initial Model

This model illustrates the interaction of active Hog1 (Hog1PP) with some components of the cell cycle machinery as shown in Supplementary Figure 1. It describes the activation and deactivation of Hog1 (v_1 and v_2). The protein Cln2 binds to Cdc28 (hereafter referred as Cdk1) (v_4). The complex Cln2Cdk1 can be degraded (v_5) and is required for phosphorylation of Sic1 in the complexes Sic1Clb5Cdk1 (v_9) and P_hSic1Clb5Cdk1 (v_{14}). Clb5 is produced (v_6), binds to Cdk1 (v_7), and later to Sic1 (v_8 and v_{12}). Rates v_{14} and v_{15} are similar to v_9 and v_{10}, denoting phosphorylation and degradation of phosphorylated Sic1 bound to the cyclin-Cdk1 complex. Clb5Cdk1 is considered the active complex coordinating DNA replication. Hog1PP acts through a dual mechanism; (a) down regulation of Cln2 by inhibiting transcription of Cln2 (v_3), and (b) direct phosphorylation of free Sic1 (v_{11}) or Sic1 bound to Clb5Cdk1 (v_{13}).
The dynamics of this network is described in Supplementary Table 1.

Supplementary Table 1. Ordinary differential system of the initial model

$$\frac{\mathrm{d}[Hog1PP]}{\mathrm{d}t} = v_1 - v_2 \qquad v_1 = k_1$$

$$\frac{\mathrm{d}[Cln2]}{\mathrm{d}t} = v_3 - v_4 \qquad v_2 = k_2[Hog1PP]$$

$$\frac{\mathrm{d}[Cln2Cdk1]}{\mathrm{d}t} = v_4 - v_5 \qquad v_3 = k_3/(1 + k_x[Hog1PP])$$

$$\frac{\mathrm{d}[Clb5]}{\mathrm{d}t} = v_6 - v_7 \qquad v_4 = k_4[Cln2]$$

$$\frac{\mathrm{d}[Clb5Cdk1]}{\mathrm{d}t} = v_7 - v_8 \qquad v_5 = k_5[Cln2Cdk1]$$

$$\frac{\mathrm{d}[Sic1Clb5Cdk1]}{\mathrm{d}t} = v_8 - v_9 \qquad v_6 = k_6$$

$$\frac{\mathrm{d}[P_c Sic1Clb5Cdk1]}{\mathrm{d}t} = v_9 - v_{10} \qquad v_7 = k_7[Clb5]$$

$$\frac{\mathrm{d}[Clb5Cdk1_active]}{\mathrm{d}t} = v_{10} + v_{15} \qquad v_8 = k_8[Clb5Cdk1][Sic1]$$

$$\frac{\mathrm{d}[Sic1]}{\mathrm{d}t} = -v_8 - v_{11}$$

$$\frac{\mathrm{d}[P_h Sic1]}{\mathrm{d}t} = v_{11} - v_{12}$$

$$\frac{\mathrm{d}[P_h Sic1Clb5Cdk1]}{\mathrm{d}t} = v_{12} + v_{13} - v_{14}$$

$$\frac{\mathrm{d}[P_h P_c Sic1Clb5Cdk1]}{\mathrm{d}t} = v_{14} - v_{15}$$

$$v_9 = k_9[Sic1Clb5Cdk1][Cln2Cdk1]$$

$$v_{10} = k_{10}[P_c Sic1Clb5Cdk1]$$

$$v_{11} = k_{11}[Sic1][Hog1PP]$$

$$v_{12} = k_{12}[Clb5Cdk1][P_h Sic1]$$

$$v_{13} = k_{13}[Sic1Clb5Cdk1][Hog1PP]$$

$$v_{14} = k_{14}[P_h Sic1Clb5Cdk1][Cln2Cdk1]$$

$$v_{15} = k_{15}[P_h P_c Sic1Clb5Cdk1]$$

The parameter values are chosen as follows:

$$k_1 = \begin{cases} 0.1, t_0 \le t \le t_1 \\ 0 \end{cases}, \quad k_2 = 0.1, \quad k_3 = 0.01, k_4 = 1, k_5 = 10, k_6 = 0.01,$$

$$k_7 = 1, \quad k_8 = 1, \quad k_9 = 0.01, \quad k_{10} = 1, \quad k_{11} = 1, \quad k_{12} = 1, \quad k_{13} = 1,$$

$$k_{14} = 0.01, k_{15} = 0.01$$

Note that the choice of parameters is completely arbitrary, at maximum a sensible guess, as we want to study the qualitative behavior of the network as at the starting stage.
The results for the simulation of the network are shown in Supplementary Figure 2.

Supplementary Information of the Final Model

Here we present the rationale that guided us to build the final model and give the detailed mathematical description of the model for the control of G_1-S transition by Sic1 and cyclins upon osmotic stress.

1. General Description

The concentrations of all relevant signal transduction molecules and components are modeled by a system of ordinary differential equations. The concentration of a certain molecule [M_i], evolving over time, is determined by the sum of the reaction rates producing (M_i) and the sum of the rates consuming (M_i) such a molecule (E1.1):

(E1.1)

$$\frac{d[M_i]}{dt} = \sum v_{production} - \sum v_{consumption}$$

The processes and regulations for osmotic stress and cell cycle progression are very complicated. Here we try to establish a “parsimonious model” and study the effect of osmotic stress on the regulation of the G_1-S transition (not the whole cell cycle). The processes outside our focus (for example, the regulation of cell cycle after S phase) are not explicitly modeled. Other processes at the border are described in coarse-grained way. In order to simplify the non-

central modules of the model and to define the interface of the system to the environment, some processes are lumped together and modeled as black boxes (functional units) according to their experimentally observed input-output behaviors (see next section). The model focuses on a detailed central system of interaction between the key players in the regulation of G_1-S transition (Cln2, Clb5 and Sic1) and their regulation by Hog1. Figure 8 in the main text shows the scheme of the mathematical model. The central core of the model describes Clb5 inhibition by Sic1, as well as all the phosphorylation processes that lead to Sic1 degradation or stabilization. After parameter estimation, this model is able to reproduce and predict well for the time course profiles of total Sic1, Cln2, Clb5 and G1-S progression under different stress scenarios (Supplementary Fig. 7-10).

Most of the molecular interactions were modeled with mass-action kinetics in the biochemical reaction equations. For some specific processes in the black boxes, we use some complicated kinetics (e.g. Michealis-Menten kinetics, Hill function) to model them (details will be presented later).
We made the following basic assumptions for the construction of the model. Other specific assumptions will be explained later: (1) Due to the common transcriptional regulation of Cln1 and Cln2 by the SBF (Swi4-Swi6 cell cycle box binding factor) and their redundancy, Cln2 tightly correlates to Cln1, and therefore Cln2 has been measured as indicator of Cln1,2 levels (hereafter referred as Cln2). Clb6 is obviated and only Clb5 has been measured, since Clb5 is the major S phase cyclin. (2) We assume activated Hog1 (Hog1PP) and Cln2-Cdc28 complex (Cln2Cdk1) are able to phosphorylate Sic1 at any state. Sic1 phosphorylation by Cln2Cdk1 leads immediately to its subsequent SCF-mediated ubiquitination and degradation. (3) Without prior knowledge, we assumed that most of the reactions are reversible. The reactions for protein production, degradation and G_1-S transition of cell cycle are irreversible.

The experimental datasets include time course data of synchronous cultures at G_1 under normal growth conditions or treated with 0.4 M NaCl at 0, 10, 20 and 30 minutes after release from α-factor. Total amounts of Cln2, Sic1, Clb5, and the percentage of cells with DNA content >1C were measured along time for all the conditions. Furthermore, Hog1PP, total amount of Cln2, and percentage of cells with DNA content >1C were measured for cells treated with 0.2, 0.6 and 0.8 M NaCl after α-factor release. The overview of the experimental design is shown in Supplementary Table 2.

Supplementary Table 2. Overview of experimental design

Time of Stress → Stress	0 min	10 min	20 min	30 min
0.2 M NaCl	X			
0.4 M NaCl	X	X	X	X
0.6 M NaCl	X			
0.8 M NaCl	X			
0 M NaCl (Control)		X		

The gray boxes on Supplementary Table 2 correspond to the data sets used for parameter estimation. The other datasets were used for the validation of the model's performance.

2. Black Boxes (Functional Units)

In this work, we focus on the impact of osmotic stress on the G_1-S transition, and more specifically, the different contribution of G_1-S cyclins regulation and Sic1 phosphorylation. We zoomed into the relevant parts of the network. Because the exact regulatory mechanism of these processes is still to be established and there is not enough quantitative data to constrain the parameter values in these processes, we reduced their complexity and simulate them by a tractable approach. Three processes of the system were lumped into black boxes and modeled as functional units according to their experimentally observed input-output behaviors, i.e. Hog1 phosphorylation, both Cln2 and Clb5 production and the progression of cells into S-G_2 phases

(percentage of cells with more than 1C of DNA). This is necessary for the model to define the system interface to the environment and cover the dynamics as good as necessary for the relevant part of the model, but as simplified as possible to determine the parameters from data. The interaction of the G_1 cell cycle control machinery and its regulation by Hog1 are described in Figure 8, and more details about the black boxes are given in the following sections.

2.1 Black Box 1: the activation of Hog1

In this model, the activation of Hog1 by the stress response pathway was simplified and lumped into a black box (Supplementary Fig. 5a). The activation rate of Hog1 is modeled as the following stress-processing function based on the experimentally observed input-output behaviors (E2.1 and E2.2):

$$v_{\mathrm{pho}}^{\mathrm{Hog1PP}} = K_{\mathrm{pho}}^{\mathrm{Hog1}}[Hog1]S(t) \qquad \text{(E2.1)}$$

$$S(t) = \begin{cases} 0 & if \;\; t < t_0 \\ stress \cdot e^{\frac{-k_{\mathrm{t}} \cdot (t - t_0)}{stress}} & if \;\; t \geq t_0 \end{cases} \qquad \text{(E2.2)}$$

$K_{\mathrm{pho}}^{\mathrm{Hog1}}$ is the phosphorylation rate constant; the stress processing function $S(t)$ is a function of time and stress representing activities of the upstream components in the signal transduction that cause the phosphorylation of Hog1; $stress$ is the strength of stress, (NaCl concentration); t_0 is the time when stress is added; k_{t} corresponds to the turn over rate constant for the stress effect on Hog1 phosphorylation.
The dephosphorylation of Hog1PP is modeled with mass-action kinetics (E2.3).

$$v_{\mathrm{depho}}^{\mathrm{Hog1PP}} = K_{\mathrm{depho}}^{\mathrm{Hog1}}[Hog1PP] \qquad \text{(E2.3)}$$

The changes in concentration of Hog1 and Hog1PP over time are described by:

$$\frac{d[Hog1]}{dt} = v_{\mathrm{depho}}^{\mathrm{Hog1PP}} - v_{\mathrm{pho}}^{\mathrm{Hog1PP}} \qquad \text{(E2.4)}$$

$$\frac{d[Hog1PP]}{dt} = v_{\mathrm{pho}}^{\mathrm{Hog1PP}} - v_{\mathrm{depho}}^{\mathrm{Hog1PP}} \qquad \text{(E2.5)}$$

We estimated the parameters in this black box with the data from Hog1PP western blots for 0.4 and 0.8 M NaCl (Supplementary Fig. 5b and c). The model is also able to reproduce Hog1 phosphorylation under other conditions, for example, cells exposed to 0.2 and 0.6 M NaCl (Supplementary Fig. 6d and e).

2.2 Black Box 2: the production of Cln2 and Clb5

Our experimental data show that there is no detectable Cln2 or Clb5 protein levels for the first 20 minutes after pheromone release, and it has been described in previous studies (de Bruin *et al*, 2004) that the activation of SBF and MBF transcriptional systems by Cln3 (Start) occurs some time after alpha factor release. Similarly to previous models from Chen K., Novak B. and Tyson J. *et al.* (Chen *et al*, 2004; Chen *et al*, 2000), cells arrested at Start should grow to a critical size to have enough Cln3 activity to overcome the repression of SBF/MBF (e.g. inhibition by Far1, Whi5), and to allow for the production of two kinds of cyclins, Cln1,2 and Clb5,6. When cells exit from G_1, the transcription mediated by SBF and MBF is turned off by Clb2, Nrm1 and other regulators. Furthermore, it was proposed long time ago and recently confirmed with new evidences by Skotheim *et al.*, that there is a Cln1,2-dependent positive feedback on the activation of SBF/MBF (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Skotheim *et al*, 2008). These regulations are shown in Supplementary Figure 6.

In the model, we take into account the regulation of SBF/MBF activity and the positive feedback by Cln2. Because of the lack of enough quantitative data for the SBF/MBF regulation and the full mechanism of their regulation is still to be established, we model these processes in a coarse-grained way. In the future, this part can be improved with the accumulation of more data and evidence about the detailed mechanisms. In both models from Chen K., *et al*. (Chen *et al*, 2004; Chen *et al*, 2000), the SBF/MBF activity is modeled through Goldbeter-Koshland function (Goldbeter and Koshland, 1981). As Chen K., *et al.*'s models include other regulations outside the focus of our work, here we model the transcription activity of SBF/MBF in another simple way as the following.

(a) Modeling of cell growth

$$\frac{d[mass]}{dt} = \frac{k_{\mathrm{g}} \cdot [mass]}{1 + w_1 \cdot [Hog1PP]} \quad \text{(E2.6)}$$

Similar as Chen K., *et al.* (Chen *et al*, 2004; Chen *et al*, 2000), in physiological conditions, it is assumed that cell size is proportional to mass, a variable indicating the overall cell size (we also assume that mass exponentially increases). It is also known that when cells are exposed to osmotic stress, they grow slower compared to normal conditions because it takes some time for cells to adapt to stress (Klipp *et al*, 2005; Warringer *et al*, 2003). Here, we describe the inhibition of the cell growth (mass) with equation E2.6. The parameter w_1 is the scaling factor for the inhibition effect of osmotic stress on mass increase.

(b) Modeling of SBF activity

For modeling the activity of SBF, we use the following equation:

$$[SBF] = \frac{A_{\mathrm{SBF,\,Cln3}} + F_{\mathrm{SBF,\,Cln2}}}{1 + I_{\mathrm{SBF,Clb2}}} \quad \text{(E2.7)}$$

where the variable $A_{\mathrm{SBF,\,Cln3}}$ describes the activation of SBF by rising Cln3 activity, which overcomes the pre-existing repression of SBF by Far1 and Whi5 (E2.8).

(E2.8)

$$A_{\mathrm{SBF,\,Cln3}} = \begin{cases} 0 & \text{if } mass < mass_{\mathrm{Cln3}} \\ \dfrac{\varepsilon_{\mathrm{SBF,Cln3}} \cdot (mass - mass_{\mathrm{Cln3}})}{J_{\mathrm{mass,Cln3}} + (mass - mass_{\mathrm{Cln3}})} & \text{if } mass \geq mass_{\mathrm{Cln3}} \end{cases}$$

The parameter $mass_{\mathrm{Cln3}}$ is used to capture the feature of delay time for SBF/MBF activation after α-factor release. $J_{\mathrm{mass,Cln3}}$ is similar to the Michaelis constant.

$F_{\mathrm{SBF,\,Cln2}}$ represents the positive feedback effect of Cln2 on the activation of SBF (E2.9).

$$F_{\mathrm{SBF,\,Cln2}} = \varepsilon_{\mathrm{SBF,Cln2}} \cdot [Cln2Cdk1] \quad \text{(E2.9)}$$

he parameter EMBED??EquBED Equation.3 $\varepsilon_{\mathrm{SBF,Cln2}}$ indicates the strength of the positive feedback.

$I_{\mathrm{SBF,Clb2}}$ represents the inhibitory effect of SBF activity by Clb2 and other regulators, which repress SBF transcriptional activity (E2.10).
(E2.10)

$$I_{\mathrm{SBF,Clb2}} = \begin{cases} 0 & \text{if } mass < mass_{\mathrm{Clb2}} \\ \varepsilon_{\mathrm{SBF,Clb2}} \cdot (mass - mass_{\mathrm{Clb2}}) & \text{if } mass \geq mass_{\mathrm{Clb2}} \end{cases}$$

$mass_{\mathrm{Clb2}}$ is the parameter to model the appearance time of Clb2 during the cell cycle progression. $\varepsilon_{\mathrm{SBF,Clb2}}$ can be interpreted as the integrated effect factor for Clb2 production rate and the scaling weight factor for the inhibition of SBF activity.

(c) Modeling of MBF activity
We use a similar way to model the transcription activity of MBF:

$$[MBF] = \frac{A_{\mathrm{MBF,Cln3}} + F_{\mathrm{MBF,Cln2}}}{1 + I_{\mathrm{MBF,Nrm1}}} \quad \text{(E2.11)}$$

The variable $A_{\mathrm{MBF,Cln3}}$ describes the activation of MBF by newly produced Cln3, which overcomes the pre-existing repression of MBF (E2.12).

(E2.12)

$$A_{\mathrm{MBF,Cln3}} = \begin{cases} 0 & \text{if } mass < mass_{\mathrm{Cln3}} \\ \dfrac{\varepsilon_{\mathrm{MBF,Cln3}} \cdot (mass - mass_{\mathrm{Cln3}})}{J_{\mathrm{mass,Cln3}} + (mass - mass_{\mathrm{Cln3}})} & \text{if } mass \geq mass_{\mathrm{Cln3}} \end{cases}$$

The variable $F_{\mathrm{MBF,Cln2}}$ represents the positive feedback effect of Cln2 on the activation of MBF.

$$F_{\mathrm{MBF,Cln2}} = \varepsilon_{\mathrm{MBF,Cln2}} \cdot [Cln2Cdk1] \quad \text{(E2.13)}$$

The variable $I_{\mathrm{MBF,Nrm1}}$ represents the repressing effect of MBF by Nrm1 and other regulators, which turn off MBF transcriptional activity (de Bruin *et al*, 2006; de Bruin *et al*, 2008).
(E2.14)

$$I_{\mathrm{MBF,Nrm1}} = \begin{cases} 0 & \text{if } mass < mass_{\mathrm{Nrm1}} \\ \varepsilon_{\mathrm{MBF,Nrm1}} \cdot (mass - mass_{\mathrm{Nrm1}}) & \text{if } mass \geq mass_{\mathrm{Nrm1}} \end{cases}$$

$mass_{\mathrm{Nrm1}}$ is the parameter to model the appearance time of Nrm1 during the cell cycle progression. The parameter $\varepsilon_{\mathrm{MBF,Nrm1}}$ can be interpreted as the integrated effect factor for the production rate of Nrm1 and the scaling weight factor for its inhibition on MBF activity.

(d) Modeling of Cln2 and Clb5 production

The production of Cln2 and Clb5 is proportional to the activity of SBF and MBF, respectively. In addition, we also consider the inhibition of Cln2 and Clb5 production by phosphorylated Hog1 (Hog1PP) in the model. Overall, the production of Cln2 and Clb5 are described as:

$$v^{\text{Cln2}} = \frac{ks_{\text{Cln2}} \cdot [SBF]}{1 + w_2 \cdot [Hog1PP]} + ks_{\text{GAL1,Cln2}} \quad \text{(E2.15)}$$

$$v^{\text{Clb5}} = \frac{ks_{\text{Clb5}} \cdot [MBF]}{1 + w_3 \cdot [Hog1PP]} + ks_{\text{GAL1,Clb5}} \quad \text{(E2.16)}$$

For the simulation of *GAL1::CLN2*, we introduced a constitutive rate $ks_{\text{GAL1,Cln2}}$ and $ks_{\text{GAL1,Clb5}}$ for the induced expression of Cln2 and Clb5 by galactose, respectively. The values of $ks_{\text{GAL1,Cln2}}$ and $ks_{\text{GAL1,Clb5}}$ are adjusted to make the desired over-expression level of Cln2 and Clb5 respectively (about 1-fold over-expression here). They are set to be 0 for other conditions (wild-type and Sic1^{T173A} mutant). In addition, we changed the specific growth rate constant k_g to match the growth rate in the medium in which *GAL1::CLN2* and *GAL1::CLB5* experiments are carried out, which is 0.0046 min^{-1}. This method was used in Chen K., *et al.* as well (Chen et al., 2004).
We also assumed that the degradation of Cln2 and Clb5 follows first-order kinetics. After parameter estimation, this model is able to reproduce the time course profiles of total Cln2 and Clb5 under different stress scenarios (Supplementary Fig. 7a-e and 7p-r,).

2.3 Black Box 3: cells going into S-G$_2$ phases

For modeling a population of cells going into S-G$_2$ phases, we simplified and lumped together all the steps regulating the firing of DNA replication into a black box that works as a functional unit. Previous studies have shown that the activity of Clb5Cdk1 is the main trigger of S phase. We assumed the rate of cells going into S-G$_2$ phases is proportional to the percentage of cells in G$_1$ phase and is regulated by Clb5Cdk1 with Michaelis-Menten-like kinetics (E2.17).

$$v^{S,G2} = \frac{V_{\max}^{\text{S,G}_2}[G_1][Clb5Cdk1]}{J_{\text{Clb5Cdk1}} + [Clb5Cdk1]} \quad \text{(E2.17)}$$

where $V_{\max}^{\text{S,G}_2}$ is the maximal rate for cells' transition into S-G$_2$ phases; J_{Clb5Cdk1} corresponds to the Michaelis constant; $[G_1]$ denotes the percentage of cells in G$_1$ phase.
After parameter estimation, this model can properly reproduce the experimental results for the percentage of cells going into S-G$_2$ phases under different stress scenarios (Supplementary Fig. 7k-o), and it predicts well for the progression of cells from G1 to S-G$_2$ under different intensities and timings of stress (Supplementary Figs. 9d and 10d).

3. Parameter Estimation

3.1 Derivation of some parameter values based on published data and models

Before implementing a global optimization of the parameter values based on our experimental data, we first derived some parameter values from published data such as the mass doubling time and half-live of some proteins.
The mass growth rate constant k_g is defined by mass doubling time (MDT) with the value of $\frac{\ln 2}{MDT}$. In the model, MDT is set with a value of 90 min for the wild type and Sic1T173 mutant (growing in YPD) and 150 min for *GAL::CLN2* and *GAL::CLB5* over-expression experiments (SD with 2 % raffinose, induced with 2% galactose), respectively, according to the reported values in the literature (Chen *et al*, 2004; Schneider *et al*, 2004).

The degradation rate constant for Cln2 ($K_{\text{deg}}^{\text{Cln2}}$) is set to be 0.14 min^{-1}, which corresponds to a half-life time of about 5 min (0.693/0.14) and this value is consistent to the experiments (Belle *et al*, 2006; Chen *et al*, 2004). Clb5 is a little bit more stable than Cln2, In our model, the degradation rate constant for Clb5 ($K_{\text{deg}}^{\text{Clb5}}$) is set with value of 0.07 min^{-1} according to the previous reported values (Chen *et al*, 2000; Seufert *et al*, 1995) (corresponding to 10 min half-life time).

The dephosphorylation rate constant of Hog1 ($K_{\text{depho}}^{\text{Hog1}}$) is taken from our previous integrative model for osmotic response, which was optimized to several experimental data sets (Klipp et al., 2005).

Because Cdk1 (Cdc28) is much more abundant than cyclins (Cross et al., 2002) and it associates rapidly with cyclins, Cdk1 is not explicitly shown in the model, its concentration and the association rate with cyclins are integrated into one parameter $K_{\text{ass}}^{\text{cdk1}}$ in the model. We choose a low dissociation rate constant value ($K_{\text{diss}}^{\text{cdk1}}$, 0.01 min^{-1}) for the dissociation rate of Cdk1 and cyclin complex and estimate the value of the association rate constant ($K_{\text{ass}}^{\text{cdk1}}$) based on the experimental data. According to Chen K., *et al*. (Chen *et al*, 2004; Chen *et al*, 2000), the binding of Clb5Cdk1 and Sic1 should be rapid, we set the dissociation rate constant ($K_{\text{diss}}^{\text{Clb5}}$) of Clb5Cdk1 and Sic1 complex to be 0.01 min^{-1}. Similarly we set the dephosphorylation rate constant ($K_{\text{depho1}}^{\text{Sic1}}$ and $K_{\text{depho2}}^{\text{Sic1}}$) of the phosphorylated Sic1 to be 0.01 min^{-1} because the phosphorylation of Sic1 is a fast switch like step (Nash *et al*, 2001). Similar as Chen K., et al., the model is not sensitive to the precise values of these parameters because 10-fold arbitrary increase or decrease for the values of these parameters ($K_{\text{diss}}^{\text{cdk1}}$, $K_{\text{diss}}^{\text{Clb5}}$, $K_{\text{depho1}}^{\text{Sic1}}$ and $K_{\text{depho2}}^{\text{Sic1}}$) doesn't significant change the model simulation results.

3.2 Global optimization of other unknown parameter values

We used a revised version of SBML-PET (a Systems Biology Markup Language based Parameter Estimation Tool) to implement the parameter estimation (Zi and Klipp, 2006). SBML-PET incorporates a global optimization algorithm (SRES) to minimize the sum of squares of differences between model simulations and the corresponding experimentally measured data sets. SRES is a stochastic ranking evolution strategy based evolutionary optimization algorithm that has a good optimization performance according to previous studies (Moles *et al*, 2003; Runarsson and Yao, 2000). We estimated other 36 unknown parameters values (including initial concentrations and kinetic parameter values) in the model with 198 experimental data points under different stress scenarios. The following data sets were used for parameter estimation (so-called in-sample data sets):

(1) Total amounts of Cln2, Sic1, Clb5 and percentage of cells going into S-G_2 phases (percentage of cells with DNA content > 1C) without stress (control) and those treated with 0.4 M of NaCl at 0, 10, 20 and 30 minutes after release from α-factor, respectively.

(2) Total amount of phosphorylated Hog1 (Hog1PP) for cells treated with 0.4 M and 0.8 M NaCl at 0 minutes after release from α-factor.

After parameter estimation, the model is able to reproduce the experimental data that were used for parameter estimation. The comparison of model simulation result with the experimental data sets that are used for parameter estimation is regarded as "in-sample fit" (Supplementary Fig. 7). In order to further validate the model's performance, we compared the model simulation results to additional experimental datasets (116 extra data points) that were not used for parameter estimation called "out-sample fit". The model simulation results also match well with the additional experimental datasets under other stress conditions (Supplementary Fig. 8).

4. Summary of the ODE system

The final model consists of 15 ODEs and 45 kinetic parameters. The changes of these state variables over time are described by the following system of ordinary differential equations E3.1-E3.15 and the accompanying equations E4.16-E4.25.

$$\frac{d[Hog1]}{dt} = K_{\text{depho}}^{\text{Hog1}} \cdot [Hog1PP] - K_{\text{pho}}^{\text{Hog1}} \cdot [Hog1] \cdot S(t) \tag{E4.1}$$

$$\frac{d[Hog1PP]}{dt} = K_{\text{pho}}^{\text{Hog1}} \cdot [Hog1] \cdot S(t) - K_{\text{depho}}^{\text{Hog1}} \cdot [Hog1PP] \tag{E4.2}$$

$$\frac{d[mass]}{dt} = \frac{k_{\text{g}} \cdot [mass]}{1 + w_1 \cdot [Hog1PP]} \tag{E4.3}$$

$$\frac{d[Cln2]}{dt} = (ks_{\text{GAL1,CLN2}} + \frac{ks_{\text{Cln2}} \cdot [SBF]}{1 + w_2 \cdot [Hog1PP]}) - K_{\text{ass}}^{\text{Cdk1}} \cdot [Cln2] + K_{\text{diss}}^{\text{Cdk1}} \cdot [Cln2\text{Cdk1}] \tag{E4.4}$$

$$\frac{d[Cln2Cdk1]}{dt} = K_{\text{ass}}^{\text{Cdk1}} \cdot [Cln2] - (K_{\text{diss}}^{\text{Cdk1}} + K_{\text{deg}}^{\text{Cln2}}) \cdot [Cln2Cdk1] \tag{E4.5}$$

$$\frac{d[Clb5]}{dt} = (ks_{\text{GAL1,CLB5}} + \frac{ks_{\text{Clb5}} \cdot [MBF]}{1 + w_3 \cdot [Hog1PP]}) - k_{\text{ass}}^{\text{Cdk1}} \cdot [Clb5] + k_{\text{diss}}^{\text{Cdk1}} \cdot [Clb5\text{Cdk1}] \tag{E4.6}$$

$$\begin{aligned}\frac{d[Clb5Cdk1]}{dt} = {} & K_{\text{ass}}^{\text{Cdk1}} \cdot [Clb5] - (K_{\text{diss}}^{\text{Cdk1}} + K_{\text{deg}}^{\text{Clb5}}) \cdot [Clb5Cdk1] \\ & + K_{\text{deg}\,4}^{\text{Sic1}} \cdot [P_c Sic1Clb5Cdk1] + K_{\text{deg}\,5}^{\text{Sic1}} \cdot [P_h P_c Sic1Clb5Cdk1] \\ & - K_{\text{ass}}^{\text{Clb5}} \cdot [Clb5Cdk1] \cdot [Sic1] + K_{\text{diss}}^{\text{Clb5}} \cdot [Sic1Clb5Cdk1] \\ & - K_{\text{ass}}^{\text{Clb5}} \cdot [Clb5Cdk1] \cdot [P_h Sic1] + K_{\text{diss}}^{\text{Clb5}} \cdot [P_h Sic1Clb5Cdk1]\end{aligned} \tag{E4.7}$$

$$\begin{aligned}\frac{d[Sic1]}{dt} = {} & v_{\text{Sic1}} - \frac{K_{\text{deg1}}^{\text{Sic1}} \cdot [Sic1]}{J_{\text{deg1}}^{\text{Sic1}} + [Sic1]} - K_{\text{pho2}}^{\text{Sic1}} \cdot [Sic1] \cdot [Hog1PP] \\ & + K_{\text{depho2}}^{\text{Sic1}} \cdot [P_h Sic1] - K_{\text{deg2}}^{\text{Sic1}} \cdot [Sic1] \cdot [Cln2Cdk1] \\ & - K_{\text{ass}}^{\text{Clb5}} \cdot [Sic1] \cdot [Clb5Cdk1] + K_{\text{diss}}^{\text{Clb5}} \cdot [Sic1Clb5Cdk1]\end{aligned} \tag{E4.8}$$

$$\frac{d[Sic1Clb5Cdk1]}{dt} = K_{\text{ass}}^{\text{Clb5}} \cdot [Sic1] \cdot [Clb5Cdk1] - K_{\text{diss}}^{\text{Clb5}} \cdot [Sic1Clb5Cdk1] - \frac{K_{\text{pho1}}^{\text{Sic1}} \cdot [Sic1Clb5Cdk1] \cdot ([Cln2Cdk1])^6}{(J1_{\text{Cln2Cdk1}})^6 + ([Cln2Cdk1])^6} - K_{\text{pho3}}^{\text{Sic1}} \cdot [Sic1Clb5Cdk1] \cdot [Hog1PP] + K_{\text{depho1}}^{\text{Sic1}} \cdot [P_c Sic1Clb5Cdk1] + K_{\text{depho2}}^{\text{Sic1}} \cdot [P_h Sic1Clb5Cdk1] \quad \text{(E4.9)}$$

$$\frac{d[P_c Sic1Clb5Cdk]}{dt} = \frac{K_{\text{pho1}}^{\text{Sic1}} \cdot [Sic1Clb5Cdk1] \cdot ([Cln2Cdk1])^6}{(J1_{\text{Cln2Cdk1}})^6 + ([Cln2Cdk1])^6} - K_{\text{depho1}}^{\text{Sic1}} \cdot [P_c Sic1Clb5Cdk1] - K_{\text{deg4}}^{\text{Sic1}} \cdot [P_c Sic1Clb5Cdk1] - K_{\text{pho5}}^{\text{Sic1}} \cdot [P_c Sic1Clb5Cdk1] \cdot [Hog1PP] + K_{\text{depho2}}^{\text{Sic1}} \cdot [P_h P_c Sic1Clb5Cdk1] \quad \text{(E4.10)}$$

$$\frac{d[P_h Sic1]}{dt} = K_{\text{pho2}}^{\text{Sic1}} \cdot [Sic1] \cdot [Hog1PP] - K_{\text{depho2}}^{\text{Sic1}} \cdot [P_h Sic1] - K_{\text{deg3}}^{\text{Sic1}} \cdot [P_h Sic1] \cdot [Cln2Cdk1] - K_{\text{ass}}^{\text{Clb5}} \cdot [P_h Sic1] \cdot [Clb5Cdk1] + K_{\text{diss}}^{\text{Clb5}} \cdot [P_h Sic1Clb5Cdk1] \quad \text{(E4.11)}$$

$$\frac{d[P_h Sic1Clb5Cdk1]}{dt} = K_{\text{pho3}}^{\text{Sic1}} \cdot [Sic1Clb5Cdk1] \cdot [Hog1PP] - K_{\text{depho2}}^{\text{Sic1}} \cdot [P_h Sic1Clb5Cdk1] - \frac{K_{\text{pho4}}^{\text{Sic1}} \cdot [P_h Sic1Clb5Cdk1] \cdot ([Cln2Cdk1])^6}{(J2_{\text{Cln2Cdk1}})^6 + ([Cln2Cdk1])^6} + K_{\text{depho1}}^{\text{Sic1}} \cdot [P_h P_c Sic1Clb5Cdk1] + K_{\text{ass}}^{\text{Clb5}} \cdot [Clb5Cdk1] \cdot [P_h Sic1] - K_{\text{diss}}^{\text{Clb5}} \cdot [P_h Sic1Clb5Cdk1] \quad \text{(E4.12)}$$

$$\frac{d[P_hP_cSic1Clb5Cdk1]}{dt}=\frac{K_{\text{pho4}}^{\text{Sic1}}\cdot[P_hSic1Clb5Cdk1]\cdot([Cln2Cdk1])^6}{(J2_{\text{Cln2Cdk1}})^6+([Cln2Cdk1])^6}-K_{\text{depho1}}^{\text{Sic1}}\cdot[P_hP_cSic1Clb5Cdk1]-K_{deg5}^{Sic1}\cdot[P_hP_cSic1Clb5Cdk1]+K_{pho5}^{Sic1}\cdot[P_cSic1Clb5Cdk1][Hog1PP]-K_{\text{depho2}}^{\text{Sic1}}\cdot[P_hP_cSic1Clb5Cdk1] \quad \text{(E4.13)}$$

$$\frac{d[G_1]}{dt}=-\frac{V_{max}^{S,G_2}\cdot[G_1]\cdot[Clb5Cdk1]}{J_{\text{Clb5Cdk1}}+[Clb5Cdk1]} \quad \text{(E4.14)}$$

$$\frac{d[SG_2]}{dt}=\frac{V_{max}^{S,G_2}\cdot[G_1]\cdot[Clb5Cdk1]}{J_{\text{Clb5Cdk1}}+[Clb5Cdk1]} \quad \text{(E4.15)}$$

$$S(t)=\begin{cases}0 & if\ \ t<t_0\\ stress\cdot e^{\frac{-k_t\cdot(t-t_0)}{stress}} & if\ \ t\geq t_0\end{cases} \quad \text{(E4.16)}$$

$$A_{\text{SBF, Cln3}}=\begin{cases}0 & \text{if } mass<mass_{\text{Cln3}}\\ \dfrac{\varepsilon_{\text{SBF,Cln3}}\cdot(mass-mass_{\text{Cln3}})}{J_{\text{mass,Cln3}}+(mass-mass_{\text{Cln3}})} & \text{if } mass\geq mass_{\text{Cln3}}\end{cases} \quad \text{(E4.17)}$$

$$F_{\text{SBF, Cln2}}=\varepsilon_{\text{SBF,Cln2}}\cdot[cln2Cdk1] \quad \text{where} \quad \text{(E4.18)}$$

$$\varepsilon_{\text{SBF, Clb2}}=\begin{cases}0 & \text{if } mass<mass_{\text{Clb2}}\\ k_{\text{SBF,Clb2}}\cdot(mass-mass_{\text{Clb2}}) & \text{if } mass\geq mass_{\text{Clb2}}\end{cases}$$

$$[SBF]=\frac{A_{\text{SBF, Cln3}}+F_{\text{SBF, Cln2}}}{1+I_{\text{SBF,Clb2}}} \quad \text{(E4.19)}$$

$$A_{\text{MBF, Cln3}}=\begin{cases}0 & \text{if } mass<mass_{\text{Cln3}}\\ \dfrac{\varepsilon_{\text{MBF,Cln3}}\cdot(mass-mass_{\text{Cln3}})}{J_{\text{mass,Cln3}}+(mass-mass_{\text{Cln3}})} & \text{if } mass\geq mass_{\text{Cln3}}\end{cases} \quad \text{(E4.20)}$$

$$F_{\text{MBF, Cln2}}=\varepsilon_{\text{MBF,Cln2}}\cdot[cln2Cdk1] \quad \text{where} \quad \text{(E4.21)}$$

$$I_{\text{MBF, Nrm1}}=\begin{cases}0 & \text{if } mass<mass_{\text{Nrm1}}\\ \varepsilon_{\text{MBF,Nrm1}}\cdot(mass-mass_{\text{Nrm1}}) & \text{if } mass\geq mass_{\text{Nrm1}}\end{cases}$$

$$[MBF] = \frac{A_{\mathrm{MBF,Cln3}} + F_{\mathrm{MBF,Cln2}}}{1 + I_{\mathrm{MBF,Nrm1}}} \tag{E4.22}$$

$$[Cln2_{total}] = [Cln2] + [Cln2Cdk1] \tag{E4.23}$$

$$[Sic1_{total}] = [Sic1] + [Sic1Clb5Cdk1] + [P_c Sic1Clb5Cdk1] + [P_h Sic1] + [P_h Sic1Clb5Cdk1] + [P_h P_c Sic1Clb5Cdk1] \tag{E4.24}$$

$$[Clb5_{total}] = [Clb5] + [Clb5Cdk1] + [Sic1Clb5Cdk1] + [P_c Sic1Clb5Cdk1] + [P_h Sic1Clb5Cdk1] + [P_h P_c Sic1Clb5Cdk1] \tag{E4.25}$$

5. Summary of Initial Conditions and Parameter Values

The concentrations of the molecules in this model are expressed in arbitrary units (a.u.). The initial concentration of total Sic1 is set to be 1. Therefore, the unit of molecule concentration is relative to the initial concentration of total Sic1 (total Sic1 at the time when α-factor is removed). The time unit of the model is minute. Therefore, the rate constants for the first-order processes have a unit of min^{-1}.

Supplementary Table 3. Initial conditions of the state variables

State variable	Initial Condition	Annotation
$Hog1$	**8.9**	estimated
$Sic1$	**1**	reference unit
$G1$	**95**	estimated by the average of the experimental data
$SG2$	**5**	estimated by the average of the experimental data
$mass$	**1**	relative to cell mass when the α-factor is removed
Others	**0**	

The estimated values for the initial condition of state variables are rounded.

Supplementary Table 4. Complete list of model parameter values

$K_{\mathrm{pho}}^{\mathrm{Hog1}}$ = 828.8	$K_{\mathrm{depho}}^{\mathrm{Hog1}}$ = 0.32 (taken from (Klipp et al., 2005))		k_{t} = 0.11
k_g = 0.0077 (0.0046 for galactose medium)		w_1 = 2.23	ks_{Cln2} = 1.74
$\varepsilon_{\mathrm{SBF,Cln3}}$ = 0.65	$\varepsilon_{\mathrm{SBF,Cln2}}$ = 0.047	$mass_{\mathrm{Cln3}}$ =1.14	$J_{\mathrm{mass,Cln3}}$ = 0.064
$\varepsilon_{\mathrm{SBF,Clb2}}$ = 12.67	$mass_{\mathrm{Clb2}}$ =1.28	w_2 = 0.49	$K_{\mathrm{ass}}^{\mathrm{Cdk1}}$ = 11.11
$K_{\mathrm{diss}}^{\mathrm{Cdk1}}$ = 0.01	$K_{\mathrm{deg}}^{\mathrm{C1n2}}$ = 0.14	ks_{Clb5} = 0.041	$\varepsilon_{\mathrm{MBF,Cln3}}$ = 2.86

$\varepsilon_{\mathrm{MBF,Cln2}}$ = 0.0097 $\varepsilon_{\mathrm{MBF,Nrm1}}$ = 109.19 $mass_{\mathrm{Nrm1}}$ = 1.67 w_3 = 0.72

$K_{\mathrm{deg}}^{\mathrm{C1b5}}$ = 0.07 v_{Sic1} = 0.0042 $K_{\mathrm{ass}}^{\mathrm{Clb5}}$ = 1804.02 $K_{\mathrm{diss}}^{\mathrm{Clb5}}$ = 0.01

$K_{\mathrm{deg1}}^{\mathrm{Sic1}}$ = 0.035; $J_{\mathrm{deg1}}^{\mathrm{Sic1}}$ = 0.25 (for the degradation of $Sic1$ independent of Cln2Cdk1)

$K_{\mathrm{deg2}}^{\mathrm{Sic1}}$ = 0.005 (for the degradation of $Sic1$; dependent on $Cln2Cdk1$)

$K_{\mathrm{deg3}}^{\mathrm{Sic1}}$ = 0.001 (for the degradation of P_hSic1; dependent on $Cln2Cdk1$)

$K_{\mathrm{deg4}}^{\mathrm{Sic1}}$ = 0.81

(for the degradation of Sic1 from $P_cSic1Clb5Cdk1$; dependent on $Cln2Cdk1$)

$K_{\mathrm{deg5}}^{\mathrm{Sic1}}$ = 0.39

(for the degradation of Sic1 from $P_cSic1Clb5Cdk1$; dependent on $Cln2Cdk1$)

$K_{\mathrm{pho1}}^{\mathrm{Sic1}}$ = 0.27; $J1_{\mathrm{Cln2Cdk1}}$ = 0.22

(phosphorylation of Sic1 from $Sic1Clb5Cdk1$ to $P_cSic1Clb5Cdk1$)

$K_{\mathrm{pho2}}^{\mathrm{Sic1}}$ = 0.0066 (phosphorylation of Sic1 from $Sic1$ to P_hSic1)

$K_{\mathrm{pho3}}^{\mathrm{Sic1}}$ = 0.27 (phosphorylation of Sic1 from $Sic1Clb5Cdk1$ to $P_hSic1Clb5Cdk1$)

$K_{\mathrm{pho4}}^{\mathrm{Sic1}}$ = 3.9; $J2_{\mathrm{Cln2Cdk1}}$ = 3.71

(phosphorylation of Sic1 from $Sic1Clb5Cdk1$ to $P_cSic1Clb5Cdk1$)

$K_{\mathrm{pho5}}^{\mathrm{Sic1}}$ = 9.59 (phosphorylation of Sic1 from $P_cSic1Clb5Cdk1$ to $P_hP_cSic1Clb5Cdk1$)

$K_{\mathrm{depho1}}^{\mathrm{Sic1}}$ = 0.01 $K_{\mathrm{depho2}}^{\mathrm{Sic1}}$ = 0.01 V_{max}^{S,G_2} = 0.027 J_{Clb5Cdk1} = 0.076

$ks_{\mathrm{GAL1,Cln2}}$ = 0 for wild type; 0.5 for about 1-fold over-expression of total Cln2

$ks_{\mathrm{GAL1,Clb5}}$ = 0 for wild type; 0.1 for about 1-fold over-expression of total Clb5

The estimated parameter values are rounded.

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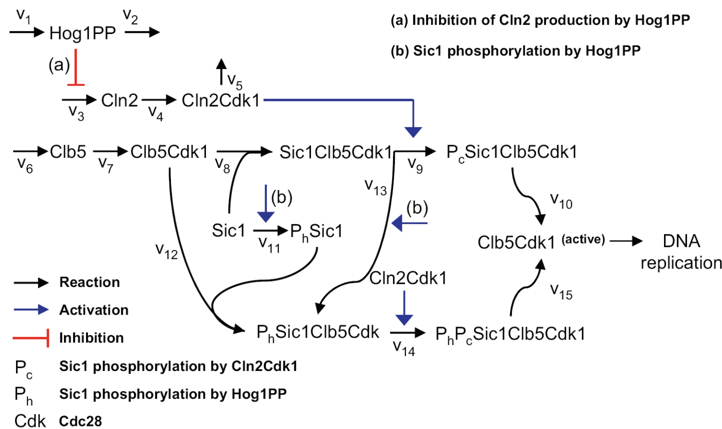
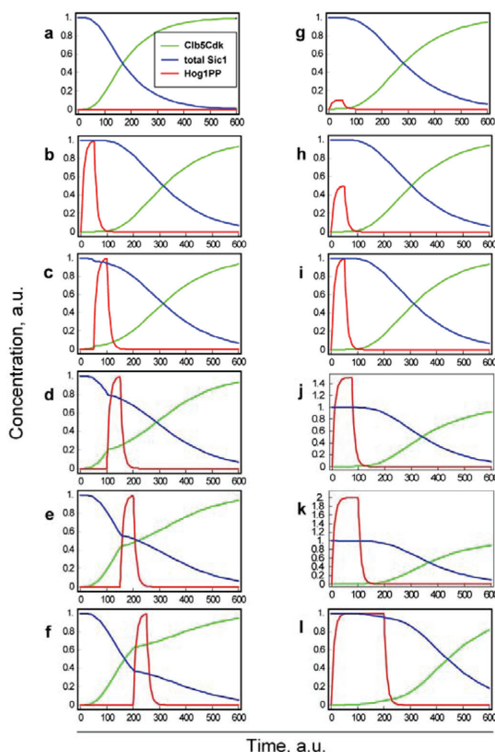
Supplementary Figures

Adrover *et al.*
Supplementary Figure1



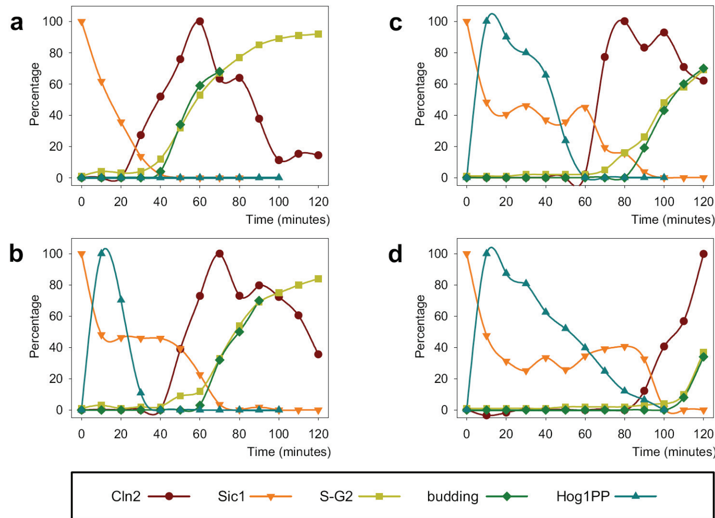
Supplementary Figure 1. Initial model of the interaction of the yeast Hog1 pathway with cell cycle control machinery.
Cln2 binds to Cdc28 (Cln2Cdk1) (v_4). The complex Cln2Cdk1 can be degraded (v_5) and is required for phosphorylation of Sic1 in the complexes Sic1Clb5Cdk1 (v_9) and P_hSic1Clb5Cdk1 (v_{14}). Clb5 is produced (v_6), binds to Cdc28 (v_7), and later to Sic1 (v_8 and v_{12}). Rates v_{14} and v_{15} are similar to v_9 and v_{10}, denoting phosphorylation and degradation of phosphorylated Sic1 bound to the cyclin-Cdk complex. Clb5Cdk1 is considered the active complex coordinating DNA replication. This model describes the activation and deactivation of Hog1 (v_1 and v_2). Hog1PP acts though a dual mechanism; (a) down regulation of Cln2 by inhibiting transcription of Cln2 (v_3), and (b) direct phosphorylation of free Sic1 (v_{11}) or Sic1 bound to Clb5Cdk1 (v_{13}).

Adrover *et al.*
Supplementary Figure 2



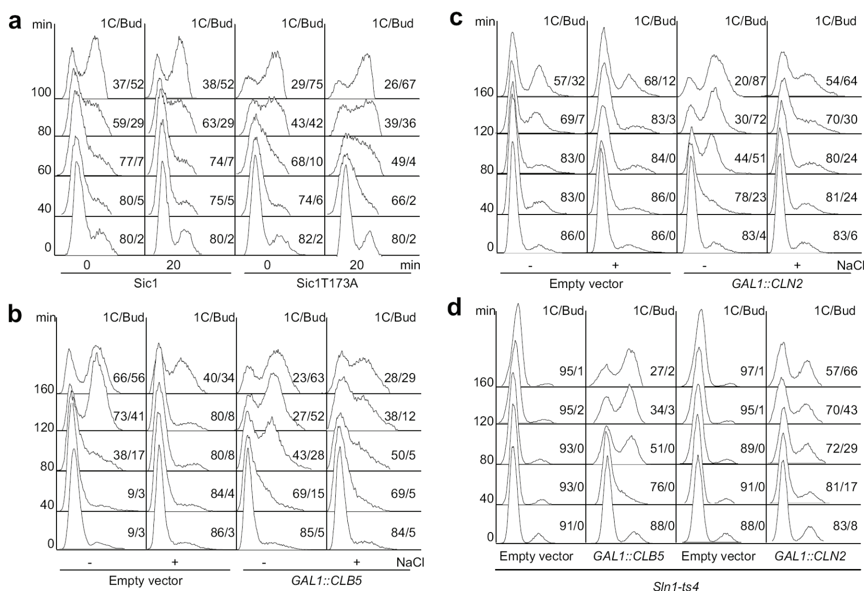
Supplementary Figure 2. Results of simulations with the initial model.
In all simulations (**a-l**); Active Hog1 (Hog1PP) is depicted in red, total Sic1 in blue and Clb5Cdc28 (Clb5Cdk1) in green. It is referred as "cross point" the time when the curves for Sic1 and Clb5Cdk1 cross each other. (**a**) Without activation of Hog1, Sic1 is degraded and Clb5Cdk1 accumulates, showing the cross point when both curves reach a value of 0,5. (**b**) The activation of Hog1 (t0) and its ulterior inactivation (t1) induces a shift of the cross point to a later time (t_0 = 0, t_1 = 50, see Supplementary Table 1). (**c**-**f**) Hog1 activations at different time points (t_0 = {0;50;100;150;200}, t_1 = t_0 + 50), reproduce the same shift of the cross point up to time 100, later activations lead to a loss of the cross point shifting. (**g**-**l**). The more intense the activation of Hog1 is, the later the cross point appears (**g**: t_0 = 0, t_1 = 50,k_1 = 0.01 ; **h**: t_0 = 0, t_1 = 50,k_1 = 0.05 ; **i**: t_0 = 0, t_1 = 50, k_1 = 0.1; **j**: t_0 = 0, t_1 = 75, k_1 = 0.15 ; **k**: t_0 = 0, t_1 =100, k_1 = 0.2 ; **l**: t_0 = 0, t_1 = 200, k_1 = 0.1), "k_1" is the phosphorylation rate of Hog1.

Adrover *et al.*
Supplementary Figure 3



Supplementary Figure 3. The timing relationship between Sic1, Cln2, replication and budding is maintained also for stronger stress.
(**a**) Sic1 is degraded before Cln2 reaches its maximal levels and ten minutes before S phase onset. YAN7 cells were synchronized for 3h with alpha-factor at 25 ºC, shifted to YPD and sampled every 10 minutes. Sic1, Cln2 proteins (expressed as percentage of their maximal levels) were determined by quantitative western blot. Phosphorylated Hog1 was detected using a specific antibody. DNA content was scored by flow cytometry, and budding index determined by microscopy. (**b**-**d**) Sic1 degradation, Cln2 production and S phase are delayed in a similar fashion when stress appears at earlier G_1 also for different strengths of osmostress. YAN7 cells were synchronized and released as in **a**, and stressed either with 0.4 (**b**), 0.6 (**c**) or 0.8 M (**d**) NaCl after release, and analyzed as above

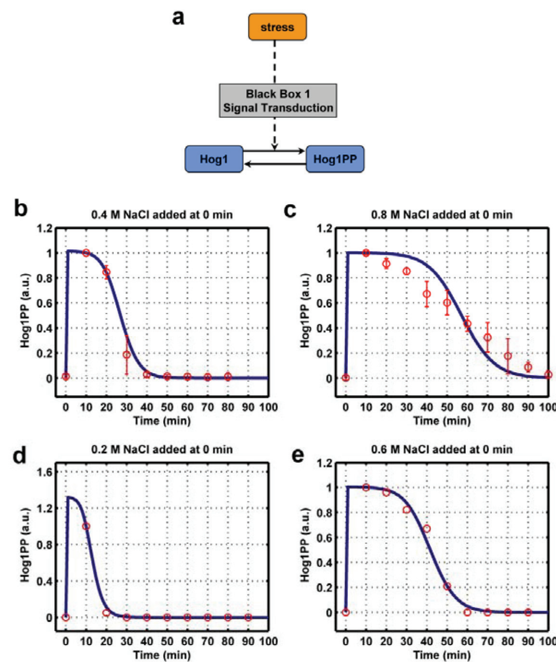
Adrover *et al.*
Supplementary Figure 4



Supplementary Figure 4. Sic1 stabilization, Cln2 and Clb5 regulation show distinct impacts on the Hog1-mediated G_1 arrest.

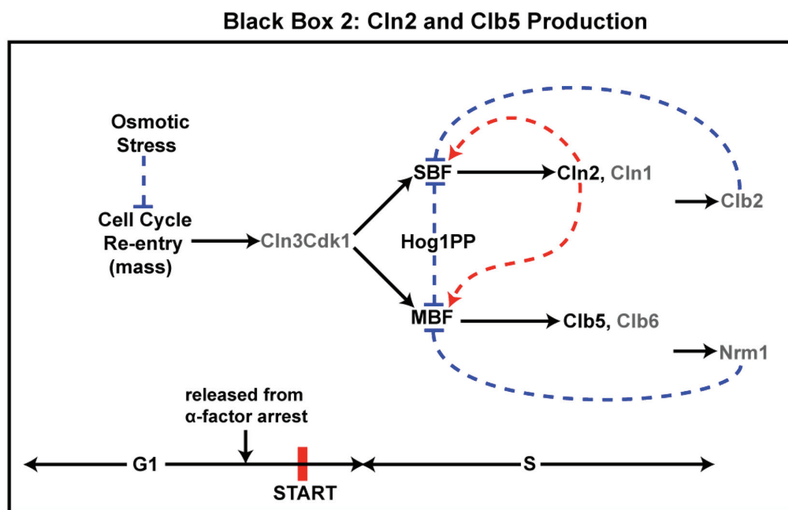
(**a**) Sic1 stabilization is only relevant when stress appears at Start. *sic1Δ* cells containing TAP-tagged Sld2 and Clb5 were transformed either wild type *SIC1* (pMZ65) or the *SIC1* mutant allele encoding *Sic1T173A* (pMZ62), released from pheromone and immediately (0) or twenty minutes later (20) were stressed with 0.4 M NaCl, DNA content was monitored by FACS analysis and G_1 population was quantified (1C) and budding (Bud) by microscopy. (**b**) Ectopic expression of Clb5 totally removes the osmostress-imposed arrest at G_1. W303 cells bearing empty plasmid or full length HA-Clb5 cloned into YCpIF16 (pMAD23) under the *GAL1* promoter were synchronized in SD medium plus raffinose and released in the presence of 0.4 M NaCl. Clb5 was induced by galactose addition 20 minutes before release from pheromone. Samples of the indicated times were taken to score DNA content and bud formation as in **a**. (**c**) Ectopic expression of Cln2 precipitates entry into S phase but does not abolish the arrest at G_1 upon osmotic shock. W303 cells bearing empty plasmid or pYES2-Cln2-HA (pCM249) were treated and analyzed as in **a**. (**d**) Ectopic expression of Clb5, but not Cln2 totally removes the blockade at G_1 imposed by hyperactivation of Hog1. TM141 cells bearing the termosentitive allele *SLN1-TS4* and empty plasmids, YCpIF16-HA-Clb5 or pYES2-Cln2-HA were synchronized with pheromone in SD medium plus raffinose at 25ºC, shifted to restrictive temperature (37ºC) and released 30 minutes later. Clb5 and Cln2 were induced by galactose addition 20 minutes before release from pheromone.

Adrover *et al.*
Supplementary Figure 5



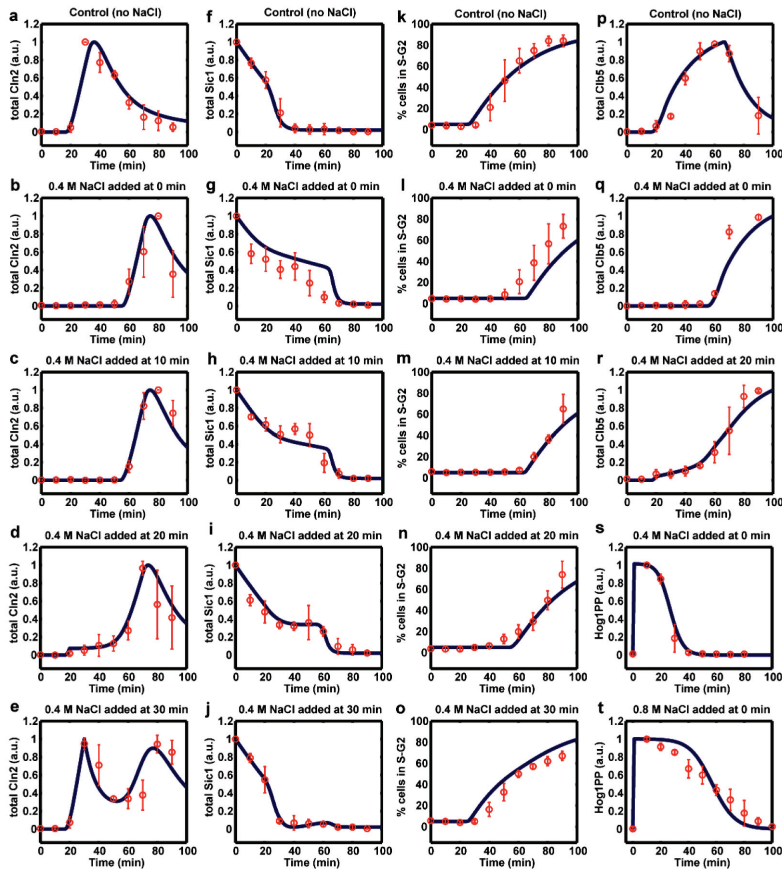
Supplementary Figure 5. The sub-module of Hog1 activation and its validation with experimental data. (**a**) Lumped black box for the Hog1 activation module; (**b** and **c**) Comparison of model simulations (blue curves) with the experimental data from treatment with (**a**) 0.4 and (**c**) 0.8 M NaCl (red circles). Error bars show s.d. (n=4); (**d** and **e**) Validation of model prediction (blue curves) by additional experimental data (not included in the model fitting) from treatments with different concentrations of NaCl (red circles, single experiment without error bar). The model simulation results have been normalized to the Hog1PP level at 10 minutes in order to compare with corresponding experimental data.

Adrover *et al.*
Supplementary Figure 6



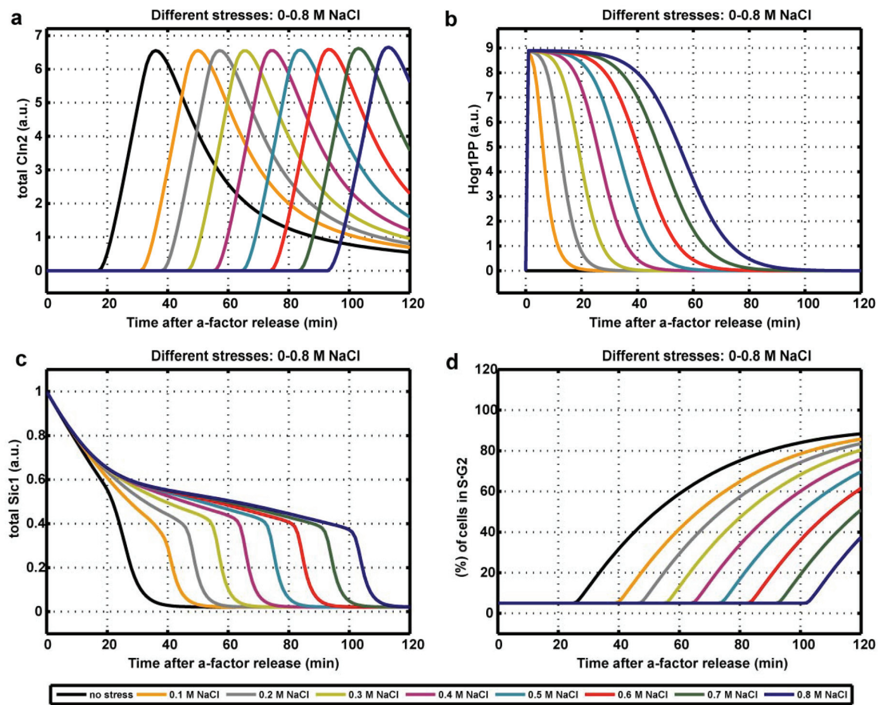
Supplementary Figure 6. The coarse-grained black box model describing Cln2 and Clb5 production. The activation of the SBF and MBF transcriptional systems and their dependency on Cln3 and the positive feedback loop of Cln1,2 are encompassed in this functional unit. The effects of mass, osmotic stress and Hog1 activity are also taken into account. Some molecules (gray) are not explicitly represented in the model, however, their regulatory effects are considered.

Adrover *et al.*
Supplementary Figure 7



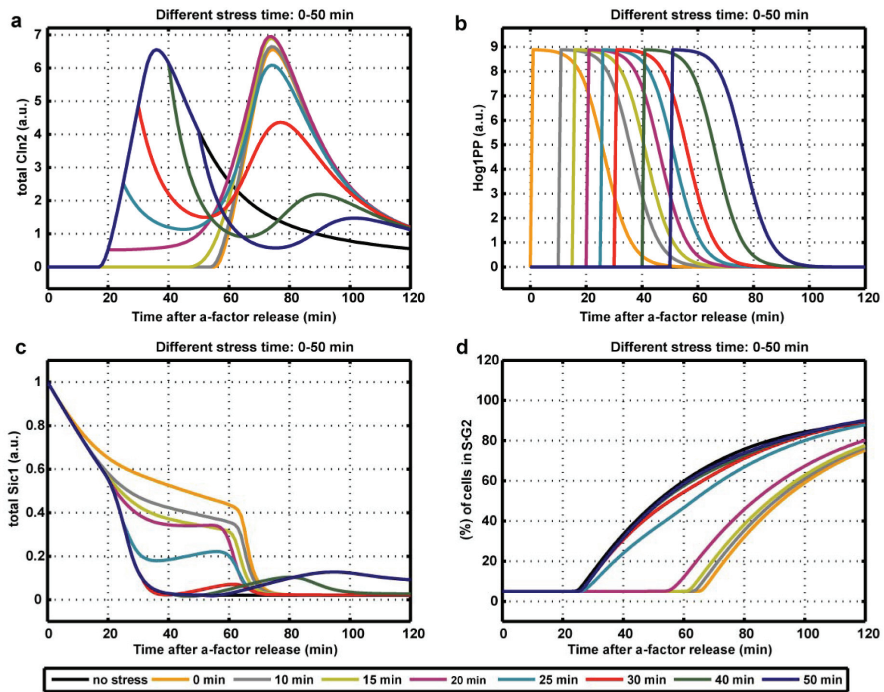
Supplementary Figure 7. Comparison of model simulation result to experimental data used for parameter estimation (In-Sample fit).
(**a**-**e**) Data comparison for total Cln2, (**f**-**j**) total Sic1, (**k**-**o**) percentage of cells in S-G_2 phases (represented by the percentage of cells with >1C of DNA), (**p**-**r**) total Clb5 and (**s**-**t**) active Hog1. In all cases, for non stressing conditions or stressing with 0.4M NaCl at different times along G_1. Blue curves are simulation results. Red circles are the average of the experimental data from 3-6 experiments. Red error bars show the corresponding standard deviation from the experiments (n=3-6).

Adrover *et al.*
Supplementary Figure 9



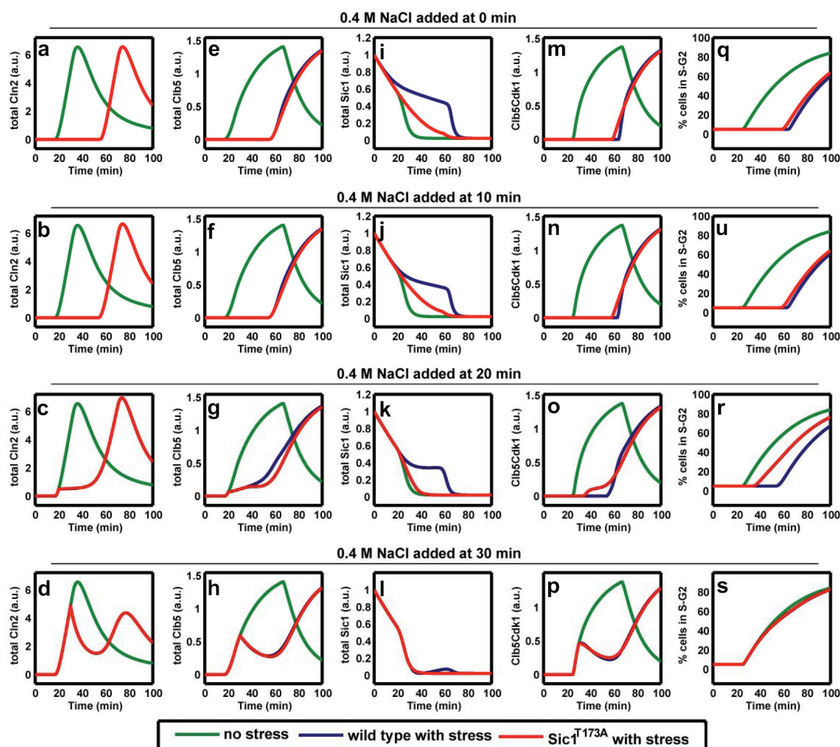
Supplementary Figure 9. Predictions of the final model for different intensities of stress.
(**a**) Simulations for Cln2. (**b**) Active Hog1. (**c**) Sic1. (**d**) Cell count in S-G_2.

Adrover *et al.*
Supplementary Figure 10



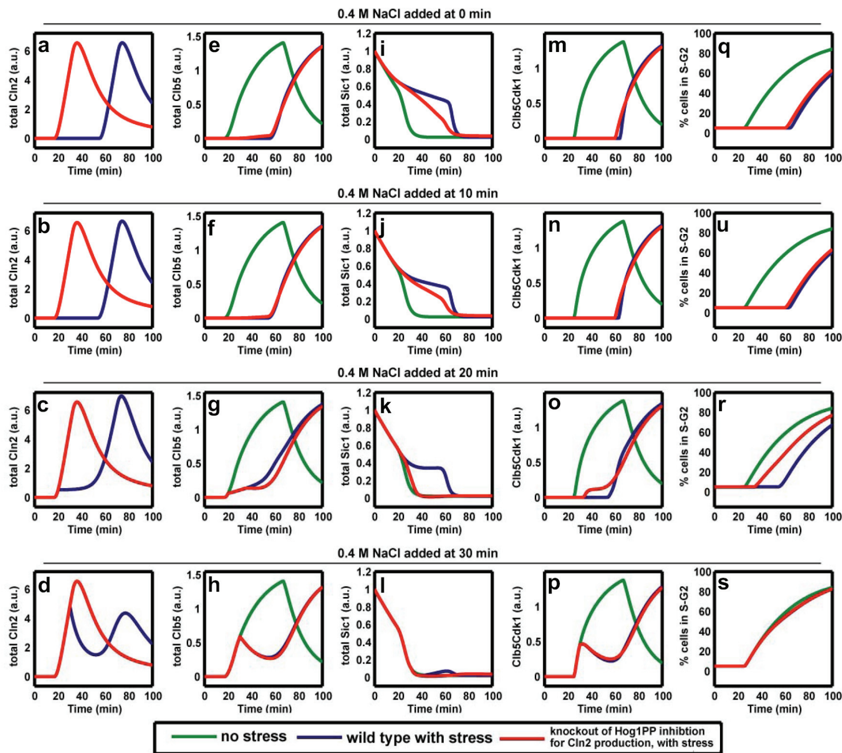
Supplementary Figure 10. Predictions of the final model for different timings of stress.
(**a**) Simulations for Cln2. (**b**) Active Hog1. (**c**) Sic1. (**d**) Cell count in S-G_2.

Adrover *et al.*
Supplementary Figure 11



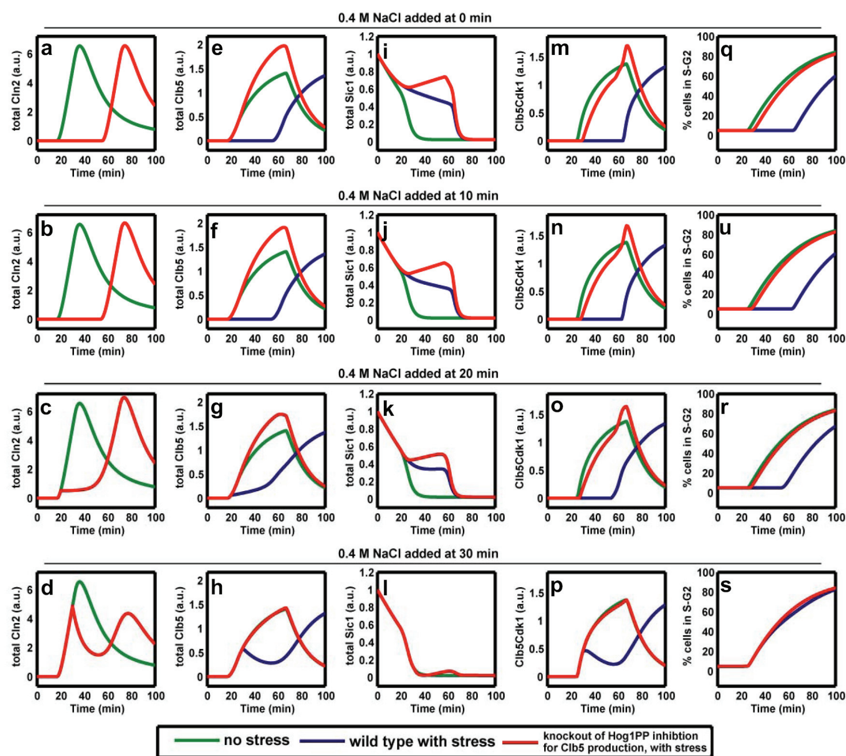
Supplementary Figure 11. Predictions for the Sic1T173A mutant.
(**a**-**d**) Predictions for time courses of total Cln2, (**e**-**h**) total Clb5, (**i**-**l**) total Sic1, (**m**-**p**) Clb5Cdk1 activity and (**q**-**s**) cell count in S-G_2. Simulations obtained with both, the unperturbed and the altered models without stress are the same for no stress (green). The unperturbed model for stress with 0.4M NaCl is depicted in blue and the perturbed model (Sic1T173A) under stress condition is shown in red. In this case, red curves and blue curves for total Cln2 are overlapped.

Adrover *et al.*
Supplementary Figure 12



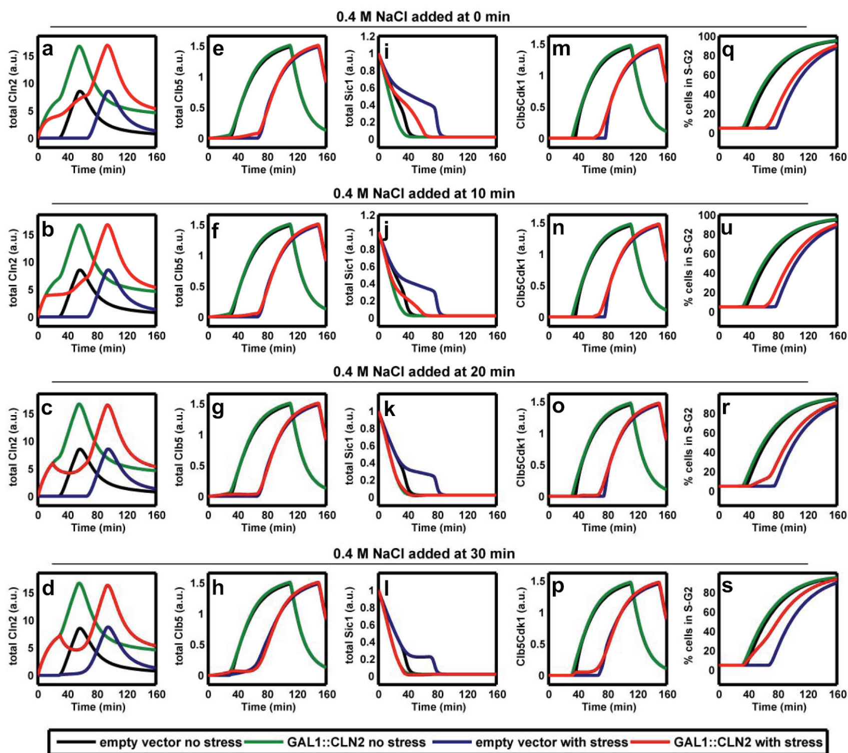
Supplementary Figure 12. Predictions for not regulated Cln2 production.
(**a**-**d**) Predictions for time courses of total Cln2, (**e**-**h**) total Clb5, (**i**-**l**) total Sic1, (**m-p**) Clb5Cdk1 activity and (**q**-**s**) cell count in S-G_2. Simulations obtained with both, the unperturbed and the altered models without stress are the same for no stress (green). The unperturbed model for stress with 0.4M NaCl is depicted in blue and the perturbed model (non-regulated Cln2 by Hog1) under stress condition is shown in red. In this case, red curves and green curves for total Cln2 are overlapped.

Adrover *et al.*
Supplementary Figure 13



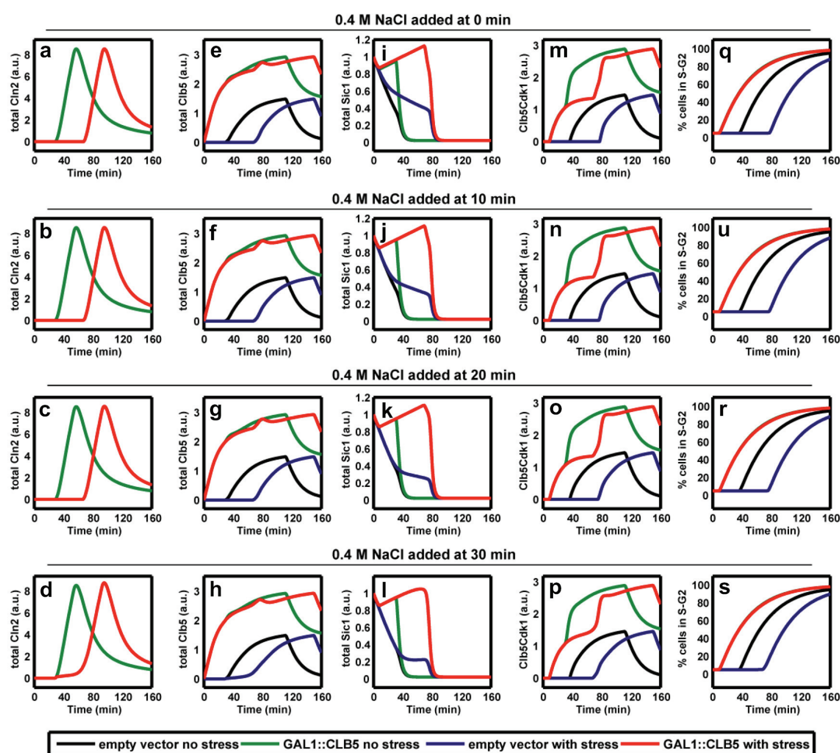
Supplementary Figure 13. Predictions for not regulated Clb5 production.
(**a**-**d**) Predictions for time courses of total Cln2, (**e**-**h**) total Clb5, (**i**-**l**) total Sic1, (**m-p**) Clb5Cdk1 activity and (**q**-**s**) cell count in S-G_2. Simulations obtained with both, the unperturbed and the altered models without stress are the same for no stress (green). The unperturbed model for stress with 0.4M NaCl is depicted in blue and the perturbed model (non-regulated Clb5 by Hog1) under stress condition is shown in red. In this case, red curves and blue curves for total Cln2 are overlapped.

Adrover *et al.*
Supplementary Figure 14



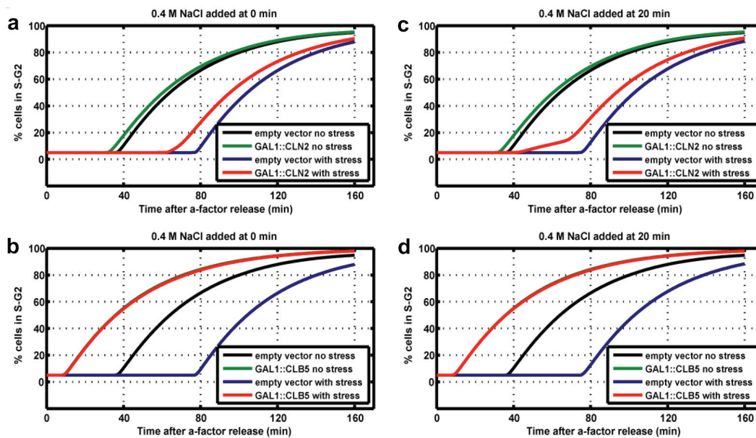
Supplementary Figure 14. Predictions for *GAL1::CLN2*.
(**a**-**d**) Predictions for time courses of total Cln2, (**e**-**h**) total Clb5, (**i**-**l**) total Sic1, (**m-p**) Clb5Cdk1 activity and (**q**-**s**) cell count in S-G_2. Simulations obtained with the unperturbed model are depicted in black for no stress and in blue for stress with 0.4M NaCl; the curves for *GAL1::CLN2* system are depicted in green for no stress and in red for stress with 0.4M Nacl.

Adrover *et al.*
Supplementary Figure 15



Supplementary Figure 15. Predictions for *GAL1::CLB5*.
Predictions for time courses of total Cln2 (**a**-**d**)**,** total Clb5 (**e**-**h**), total Sic1 (**i**-**l**), Clb5Cdk1 activity (**m**-**p**) and cell count in S-G2 (**q**-**s**). Simulations obtained with the unperturbed model are depicted in black for no stress and in blue for stress with 0.4M NaCl; the curves for *GAL1::CLB5* system are depicted in green for no stress and in red for stress with 0.4M NaCl. In this case, the red curves and blue curves for total Cln2 are overlapped, black and green curves for total Cln2 are overlapped as well.

Adrover *et al.*
Supplementary Figure 16



Supplementary Figure 16. Predictions of cell cycle progression for overexpression of cyclins upon different times of stress.
All panels show predictions for time courses of the percentage of cells in S-G_2 obtained with the unperturbed model (wild type) under normal conditions (black), or stressing with 0.4M NaCl (blue) immediately (**a**-**b**) or 20 minutes (**c-d**) after release from alpha-factor; and simulations for different perturbations of the model; ectopically expressed Cln2 (**a** and **c**), and ectopically expressed Clb5 (**b** and **d**) under normal conditions (green) or stressing with 0.4M NaCl (red). Note that for panels **b** and **d** the green and red curves overlap.

A quantitative approach reveals that the Hog1 SAPK regulates G1-S progression upon stress by independently controlling Clns, Sic1 and Clb5

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Regulation of cell cycle progression is also orchestrated as a response to external stimuli and is essential for cell adaptation to changing extracellular conditions (Kyriakis and Avruch, 2001). In budding yeast, the Stress-Activated Protein Kinase (SAPK) Hog1 triggers a whole cellular program for adaptation to changes in external osmolarity (Hohmann, 2002). In response to osmostress, the regulation of progression from G1 to S phase involves the down-regulation of *CLN1,2* and the stabilization of Sic1 through direct phosphorylation by the Hog1 MAPK. It has been proposed that Hog1 may delay Clb5 transcription as well (Escote *et al*., 2004; Zapater *et al*., 2005). Up to date, only basic mechanistic properties of the system have been described. However, specific dynamic properties, putative differential roles of these cell cycle regulators or their real contribution on cell cycle control upon osmotic stress are yet to be described. Computational modelling has shown to be instrumental to elucidate regulatory principles of cell cycle (Barberis *et al*., 2007; Chen *et al*., 2004; Novak *et al*., 2007). For this reason, we took profit of computational analyses as effort to further understand the dynamics of the mechanistically described control of G1 by Hog1. We performed *in vivo* quantitative experiments complemented with mathematical modelling, to create the first model that defines the impact of a MAPK on the cell cycle machinery.

Quantification and modelling of the impact of Hog1 on the Cell Cycle arrest at Start

Here we modelled the interaction of Hog1 with the basic cell cycle machinery that governs the G1-S transition, this is to say, Cln1,2, Clb5,6 and Sic1. In a first approximation, and starting only from knowledge extracted from previous works, we built a qualitative model that linked Hog1 activity to cell cycle progression, taking into account the already known regulators. This initial model aimed only to study qualitative properties of the network and, rather surprisingly for experimentalists, the simulations brought up to two important properties of the performance of the system. The model described the effect of the timing and strength of osmotic stress on cell cycle progression. On the first place, the arrest of cell cycle was similar at any stage of G1 in which Hog1 was activated, until a critical point. Thus, indicating cells should be capable to delay progression to the same extent in response to stress at any stage of G1. This observation seemed at first quite unexpected, for, from a logical point of view, one would expect cells arrested differentially when having high levels of Sic1 and no Cln1,2 (before Start), compared to the time when they have fewer Sic1 molecules and Cln1,2 is being produced (nearby Start). On the second place, the simulations also suggested that an increase on Hog1 activation would lead to a longer cell cycle delay and, consequently, the period of Hog1 activity should determine the timing of cell cycle progression. Again, this was not an obvious prediction, as it could be thought that cells would need the similar time to adapt to hyperosmolarity. But it rather seems Hog1 keeps cell cycle progression still as long as it is active.

The first simulations served us to design simple physiological experiments to investigate the dynamics of the G1-S regulation upon osmotic stress with no alteration of the regulatory machinery of the cell cycle. In order to get this dynamic information from the regulators of G1 upon osmotic stress, we quantified along time Hog1 activation and its subsequent cell cycle arrest, and found that, indeed, exposure of cells to increasing amounts of NaCl resulted in longer Hog1

phosphorylation and a longer arrest in G1, with a strong correlation between the period Hog1 remained active and the time of arrest at G1. These *in vivo* results, did not only confirm modelling was instrumental to us, but also served to quantitatively establish the relationship between Hog1 activation and its resulting arrest in G1. These data were integrated in a second quantitative model that helped us to find out the reason why both cyclins and the inhibitor Sic1 are regulated in parallel.

A comprehensive and quantitative mathematical model needed a rather extensive set of data for parameter estimation. For this, we collected time course data sets, stressing cells at different times after release from pheromone and in response to different strengths of osmostress. In this experiments the dynamics of total Cln2, Clb5 and Sic1 protein levels were measured, along with DNA content and budding index, in addition to phosphorylated Hog1. An initial analysis of these data, pointed out that Sic1 degradation was initiated before Cln2 appears and therefore, under normal conditions Cln1,2 activity might rather account for degradation of the residual Sic1 at Start, than being responsible for the destruction of the whole pool of Sic1, when cells are released from alpha-Factor. Also that Start coincided with the time that cells loose the ability to arrest upon osmostress, and that Sic1 levels seemed to correlated better with the onset of DNA replication, meanwhile Cln2 did correlate with budding, as expected. Thus it originally seemed that these two regulators are coordinated in time by Hog1 in order to keep the concomitancy of replication and morphogenesis.

Withstanding on its own, the mathematical model makes judgment of the relevance of each component of the G1 arrest (Cln2, Clb5 and Sic1), as it is itself an *in silico* tool to study the result of knocking out of the action of Hog1 on each of these specific components. The model predicts the role of Sic1 stabilization is to modulate cell cycle only at Start, for it also predicts that before Start, Clb5 production is tightly down-regulated and therefore the presence of its inhibitor, Sic1, is irrelevant. A similar, but not equal, role is bestowed to Cln2, since cells deficient on the down-regulation of *CLN2* displayed a

comparable arrest when stressed after release and partially maintained their ability to arrest when stressed at Start. Thus, both the down-regulation of *CLN2* and stabilization of Sic1 solely displayed a clear defect on cell cycle arrest when cells were stressed at Start. These simulations gave an indication that before Start, a third mechanism must be responsible of properly delaying entry into S phase when Cln1,2 and Sic1, two well-established regulators, are misguided. If truth be told, an initial version of the quantitative model poorly fitted data for Cln2, Sic1 and cell cycle progression, until it was updated with the inhibition of Clb5 production by Hog1. As a matter of fact, when the down-regulation of Clb5 is simulated non-existing in response to stress, using the final version of the model, cells showed a very poor ability to arrest. Therefore, the model predicted that the down-regulation of Clb5 by Hog1 is most critical to arrest at any stage of G1, and indicated that the different cell cycle regulators under control of Hog1 have distinct temporal roles.

Cln2, Clb5 and Sic1 have different *in vivo* contributions to the osmostress-imposed delay at Start

Based on the mathematical model predictions, we then tried to dissect *in vivo* the individual contribution of the Hog1-mediated down-regulation of Cln1,2 and stabilization of Sic1. We found that in both cases *sic1*Δ cells and cells expressing *CLN2* under heterologous promoter, were able to delay, at least to some extent, entry into S phase in response to osmostress. This is to say, neither down-regulation of *CLN2* nor the stabilisation of Sic1 account for the totality of the cell cycle arrest mediated by Hog1. This finding is consistent with previous results, but with the difference that now these two mechanisms do not seem to be just additive, as previously thought due to the lack of dynamic information of the system (Escote *et al.*, 2004). Additionally, we found indications that this is unlikely to be the case, because over-expression of *CLN2* in a *sic1Δ* background does not abolish the arrest, and cells behave similarly to when over-expressing *CLN2* in wild type cells. This also confirms the result from

the simulations that indicate there ought to be an additional mechanism to delay cell cycle upon Hog1 activation; the direct down-regulation of Clb5. We show in this work that, indeed, Clb5 production is strongly delayed in response to stress, as described previously for the case of Hog1 hyperactivation or heat shock (Escote *et al*., 2004; Li and Cai, 1999). In fewer words, we bestow restricted roles to the already described regulators rather than describing new mechanisms of the G1 arrest in response to osmotic stress, but giving new insights in their functionality and confirming Clb5 down-regulation as part of the mechanism triggered by Hog1 to control passage through Start.

Concerning the temporal roles we found associated to Cln1,2 and Sic1, we can distinguish two distinct scenarios concerning the regulation of the G1-S transition in osmotic stress; G1 (before Start), and Start. Before Start, during the time in G1 when G1 and S cyclins may still be down-regulated, Sic1 seems irrelevant for the osmostress-imposed arrest, as indicated by the fact that *sic1*Δ cells show almost the same ability to delay DNA replication as wild type cells. But during Start, a leaky production of Clb5 turns Sic1 into an important regulator. When measuring Sld2 phosphorylation, as indication of Clb5 activity, we observed that *sic1*Δ cells or cells containing a mutant allele encoding $Sic1^{T173A}$, which cannot be phosphorylated by Hog1, were neither able to properly delay onset of Clb5 activity nor DNA replication and showed a partial progressive phosphorylation of Sld2 when stressed at Start, but not before. Thus, and correspondingly with the simulations, the role of Sic1 is to restrict the activity of newly produced Clb5 when cells are stressed at Start, when the inhibition of cyclin production by Hog1 is not tight enough, but the presence of Sic1 before this point seems immaterial, for Clb5 is not even present in the cell.

Similarly to Sic1, Cln2 regulation is also more relevant at Start to stop cell cycle. Cells ectopically expressing *CLN2* show lower capacity to delay replication when stressed at a moment when Cln2 is already present (artificially induced Start), compared to when stressed before Cln2 levels rise due to the ectopic expression (G1 previous to Start), correspondingly with the simulations, were cells arrest less when

simulating *CLN2* over-expression. Although we have not measured it, this is likely to be due to the fact that the role of Cln2 in regulating replication is through the degradation of Sic1, contributing to the relieve of Clb5 inhibition. It could be, also, that the expression of *CLN2* induces transcription of *CLB5* through a positive feedback loop, even during osmostress, but this is unlikely to be the case for the reasons discussed hereafter.

Besides *CLN2*, we now know *CLB5* is also down-regulated in a time-related fashion as well. When quantifying Clb5 levels we detected that meanwhile *CLB5* was totally repressed when stress was applied before Start, a leaky production of Clb5 was observed when cells were stressed at Start, although it did not result in a loss in arrest capability, probably because these cells were still able to stabilize Sic1 (discussed above). We also found the down-regulation of *CLB5* is the most critical event for cells to delay replication upon osmotic shock at any stage of G1 and even at Start. Cells ectopically expressing *CLB5* show no capacity at all to delay entry into the replicative state, in correlation with the results from the simulations. It may be argued, though, that by over-expressing *CLB5* the ability of Sic1 to inhibit the whole pool of this cyclin is overcome, and therefore the possible implication of Sic1 in this mechanism is masked. But, we now have two indications against this idea; on one hand the over-expression of *CLB5*, although resulting in similar levels of protein than when it is expressed from the endogenous promoter, produces the very exact effect in *sic1Δ* cells and in wild type cells; and on the other hand the simulations also indicate the non regulated Clb5 by Hog1 has the same effect at any stage of G1, both when Sic1 is relevant and when it is not.

In addition to these temporal roles, our results clearly show one primordial task of the Hog1 regulation on *CLN1,2* and *CLB5* is to coordinate cell cycle events. Over-expression of *CLB5* precipitates replication but not budding, upon osmotic stress. In contrast, over-expression of *CLN2* promotes budding but not replication, under the same stressing conditions. Thus, it seems the main role of Hog1-mediated down-regulation of *CLN2* is to coordinate the arrest in

morphogenesis with the one observed in replication, rather than delaying replication by incising on Sic1 degradation.

Clb5 protein production is regulated independently of Cln2 during osmotic stress

Previous data showed that hyper-activation of Hog1 resulted in a down-regulation of *CLB5* expression (Escote *et al.*, 2004). It has been proposed that the expression of Clb5 is under the control of a positive feed-back loop exerted by G1 cyclins (Skotheim *et al.*, 2008). Therefore *CLB5* down-regulation could be a consequence of *CLN2* down-regulation. In order to investigate this, we quantified Clb5 levels in response to osmostress and found that, as commented before, *CLB5* is also down-regulated, but this same delay in Clb5 production happened when we forced the G1 cyclin loop by over-expressing *CLN2*, or when we interrupted such loop by deleting *WHI5*, a mediator of the positive loop of *CLN1,2* on the transcription of the SBF/MBF-regulated genes (Skotheim *et al.*, 2008). The same capacity to delay Clb5 appearance, and accordingly DNA replication, was observed in *whi5Δ* cells than wild type cells. Moreover, it is known that the sustained activation of Hog1 by inactivation of a thermosensitive allele of the osmosensor *SLN1* (*sln1^{ts4}*) induces a permanent arrest in G1. We found that upon *sln1^{ts4}* inactivation, the over-expression of *CLB5* totally abolished the Hog1-imposed arrest in replication onset, whereas over-expression of *CLN2* only had a minor effect, hinting that the regulation of Clb5 and subsequently DNA replication is likely to be independent of the regulation over Cln2 upon osmotic shock. Thus, in response to osmostress, Hog1 plays a direct regulation on *CLB5* that is independent of the positive feedback loop initiated by *CLN3* and mediated by *CLN*1,2.

I have personally contributed to the design of both mathematical and experimental work, as well as the writing of this scientific communication. I have carried out all the experimental work, from strain construction, data obtaining, processing and analysis. The group of Dr. Klipp has developed the modelling part.

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Control of cell cycle progression by stress-activated protein kinases (SAPKs) is essential for cell adaptation to extracellular stimuli. In budding yeast, the SAPK Hog1 regulates G1 to S phase progression in response to osmostress by the dual targeting of cyclin transcription and the B-type cyclin inhibitor Sic1 (Escote *et al*., 2004; Zapater *et al*., 2005). In this work, we have found that survival to osmostress also requires regulation of G2 progression in a similar fashion to the Hog1 regulation of Start. Hog1 triggers a dual mechanism that involves the down-regulation of *CLB2* and the phosphorylation of Hsl1, which results in delocalization of Hsl7 from the septin ring and ultimately in Swe1 accumulation. Here we propose a new mechanism that integrates a MAPK pathway and a cell cycle checkpoint to regulate cell cycle progression upon osmotic stress.

Hog1 mediates a G2 cell cycle arrest in a Swe1 dependent manner.

In response to osmostress, Hog1 delays entry into S phase in a mechanism that involves Sic1 (Escote *et al*., 2004). It has also been reported that osmostress induces an arrest in G2 in a Hog1 dependent manner (Alexander et al.). After the observation that, upon osmotic stress, cells delay their passage through cytokinesis, as monitored by DNA content analyses, we wanted to characterise the involvement of Hog1 in such delay. For this, we genetically activated the HOG pathway either using a thermosensitive allele of *SLN1* (*sln1*^{ts4}), over-expression of a hyperactive allele of SSK2

(*ssk2ΔN*), or over-expressing a hyperactive allele of the MAPKK PBS2 ($Pbs2^{DD}$). Upon Hog1 activation, a large proportion of cells synchronized in G2 remained still as budded (buds with apical growth) with 2C of DNA and pre-mitotic spindles, in contrast to *hog1Δ* cells or wild type cells that did not express any of these hyperactive alleles, that did not arrest cell cycle. These effects were reproduced during osmostress, and it was observed that both delays in G1 and G2 had a comparable extent for a given intensity of osmotic stress. Therefore, the activation of Hog1 results in arrest in G2 before mitosis, alike in G1 before S phase.

It has been proposed that *SWE1* is required for the delay of the onset of mitosis induced by osmostress, although through some independent mechanism from the morphogenesis checkpoint (Alexander *et al*., 2001). But it has also been reported that osmotic stress impinges on cytoskeleton function, which is held under check by the morphogenesis checkpoint and *SWE1* (Chowdhury *et al*., 1992). It could be, then, that the morphogenesis checkpoint mediates the arrest in G2 upon osmostress, instead of Hog1. For that reason, we decided to study the possible implication of *SWE1* in the Hog1-mediated arrest in G2 by hyperactivating the pathway using $sln1^{ts4}$, instead of using osmotic stress. We observed that, upon shifting $sln1^{ts4}$ cells to restrictive temperature, cell cycle was detained in G2 and this blockade was abolished by deletion of *HOG1*, although it was only partially lost in *swe1Δ* cells, indicating that some other mechanism ought to be triggered by Hog1, besides the one that implicates Swe1.

In G2, the reversible phosphorylation on tyrosine 19 of Cdc28 results in the inhibition of its Clb2-specific CDK activity until proper cytoskeleton formation and spindle positioning (Barral *et al*., 1999; Cid *et al*., 2002; Lew and Reed, 1995; McMillan *et al*., 1999). This phosphorylation avoids Clb2-Cdc28 activation and spindle elongation during S phase/G2 (Amon *et al*., 1992). It is mediated by Swe1 and opposed in mitosis by the Mih1 phosphatase (Booher *et al*., 1993; Russell *et al*., 1989). We did not test the levels of phosphorylated Cdc28 on this site, but we observed that the effects

of Hog1 activation in G2 were exacerbated in *mih1Δ* cells, giving an indirect indication that Swe1 and its mediated phosphorylation on Cdc28 have, indeed, a prominent role in this arrest. In fact, deletion of *SWE1* renders cells osmosensitive, with a stronger phenotype than in the case of *SIC1* deletion, which reinforces the idea of *SWE1* plays an important role in cell cycle control during osmoadaptation. Moreover, upon osmostress, a proportion of *swe1Δ* cells appeared as binucleated cells as a result of a failure in splitting nuclei between mother and daughter cells. Most probably, this is due to a failure in delaying spindle elongation and thus carrying out karyokinesis before spindle positioning. Taken together, Hog1 delays cell cycle in G2 through, at least, *SWE1*, to assure cell viability upon osmostress.

Hog1 activation results in Clb2 down-regulation and Swe1 stabilization.

After observing that *SWE1* deletion did not totally abolish the arrest in G2 upon osmotic shock, we tested if Clb2 levels could be also down-regulated, similarly to what happens with other cyclins. The activation of Hog1 resulted in both the dropping of the levels of mRNA and protein of *CLB2*. We observed, as well, that Clb2-Cdc28 activity was strongly inhibited. Accordingly, only wild type cells but not *hog1Δ* or *swe1Δ* cells showed elongated buds upon Hog1 sustained activation. This morphology has been associated to defects in Clb2 function (Fitch *et al*., 1992). This down-regulation, though, seems unlikely to be the main effector that mediates G2 arrest upon transient osmotic stress. Since the observed reduction in activity of Clb2-Cdc28 did not greatly change when hyperactivating Hog1 in *swe1Δ* cells, compared to when Hog1 was activated in wild type cells. Taken together, Hog1 down-regulates the levels and activity of *CLB2*, although, Swe1 might play a much more prominent role than this down-regulation in the delay in G2, at least for the conditions tested.

It is described that the levels of Swe1 are critical to regulate Cdc28-Clb2 activity (McMillan *et al*., 1999; Sia *et al*., 1996). In G2, Swe1 protein levels depended on its phosphorylation state, which triggers its ubiquitination and ulterior degradation (McMillan *et al*., 1999; Tyers and Jorgensen, 2000). After the observation that the down-regulation of Clb2-Cdc28 activity was strongly dependent on Swe1, we decided to investigate the phosphorylation state and stability of this inhibitor throughout G2 in high osmolarity conditions. We first found that osmostress delayed Swe1 phosphorylation in comparison to non-stressing conditions, and accordingly, Swe1 degradation was also delayed. Moreover, Swe1 accumulates in asynchronous cultures subjected to osmotic stress or Hog1 hyperactivation by expression of *pbs2*^{DD}. Therefore, the activation of Hog1 also results in lower Swe1 phosphorylation and its subsequent stabilization.

Hog1 phosphorylation on Hsl1 induces Hsl7 delocalisation and Swe1 stabilisation.

Swe1 degradation is accomplished specifically at the bud neck. The complex Hsl1-Hsl7 tethers Swe1 to the septin ring of the bud neck, where it is mainly phosphorylated by the polo-like kinase, ubiquitinated and degraded (Lew, 2003). This complex is part of the morphogenesis checkpoint, which, in response to morphogenetic defects, delays tethering of Swe1 to the bud neck and therefore postpones its degradation (Lew, 2003).

We observed that, unlike the case of Sic1, Hog1 does not phosphorylate Swe1 *in vitro*. For that reason, we thought Hog1 might be interfering with the mechanism in charge of Swe1 degradation to promote its stabilisation. We examined the localisation of septins at the bud neck, which proved to remain unchanged upon Hog1 activation and osmotic shock. Therefore, Swe1 stabilisation was not due to cytoskeleton disorganization and triggering of the morphogenesis checkpoint, and thus, it must be through some specific mechanism elected by Hog1. It has also

been described that the disruption of the interaction between Hsl1 and Hsl7 or the loss of function of Hsl1 results in slower degradation rate of Swe1 (Barral *et al*., 1999; Lew, 2003; Shulewitz *et al*., 1999). We then checked whether the localisation at the bud neck of Hsl1 and Hsl7 was affected by Hog1 activation. Like the case of septins, Hsl1 did not change localisation upon osmostress, but Hsl7 did so, migrating from the septin ring to patches nearby the cytoplasmic membrane. Moreover, the exclusion of Hsl7 from the bud neck correlated in time with Hog1 activation. It is also reported that when Hsl7 delocalises from the bud neck, it is rapidly dephosphorylated (Sakchaisri *et al*., 2004). Correspondingly, Hog1 activation results in rapid Hsl7 dephosphorylation.

After the observation that Hsl7 delocalizes from the bud neck upon Hog1 activation, we then studied whether the MAPK interacted with Hsl1 or Hsl7. Co-precipitation assays demonstrated that Hog1 binds to Hsl1 but not Hsl7. And this led us to check whether Hog1 was able to phosphorylate any of these proteins. *In vitro* kinase assays showed that whereas Hog1 was not able to phosphorylate Hsl7 it did phosphorylate Hsl1. Furthermore, Hsl1 showed to be phosphorylated *in vivo* in a Hog1 dependent manner upon osmotic shock. Therefore, Hog1 directly interacts and phosphorylates Hsl1 to delocalise Hls7 and Swe1 from the bud neck, which leads to Swe1 accumulation.

Phosphorylation on Hsl1 *S1220* by Hog1 is critical for osmoadaptation.

Hsl7 recruitment to the bud neck requires its binding to the C-terminal domain of Hsl1 (Shulewitz *et al*., 1999). *In vitro* kinase assays indicated that Hog1 phosphorylated Hsl1 at Serine 1220, within the Hsl7-binding domain. As previously commented, the localisation of Hls7 was altered in response to activation of the Hog1. But the localisation of Hsl7 did not change in cells containing a mutant allele of Hsl1 that cannot be phosphorylated by Hog1 ($Hsl1^{S1220A}$). Moreover, these cells did not experience a lost of

interaction between Hsl1 and Hls7 when subjected to osmostress, which corresponded to the inability to stabilize Swe1. All together, proves the phosphorylation of Hsl1 at S1220 by the Hog1 to be responsible for the delocalisation of Hsl7 upon osmotic stress. And correspondingly, $Hsl1^{S1220A}$ containing cells, in response to Hog1 activation, were unable to arrest in G2 becoming more sensitive to osmostress than wild type cells.

In this work, we showed Hog1 regulates G2 in an analogous manner to its regulation of G1. It is an interesting observation, though, that this MAPK takes profit of pre-existing cell cycle regulatory mechanisms, like Sic1 and components of the morphogenesis checkpoint, in order to integrate stress signals to the machinery that controls entry into mitosis and assure viability. Therefore, the engagement of a MAPK in the instrument that controls a normal cell cycle, represents a novel mechanism of cell cycle control.

My personal contribution to this work has been the technical assistance at multiple levels of the experimental work, and specially in demonstrating that Hog1 interacts and phosphorylates Hsl1, as well as the biological relevance of the phosphorylation on Hsl1 in the localization of Hls7, cell cycle progression and loss of interaction of Hsl1 with Hsl7.

GLOBAL DISCUSSION

Hog1 controls G1 transition by a dual mechanism that involves regulation of cyclin expression and the accumulation B-type cyclin inhibitor Sic1. In G2, Hog1 is also controlling cell cycle progression by down-regulating Clb2 transcription, and accumulation of its inhibitory kinase Swe1 trough the phosphorylation of the Septin Dependent Kinase (SDK) Hsl1. In both cases, the stabilization of the inhibitor is achieved by both a phosphorylation that interferes somehow in its degradation, and a drop in CDK activity that targets this inhibitor for degradation. The capability of a single MAPK to regulate different stages of cell cycle seems obvious from the fact that cells might be subjected to stress at any stage of their cell cycle. Therefore there ought to be mechanisms to adapt before progressing into more sensitive phases of the cell cycle. The Hog1 MAPK shows competence to regulate both G1 and G2, through two-independent checkpoints, this is, the trigger of Start and the Morphogenesis Checkpoint. In fact, cells unable to regulate some of these components under the control of Hog1 become growth-sensitive in high osmolarity conditions. Therefore, it seems that the coordinated action of a MAPK over the cell cycle machinery is orchestrated at different steps of cell cycle in response to stress.

There could be several explanations for the selective pressure of having a dual control system like the ones above described. If the single stabilization of Sic1 or Swe1 were rather permanent, the down-regulation of CDK activity to further stabilize them would seem unnecessary. But, on the contrary, as the stability of these inhibitors also depends on the cyclins, by delaying the onset of activity of such cyclin-CDK complexes, these inhibitors should be stabilized, and therefore it would be the direct stabilization of such proteins what would seem unnecessary. Be one case or the other, neither the stabilization of inhibitors, nor regulation of cyclins account by themselves for the totality of the arrest in both phases (G1 and G2). At a first thought, it may seem that the advantage of this dual effect over the inhibitors would be an increase of the efficiency of two additive mechanisms over Sic1 and Swe1 stabilization, either to increase the astringency of the signal that would induce a blockade of cell cycle in order to assure the non-

interference on the normal cell cycle without stress, or by providing redundancy on a key regulator by controlling it through different ways that split functionality. This is to say, they either have distinct temporal roles, they contribute to coordinate other processes than DNA replication or karyokinesis, or they have different reaction times.

As just commented, the control of Hog1 at G2 is surprisingly similar to the one described in G1. In both cases it consists of a dual mechanism that involves the down-regulation of one or more cyclins and stabilization of an inhibitor. Even though, the reasons for this kind of architecture to be like this, might differ from one phase to another. *CLN2* and *CLB5* are down-regulated within minutes and at the same time Sic1 stabilization occurs (Escote *et al.*, 2004). On the other hand, we still have no proofs of *CLB2* levels and associated CDK activity being down-regulated at the same time as Swe1 is stabilized. Thus, rather than having distinct temporal roles like the regulators of G1, this dual mechanism in G2 might be a two speed redundant mechanism, robust enough to avoid transition into mitosis without adaptation for any given strength (or length) of stress. And consisting of a fast short-term effect, the stabilization of Swe1; and a stronger long-term effect, the down-regulation of Clb1,2.

After this work, the reason why the control of G1 by Hog1 consists of a dual mechanism seems clearer. The modelling efforts have been instrumental to find that the different components controlled by the SAPK play distinct temporal roles. This differential contribution of each component along time probably makes cells competent to respond to osmostress at any step of the transition into S phase. At a time when Start events are underway, a tight control of cyclin activity is no longer possible, and therefore, being able to further inhibit such activity, most likely brings in an extra advantage. Another, more evident advantage of such a mechanism is that the SAPK keeps coordination between replication and morphogenesis by affecting at the same time *CLN1,2* and Clb5 activity, and thus assuring the concomitancy of these two

processes during the osmostress-imposed arrest. Moreover, the results here displayed, shed light on the interplay of the dynamics of a MAPK activation and the cell cycle control machinery. It is now clear that there is a direct correlation between Hog1 activity and the cell cycle blockade for many different conditions, indicating that the blockade of cell cycle at G1 by Hog1 is a rather strict mechanism, instead of just being a short transient delay of events.

Given these similarities, a modelling approach might be interesting to try to elucidate the reasons why the regulation of G2 by the Hog1 MAPK is also structured like it is. Several parameters could be measured in the effort of modelling the impact of Hog1 in G2, from the delay of Swe1 phosphorylation and degradation, Hsl7 localisation, or the dynamics of phospho-tyrosine Cdc28, to spindle elongation, in response to osmotic stress. Once built, such model could be useful to test the hypothesis stated above, and see if it is really a two-speed mechanism, or there is a temporal share of responsibilities. It could also be studied the implication of the different regulatory loops triggered by Clb2-Cdc28 that regulate *CLB2* expression itself and the self activation by contributing to the degradation of Swe1. It could also be useful to investigate the implication of other regulators of the G2-M transition; for instance *CDC5*, *MIH1*, *CDH1*, *CDC20*, *SIC1* or *SWI5*; as all have proven to be necessary for passage through mitosis and also to integrate checkpoint signals.

Whichever is the case, up to now we still have a poor understanding of the molecular mechanisms underlying the down-regulation of cyclins by osmostress. We have elucidated only the biochemistry of the Hog1-mediated inhibitor stabilisation and only genetically or functionally described the delay of the onset of *CLN2*, *CLB5* and *CLB2* transcription. We are still missing the effect of Hog1 on the inhibition of the SBF and MBF transcriptional regulators, ignoring if this MAPK directly phosphorylates these transcription factors, it affects localization of the transactivator *SWI6*, or their inhibitor *WHI5*, for instance. The mechanism Hog1 down-regulates *CLB2* is not better understood, *FKH1,2*, *MCM1* or

NDD1, would seem at a first glance, good candidates to be affected by Hog1 upon osmotic stress. Finally, one should keep in mind that the strongest phenotype of *SIC1* deletion is in mitosis, these cells have problems completing cytokinesis. Therefore another interesting issue to be studied is the implication of the most probable accumulation of Sic1 due to Hog1 activation nearby Mitosis.

Complementary strategies to keep on research of cell cycle control by stress, would be the screening for other mechanisms in other phases of cell cycle, for instance, we now have evidence that replication is slowed down by Hog1, although we are still missing the molecular mechanism underlying this effect. Or to investigate the possible impact of other SAPK or stresses on cell cycle control, as the case of research in *Schizosaccharomyces pombe*, where a single SAPK, Sty1, triggers different effector mechanisms as a response to different stimuli, for instance the election of different subsets of genes to be transcribed in response to different stimuli (Chen *et al*., 2003).

It has been demonstrated that osmotic imbalances results in the activation of the Hog1 homolog p38 SAPK in mammalian cells. Unpublished observations from our group, indicate that mammalian cells arrest in G1, G2 and mitosis upon osmostress, in accordance to already published data (Ambrosino and Nebreda, 2001; de Nadal *et al*., 2002; Dmitrieva *et al*., 2002; Sheikh-Hamad and Gustin, 2004). Up to date, several targets for the SAPKs have been described, but the complex regulation of mammalian cell cycle makes it difficult, with the actual knowledge, the assessment of cell cycle regulation as it is done in yeast cells. In consequence, during the upcoming years, further Systems Biology-based research on cell cycle control by extracellular stimuli in budding and fission yeast will be instrumental for the elucidation of regulatory principles beyond the scope of classical molecular biology strategies.

CONCLUSIONS

1. There is a tight correlation between the timing of Hog1 activity and the cell cycle blockade along late G1

2. The down-regulation of *CLN2* and the stabilization of Sic1 do not account for the totality of the G1 arrest upon osmostress

3. The Hog1-mediated down-regulation of cyclins and Sic1 stabilization show to have distinct temporal roles in the arrest at G1

4. Sic1 stabilization by Hog1 becomes relevant only at Start

5. Hog1 coordinates morphogenesis and DNA replication by mediating a concomitant down-regulation of *CLN2* and *CLB5*

6. The down-regulation of the B-type cyclin *CLB5*, but not *CLN2*, upon osmostress is determinant for the delay in replication onset

7. The down-regulation of *CLB5* by Hog1 is independent of the regulation of *CLN2* by this SAPK

8. The Hog1-mediated arrest at G2 involves the down-regulation of *CLB2* and the stabilization of Swe1

9. Hog1 directly phosphorylates Hsl1 at Ser1220 within the Hsl7 docking site, thus integrating osmostress-responsive signalling to the morphogenesis checkpoint

10. The phosphorylation of Hsl1 by Hog1 is responsible for the delocalization of Hsl7 from the bud neck and the subsequent Swe1 stabilization

11. The phosphorylation of Hls1 by Hog1 is critical for the observed G2 arrest and cell survival upon osmotic stress

12. Hog1 induces transient arrests of cell cycle both at G1 and G2 phases to assure cell survival upon osmostress

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