Control of Transcription by the Stress Activated Hog1 Kinase

Mariona Nadal Ribelles

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Treball dirigit pel Dr. Francesc Posas Garriga i la Dra. Eulàlia de Nadal Clanchet

Departament de Ciències Experimentals i de la Salut (DCEXS)

Programa de Doctorat en Biomedicina de la Universitat Pompeu Fabra

DON'T QUIT

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SUMMARY

A fundamental property of living cells is the ability to sense and robustly respond to fluctuations in their environment. In budding yeast (*Saccharomyces cerevisiae*) changes in extracellular osmolarity are sensed by the HOG pathway, which evokes the program for cell adaptation required for cell survival.

Genome-wide profiling of gene expression in response to stress has revealed a prominent role for the Hog1 SAPK as master regulator of transcriptional reprogramming. Previous studies showed that Hog1 commands gene expression through several mechanisms that control the different steps of the transcription cycle (de Nadal and Posas, 2010). The aim of this thesis was to further characterize the molecular mechanisms by which Hog1 regulates gene expression upon osmostress.

A genome-wide genetic screen lead to the identification of several activities required for regulation of gene expression. Here we describe the characterization of a novel substrate for the SAPK whose activity is required for proper transcription initiation and elongation in response to stress.

While genetic and biochemical studies have been proven to be very effective at dissecting the molecular mechanisms used by Hog1, this thesis also aimed to globally characterize the role of Hog1 in reprogramming the transcriptome of S. *cerevisiae* under osmostress conditions. By the combination of molecular approaches coupled to genome-wide techniques (ChIP-seq, MNase-seq and Tiling arrays) we have been able to fully characterize the localization of the key components that drive osmoresponsive transcription, providing for the first time a complete picture of the

transcription process. The high resolution of the genome-wide approaches, has allowed us to identify new transcriptional roles for the SAPK such as the targeting of RNA Pol III machinery, and the regulation of a novel class of functional long noncoding RNAs (lncRNA). In summary, results presented in this thesis provide novel insights into the mechanisms by which the Hog1 SAPK modulates gene expression.

Una propietat cel·lular fonamental és l'habilitat de detectar i respondre de forma robusta a les fluctuacions en el seu entorn. En cèl·lules de llevat (*Saccharomyces cerevisiae*), els canvis en l' osmolaritat extracel·lular són detectats per la via de senyalització de HOG, que coordina el procés d'adaptació cel·lular imprescindible per sobreviure a un estrès osmòtic.

La caracterització del transcriptoma en resposta a estrès osmòtic ha permès identificar l'important paper de Hog1 SAPK com a màxim regulador de la reprogramació transcripcional. Estudis previs han demostrat que Hog1 controla l'expressió gènica a través de varis mecanismes, que afecten a les diferents etapes del procés de transcripció (de Nadal and Posas, 2010). L'objectiu d'aquest estudi és identificar i caracteritzar els mecanismes moleculars utilitzats per Hog1 per regular l'expressió gènica en resposta a estrès osmòtic.

Fent servir un crivatge genètic a gran escala dissenyat per identificar activitats necessàries per la regulació de l'expressió gènica en resposta a estrès osmòtic, hem identificat un nou substrat de Hog1, l'activitat del qual és requereix tan per la iniciació com l' elongació de la transcripció.

Mentre que els estudis genètics i bioquímics han demostrat ser molt eficients en identificar els mecanismes utilitzats per Hog1, en aquest treball també ens hem centrat en caracteritzar el paper global de Hog1 en la reorganització del transcriptoma de *S. cerevisae* en condicions d' estrès osmòtic. Mitjançant la combinació de tècniques moleculars amb tècniques de seqüenciació (ChIP-seq, MNase-seq, Tiling arrays) hem definit el posicionament en el

genoma dels components claus que regulen la transcripció, oferint per primera vegada una visió general del procés de transcripció en resposta a estrès osmòtic L'alta resolució d'aquestes técniques ens ha permès identificar noves dianes transcripcionals de Hog1, com és la regulació d'una altra maquinària transcripcional (RNA Pol III) i la regulació de la transcripció de una nova classe de RNAs no codificants (lncRNAs).

En conjunt, els resultats presentats en aquesta tesi proporcionen una nova visió dels mecanismes per els quals Hog1 modula l'expressió gènica.

PREFACE

Over the last twenty years a huge effort has been directed towards understanding the properties of the signaling pathways that coordinate adaptive responses. Activation of MAPK affects virtually all cellular processes, among them the readjustment of the transcriptional program has been extensively studied. In *S.cerevisiae*, alterations in the external osmolarity activate the stress-activated Hog1 MAPK, the functional homologue of the mammalian JNK and p38 MAPKs. The fundamental mechanisms that regulate transcription are highly conserved from yeast to humans, hence making Hog1 a perfect model to understand how MAPKs control gene expression in eukaryotes.

In this PhD project we have taken advantage of classical molecular techniques and genome-wide approaches to understand the mechanisms that underlie Hog1-mediated transcription. Our findings provide a comprehensive view of the complex process by which SAPKs rapidly induce and fine-tune transcription in response to adverse conditions.

From a genetic screen designed to identify essential proteins required for proper transcription in response to osmostress, we have identified a novel enzymatic activity required for Hog1-mediated transcription. Recently, the use of 'Tiling arrays' and ChIP-seq technologies have identified the limits, the topology and the quantity of the real coding and non coding genome of *S. cerevisiae*. We have used these genome-wide techniques to profile the transcriptome and to position the key components of transcription in response to osmostress (Hog1, RNA Pol II and nucleosomes). We have demonstrated a novel role of Hog1 in the regulation of the

transcription of the noncoding genome. Also, we have quantitatively and qualitatively extended the regulatory role of Hog1 in protein-coding genes.

In general, this work offers new insights and an integrated view of the mechanisms by which the Hog1 SAPK regulates gene expression. Despite that many of the components required for transcription reprogramming under osmostress have been identified, a complete picture of the process is far from complete. Further studies on novel Hog1 substrates, single cell and genome-wide analyses, will probably help to further understand the osmoadaptive response at the transcriptional and posttranscriptional level.

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INTRODUCTION

1. STRESS SIGNALING

1.1 Yeast and its environment

Yeasts include a wide variety of single celled eukaryotic fungi that live as saprophytes on plants or animal material. These habitats are often rich in simple organic carbon, liquid or very high in moisture, acid or occasionally alkaline and nutritionally complex. As carbon and energy source, they preferentially catabolize sugars, polyols, alcohols, organic acids and amino acids (Spencer and Spencer, 1997).

Niches where yeasts live are dynamic environments that frequently and suddenly fluctuate in temperature, oxidative or osmolarity conditions, among many others. These changes lead to stressful situations that disrupt cellular homeostasis and physiology. In particular, the budding yeast *Saccharomyces cerevisiae* is found mainly on fruits, flowers and other sugar rich substrates. Depending on their availability, fermentable sugars such as glucose, galactose, fructose, maltose and raffinose as well as non fermentable substrates like acetate, ethanol or glycerol can be used as carbon sources. *S. cerevisiae* cells can rapidly adapt to the changes in nutrient, temperature, pH, radiation, oxygen concentration and water activity (Hohmann, 2002). Amazingly, they can grow in temperatures from freezing to 55ºC, in pH ranging from 2.8 to 8, and almost complete drying is tolerated (dry yeast). They can support growth and fermentation under extremely harsh conditions such as media containing up to 20% ethanol (Hohmann and Mager,

2003), or 40% glucose which represents a dangerous osmotic condition.

The adaptive capacity of yeast requires different mechanisms to maximize cell survival. Consequently, yeast cells have evolved sophisticated mechanisms to sense and integrate the changes in their environment and rapidly correct internal parameters accordingly. This process known as adaptive response, and the associated molecular mechanisms are mainly controlled by intracellular signaling pathways.

1.2 MAPK Pathways

MAP kinase pathways are key signaling pathways important to transduce extracellular insults. They are one of the most widespread and well conserved mechanisms for signal transduction among lower and higher eukaryotes. All eukaryotic cells posses several signaling MAPK pathways, which are specifically activated depending on the stimuli. Therefore, MAPK pathways allow cells to respond coordinately and specifically to multiple environmental signals (Kyriakis and Avruch, 2001).

MAPKs involved in stress responses belong to a family of proteins known as stress-activated protein kinases (SAPKs), and are responsible for the transduction of stress signals in eukaryotic cells. Adaptive responses coordinated by SAPKs cover almost any aspect of cell physiology from regulation of gene expression, protein homeostasis, cell cycle progression and metabolism among others (Chang and Karin, 2001).

1.2.1 Modular organization of MAPK signaling pathways

Canonical MAPK pathways are conserved kinase cascades organized in modules containing three sequentially activating protein kinases that transmit signals by sequential phosphorylation events in a hierarchical way (Figure 1): The MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK.

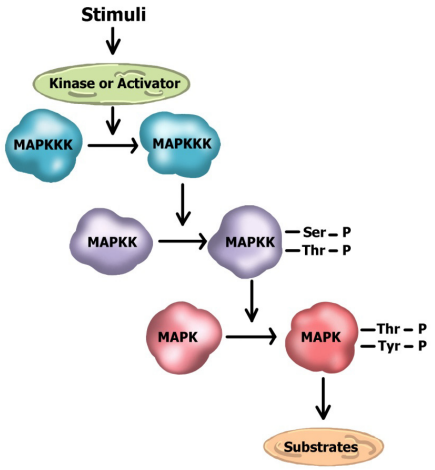

Figure 1. Schematic diagram of a canonical MAP kinase module. The core module of MAPK pathway is composed by three kinases that are sequentially activated by phosphorylation.

MAPKKKs contain a regulatory domain located at the N-terminal part and a catalytic Ser/Thr kinase domain at the C-terminus. Activation of the MAPKKK is usually achieved by either phosphorylation by upstream kinase or through the interaction with other proteins, which releases the autoinhibition exerted by the N-terminal domain resulting, in some cases, in autophosphorylation.

MAPKKs are phosphorylated by MAPKKKs on serine and threonine residues within a conserved loop at the N-terminal lobe of the kinase domain. Subsequently, the MAPKK phosphorylates the MAPK at a conserved Thr-X-Tyr in the activation loop. The dual phosphorylation activates the MAPK causing a conformational change as well as an increase in nuclear localization. Then, activated MAPK phosphorylates its downstream targets at serine/threonine followed by proline (S/TP sites). Substrates of the MAPK include: transcription factors, cell cycle regulators, phosphatases, translational regulators, MAPK activated protein kinases (MAPKAP) and other classes of proteins. Hence, MAPK signaling allows to regulate metabolism, cell cycle progression, cellular morphology and gene expression to properly adapt to external stimuli (Chen and Thorner, 2007).

1.2.2 Yeast MAPK pathways

Components belonging to MAPK signaling cascades can be identified by conservation of the T-X-Y domain together with sequence similarities. Based on genetic analyses, studies in transcriptional output upon stimulation five MAPK pathways have been allocated to five different MAP Kinase cascades (Hohmann, 2002a; Qi and Elion, 2005) (i) the mating pheromone response pathway (Fus3), (ii) the filamentous growth or pseudohyphal development pathway (Kss1), (iii) the cell wall integrity pathway (Slt2/Mpk1), (iv) the spore wall assembly pathway (Smk1), and (v) the HOG pathway (Hog1) (Figure 2).

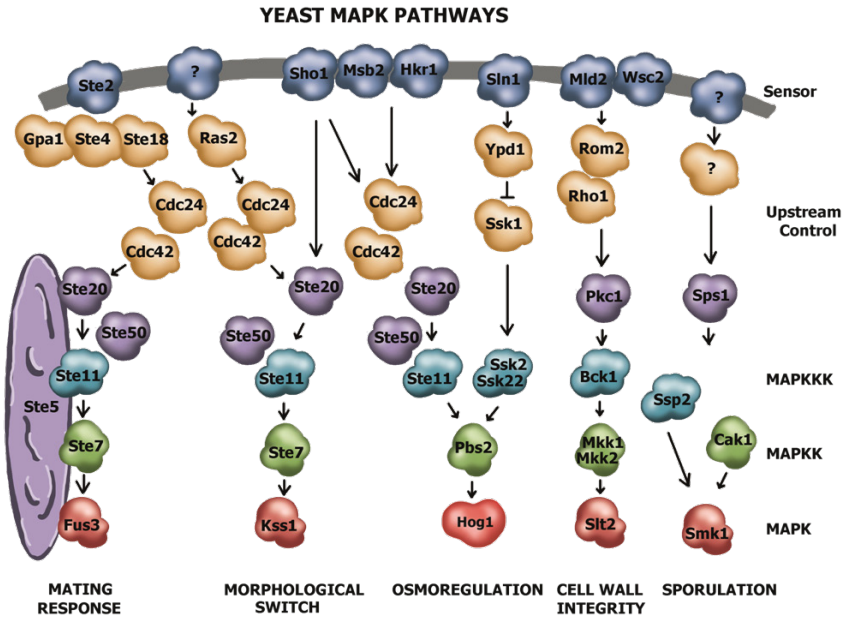

Figure 2. Scheme of yeast MAPK pathways. *S. cerevisiae* contains five MAPK pathways: mating response, morphological switch, osmoregulation, cell wall integrity and sporulation.

These MAPK pathways play different roles in response to developmental and external inputs. Cell surface remodeling during shmoo formation of pseudohyphal growth is controlled by the mating pathway and the development pathway. The HOG pathway and cell integrity pathway are responsible for maintenance of adequate turgor pressure during osmotic changes. Sporulation is regulated by the Smk1 pathway (Krisak et al., 1994).

In spite of the five MAPK are encoded in the *S. cerevisiae* genome, only four MAPKKs are required to control its activity. Likewise, four MAPKK are controlled by several MAPKKK. In

yeast, as in higher eukaryotes, MAPKKK and MAPKK can control more than one single MAPK giving rise to a highly complex signaling network that ensures an appropriate response to each stimulus (Qi and Elion, 2005).

1.2.3 Signaling specificity in MAPK pathways

The reuse of signaling components between different MAPK pathways is common. For example, different MAPK pathways within the same organism can share protein kinases that are triggered by various signaling inputs. This can eventually lead to cross-talk among the signaling pathways. Up to date, there is not a unique mechanism to prevent cross-talk.

Scaffold proteins physically sequester components of the MAPK pathway into separate pools spatially restricting the interactions between signaling molecules. An example of a prototypical scaffold protein is Ste5, which is activated in response to pheromone treatment (Printen and Sprague, 1994). Ste5 is recruited to plasma-membrane where it undergoes conformational changes that release autoinhibition and promotes efficient phosphorylation of Fus3 by Ste7, and signal propagation (Zalatan et al., 2012). Although the usage of scaffold proteins is a commonly used mechanism, there are other strategies that maintain robustness and efficiency: docking interactions, cross-pathway inhibition and kinetic insulator are some of the strategies used to prevent cross-talk (Saito, 2010).

Post-translational modifications other than phosphorylation are emerging as regulators of signaling specificity. Sumoylation, acetylation and ubiquitylation, among others have been recently

reported to regulate strength and fidelity of signaling. For example, proper ubiquitylation-deubiquitylation of Ste7 by the ubiquitin ligase $SCF4^{cdc4}$ and ubiquitin protease Ubp3 allows correct activation of Fus3 and prevents crosstalk with Kss1 respectively (Hurst and Dohlman, 2013). Taken together, cells have evolved complex signaling architectures that are tightly regulated to ensure proper gating of signaling to each MAPK pathway.

1.3 Osmostress

A common stress that yeast cells face is a sudden change in osmotic pressure, which represents a rapid change in water activity. Water activity is defined as the chemical potential of free water in solution, and appropriate levels are essential to favor biochemical reactions and cell volume. Under normal growth, cytosolic water activity is lower than its surrounding environment. Two opposed scenarios need to be considered: hypo-osmotic shock which causes a rapid water influx leading to swelling and increased membrane turgor pressure; on the other hand, hyper-osmotic shock results in massive water outflow provoking cell shrinking.

Water movement occurs within seconds. Hence, the signaling mechanisms that mount an adaptive response to osmotic changes have to be ready to rapidly react at all moments. Adaptation is an active process based on sensing osmotic changes and triggering the accumulation of chemically inert osmolytes such as glycerol. An increase in glycerol balances intracellular osmolarity with the surrounding medium and causes an inside-directed driving force of water that restores cell volume (Hohmann, 2002a).

1.3.1 Signaling pathways involved in osmoadaptation

In the budding yeast, several pathways have been shown to be activated by changes in external osmolarity. The best characterized osmoresponsive system is the HOG pathway (**H**igh **O**smolarity **G**lycerol pathway). Scientific interest in the osmoadaptation field arouse with the discovery, in 1993, that a MAPK cascade was essential to adapt to osmostic upshift (Brewster et al., 1993). Mutants defective in activation of the HOG pathway cannot survive in high osmolarity medium, thus, confirming that its cellular role is to orchestrate the osmostress response. However, other pathways signaling pathways are activated upon changes in osmolarity: the protein kinase A pathway and the phosphatidylinositol-3,4-biphosphate pathway.

Studies on the role of PKA during exponential growth under osmostress suggest that low PKA activity causes a change in protein expression that resembles what is observed in osmotically stressed cells, and thus PKA is an important determinant of osmotic shock tolerance (Norbeck and Blomberg, 2000). On the other hand, the PKA pathway mediates a general stress response observed under essentially all stress conditions, such as oxidative stress, nutrient starvation, heat shock and osmostress (Marchler et al., 1993; Ruis and Schüller, 1995). Therefore, PKA probably does not directly respond to osmotic changes. In fact, regulation of PKA under stress is not fully understood.

Additionally, the production of phosphatidylinositol-3,4-biphosphate seems to be stimulated by osmotic upshift. This

molecule could serve as a second messenger in osmostress signaling (Dove et al., 1997).

Much less is known about the response to osmotic downshift. Cell swelling is counteracted by opening glycerol channel (Fps1) and therefore decreasing internal osmolarity (Hohmann, 2002b). Cell wall integrity pathway is rapidly activated, although the physiological relevance is still poorly understood (Davenport et al., 1995).

2. OSMOSTRESS SIGNALING: THE HOG PATHWAY

Among the different pathways activated in response to osmostress, the HOG pathway is the most important signal transduction pathway. Activation of the MAPK pathway elicits the program for cell adaptation required for survival. Osmoadaptive responses include: metabolic regulation, cell cycle control, translation and reprogramming of gene expression (de Nadal et al., 2002). The Hog1 SAPK is a prototype of SAPK equivalent to the mammalian p38 MAPK. Strong functional conservation between HOG and p38 pathways is illustrated by the fact that p38 MAPK can rescue osmosensitivity of *hog1* mutants (Derijard et al., 1995; Galcheva-Gargova et al., 1994; Sheikh-Hamad and Gustin, 2004).

2.1 Components and organization

The HOG pathway is composed by membrane-associated osmosensors, and intracellular signaling pathway whose core is the Hog1 (MAPK) that has cytoplasmic and nuclear targets (Saito and Posas, 2012). The architecture of the pathway has been determined

during the last 20 years through genetic screens, epistasis analysis, complementation analysis and analogies to other pathways.

Schematically, the central core of the HOG pathway comprises a layer of three MA PKKK, Ssk2, Ssk22 and Ste11 that are responsible for the activation of the MAPKK Pbs2. Activated Pbs2 then phosphorylates and activates Hog1 MAPK (Figure 3).

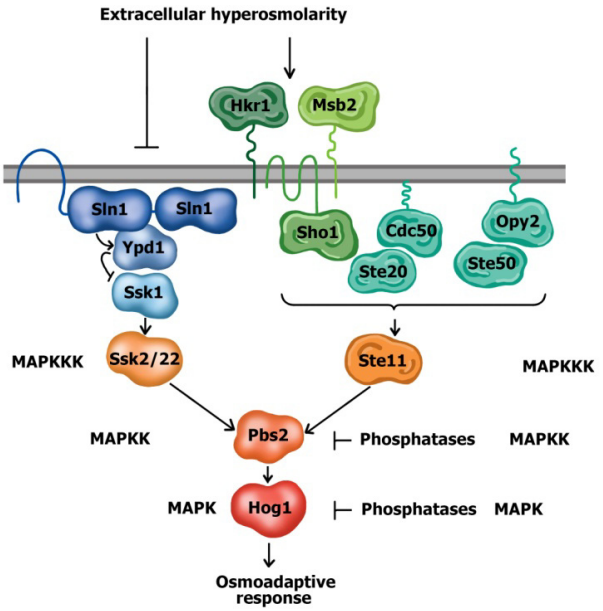

Figure 3. Outline of the HOG pathway. Two independent upstream osmosensing mechanisms lead to the specific activation of the MAPKKKs (Ste11 and Ssk2/22) that converge to activate the MAPKK (Pbs2). Activated Pbs2 phosphorylates Hog1 that coordinates the adaptive response.

Upstream activation of the pathway involves two functionally redundant but mechanistically distinct mechanisms that activate the MAPKKKs. The first mechanism is the Sln1 two-component sensor (Maeda et al., 1994; Posas et al., 1996) while the

second branch contains the Sho1 transmembrane protein. Signaling emerging from either branch converges at a single MAPKK, Pbs2, which specifically activates the Hog1 MAPK. Activated Hog1 accumulates in the nucleus where it regulates transcription and cell cycle. Although localization is mainly nuclear, there are also Hog1 targets in the cytoplasm.

2.2 Osmosensing mechanisms

The role of osmosensors is to monitor fluctuations in external osmolarity and activate downstream signaling pathways for osmoadaptation. Changes in extracellular osmolarity can be sensed directly or indirectly by tracking changes in physical properties of the cell structure. Both direct and indirect sensing mechanisms seem to be present in the HOG pathway.

2.2.1 The Sln1 branch

The Sln1 osmosensor is a variation of the bacterial two-component osmosensors. In general, two-component sensing mechanisms, as the name indicates, require two proteins: first, a sensor histidine kinase (SHK) with an extracellular input domain, a cytoplasmic HK domain, and a histidine auto-phosphorylation site. Second, a cytosolic response-regulator protein with an output domain and receiver domain. Upon environmental stimulus the sensor protein is auto-phosphorylated at the histidine residue in its HK domain, and then the phosphate group is transferred to an aspartic residue on the receiver (REC) domain, resulting on its activation. This is referred as a His-Asp phosphorelay mechanism (Saito, 2001).

There are more complex phosphorelay mechanisms, in particular the Sln1 branch is a good example of complex two component system (Posas et al., 1996; Saito, 2001). The Sln1 branch requires the primary osmosensor, Sln1 that contains a histidine kinase and a receiver domain, the phosphorelay protein (Ypd1) and the response-regulator (Ssk1) (Maeda et al., 1994; Posas et al., 1996).

Under basal conditions Sln1 is auto-phosphorylated, this phosphate is transferred to aspartic residue, then to Ypd1 (His residue) and finally to Ssk1, hence creating a multistep phosphorelay mechanism (His-Asp-His-Asp). Phosphorylated Ssk1 represses the activity of the MAPKKK (Ssk2, Ssk22) inhibiting signaling through the cascade. At high osmolarity, the Sln1 histidine kinase is inhibited and prevents phosphorelay to Ypd1, this leads to a rapid dephosphorylation of Ssk1 (Horie et al., 2008; Posas and Saito, 1998). Accumulation of unphosphorylated Ssk1 induces Ssk2 and Ssk22 to autophosphorylate and activate, which leads to Hog1 MAPK phosphorylation.

2.2.2 The Sho1 branch

Unlike the Sln1 branch, which is a variation of a well understood system, the Sho1 branch is an alternative sensor mechanism, less understood, that activates Pbs2 (Maeda et al., 1995) through the Ste11 MAPKKK. Sho1 contains four transmembrane domains and a COOH-terminal cytoplasmic region a SRC homology 3 domain (SH3) which serves to bind proline rich motifs such as the N-terminal part of Pbs2 (Maeda et al., 1995).

This branch is regulated by two mucin-like transmembrane sensors Hkr1 and Msb2, which interact with Sho1 in response to stress (de Nadal et al., 2007; Tatebayashi et al., 2007). Upon osmostress Msb2/Hkr1, which are highly glycosilated, interact with Sho1. This association leads to activation of PAK-like kinases Ste20 and Cla4 by inducing their association with the membrane bound G-protein Cdc42 (Lamson et al., 2002). Activated Ste20/Cla4 phosphorylates Ste11 (MAPKKK) which in turn phosphorylates and activates Pbs2. This is brought together through Sho1 (Raitt et al., 2000; van Drogen et al., 2000).

Opy2 is a single-path transmembrane protein with three independent cytoplasmic Ste50 binding sites (CR-A, CR-B and CR-D). Interestingly, Opy2-Ste50 interaction regulates activation of the HOG and filamentous pathway through the A and B domain respectively. This indicates that in nutrient limiting conditions the A domain will be preferential and signaling through the filamentous pathway occurs. In high glucose, Ste50 is bound mainly through the B domain and so, ready to activate the Sho1 branch of the HOG pathway (Yamamoto et al., 2010).

The relevance of the Ste50-Opy2 binding regulation is shown by the fact that three MAPK (Fus3, Kss1 and Hog1) regulate this interaction by direct phosphorylation of Ste50. Taken together, the main function of Opy2 is to serve as a membrane anchor for the Ste11 MAPKKK through the binding to Ste50. This is a clear example of how cells fine tune the integration of extracellular stimuli to the signaling pathways.

The MAPKKK Ste11 is used in three functionally distinct MAPK pathways in yeast (Posas and Saito, 1997) (see Figure 2). For example Ste11 upon hormone stimulation interacts with Ste5, a pheromone-specific component of the mating pathway (Chol et al., 1994; Inouye et al., 1997; Marcus et al., 1994; Printen and Sprague, 1994). In the HOG pathway Pbs2 serves as scaffold protein interacting with Ste11 and Sho1 (Maeda et al., 1995; Posas and Saito, 1997). It is worth noting that in wild type cells, neither the HOG pathway is activated by alpha-factor stimulation nor is the mating pathway activated by osmostress. Thus, indicating the isolation of Ste11 in different complexes.

2.2.3 Specific roles of Sln1 and Sho1 branches

Genetic evidence suggests that the upstream branches of the HOG pathway operate independently of each other; blocking one branch of the pathway still allows rapid Hog1 phosphorylation and cells are apparently fully resistant to high osmolarity conditions.

Even though both branches converge at the Pbs2, they do not have a redundant role. Kinetics of Hog1 phosphorylation using mutants from each branch revealed different behavior of the two branches upon stress (Maeda et al., 1995). Sln1 branch is more sensitive than the Sho1. The Sho1 branch seems to operate in and ON-OFF switch, while the Sln1 has a linear dose response up about 400 mM NaCl. Single cell analysis has confirmed the difference in sensitivity of the two branches. Sln1 branch is capable of fast integration of repeated stimuli, while the Sho1 branch does not (Hersen et al., 2008).

Systems biology analysis of the pathway dynamics together with mathematical modeling showed intrinsic signaling through the HOG signaling activity mediated solely through the Sln1 branch and counteracted by Hog1 kinase activity (Macia et al., 2009). This distinct signaling properties and responsiveness may reflect the different mechanisms of stimulation.

2.2.3 Signaling through the HOG pathway

Any of the MAPKKK can activate Pbs2 by phosphorylation at Ser514 and Thr518. Pbs2 is a cytoplasmic protein and when activated phosphorylates its substrate, the Hog1 MAPK. Dual phosphorylation at the conserved Thr174 and Tyr176 is necessary and sufficient for Hog1 activation and rapid concentration in the nucleus (Brewster et al., 1993; Ferrigno et al., 1998).

Mutations in Thr174 and Tyr176 prevent nuclear accumulation of Hog1 (Ferrigno et al., 1998), although phosphorylation itself as the one observed phosphatase mutants (*ptc1* and *ptc2*) do not accumulate Hog1 in the nucleus (Mattison and Ota, 2000). The precise role of Hog1 catalytic activity for its nuclear translocation remains unclear, since different Hog1 inactive mutants result in either deficient import (D144A) or fail to be exported out of the nucleus (K52R or K52M) (Ferrigno et al., 1998; Westfall and Thorner, 2006).

Nuclear import of Hog1 that lacks a NLS is dependent, at least in part, of Gsp2 and the importin Nmd5 while nuclear export requires the activity of the nuclear export signal (NES) receptor Xpo1/Crm1 (Ferrigno et al., 1998). Nuclear accumulation of Hog1

is necessary to phosphorylate downstream targets such as transcription factors and cell cycle regulators.

2.3 Modulation and feedback control of the HOG pathway

The use of phosphorylation cascades allows rapid and reversible activation of signaling pathways. The HOG pathway is transiently activated reaching its peak 5 minutes after osmostress and Hog1 is dephosphorylated at 30 minutes under mild osmotic shock (0.4M NaCl) (Maeda et al., 1995). Maximum amplitude is achieved at low stress concentrations but duration is extended in a dose-dependent manner (Macia et al., 2009). This negative regulation indicates that signaling is not only regulated by upstream components, and that several negative feedback mechanisms exist to bring the system down to pre-stimulation levels.

Probably, the most important mechanism for successful adaptation is the reestablishment of osmotic balance by internal accumulation of glycerol (Brewster et al., 1993). Hog1 induces transcription of *GPD1*, a key enzyme for glycerol accumulation, but this mechanism is too slow to account for pathway downregulation (Hirayarna et al., 1995). A faster mechanism to regulate glycerol concentration is modulation of glycerol channel (Fps1) and enzymes involved in glycerol synthesis (Bouwman et al., 2011; Dihazi et al., 2004; Mollapour and Piper, 2007; Westfall et al., 2008).

Other mechanisms for proper pathway downregulation include two phosphotyrosine phosphatases (Ptp2 and Ptp3) and the phosphoserine/threonine phosphatases (Ptc1 to Ptc3) that target Thr174 (Warmka et al., 2001). Hog1 induces transcription of Ptp3

(Jacoby et al., 1997) thus, triggering a gene expression dependent negative feedback. However, as it happens with the transcriptional control of glycerol production, transcriptional upregulation of phosphatases cannot account for the downregulation kinetics of the pathway.

Although Ptp2 and Ptp3 are partially redundant, they mainly dephosphorylate Tyr176. Dephosphorylation Ptp2 seems to be more important possibly because Ptp2 mainly colocalizes with phosphorylated Hog1 in the nucleus (Mattison and Ota, 2000). Members of the type 2 Ser/Thr phosphatases family dephosphorylate Thr174. Of these, Pct1-mediated dephosphorylation is the most important for deactivation since *ptc1* mutant cells still display high Hog1 activity after 1 hour of exposure to stress (Warmka et al., 2001). Specific targeting of Ptc1 towards Hog1 is due to the fact that Ptc1 is recruited to the Pbs2-Hog1 complex by Nbp2 (Mapes and Ota, 2003). Negative feedback by phosphorylation of upstream components such as Sho1 or Ste50 has been reported, and Hog1 phosphorylation is diminished in cells expressing phosphomimetic Sho1 (Hao et al., 2007; Hao et al., 2008).

Signaling through the HOG pathway is not “ON” only when cells face osmostress, but there is rather a dynamic basal signaling through the pathway. This internal negative feedback requires Hog1 kinase activity and it only involves the Sln1 branch (Macia et al., 2009). This property is not unique to the HOG pathway since the Fus3 and Kss1 MAPK pathways also display high basal signaling

allowing rapid and sensitive response to environmental changes (Macia et al., 2009).

Although mechanisms that activate the HOG pathway are essential for proper adaptation, negative feedback mechanisms are also important for survival. Sustained activation of Hog1 leads to an increase of reactive oxygen species (ROS) that will finally lead to cell death (Vendrell et al., 2011).

3. PHYSIOLOGICAL ROLES

Hog1 orchestrates the adaptive response to osmostress, which includes modulation of several aspects of cell biology essential for survival, ranging from metabolic adaptation, protein synthesis, cell cycle progression and transcriptional reprogramming (Figure 4).

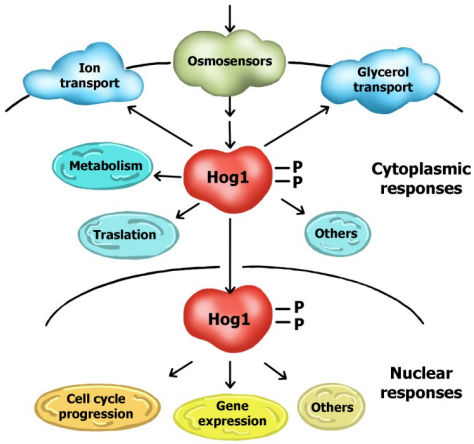

Figure 4. Functions of Hog1 in the osmoadaptive response. Once activated, Hog1 controls cytoplasmic and nuclear adaptive responses. Cytoplasmic responses include control of glycerol transport and ionic fluxes, translation, and regulation of metabolic enzymes. Nuclear functions include reprogramming of gene expression and cell cycle control.

3.1 Metabolic adaptation

Reestablishment of osmotic balance is mainly achieved by the accumulation of compatible osmolytes such as glycerol and threalose (Albertyn et al., 1994; Proft and Struhl, 2004), and occurs within the initial minutes upon stress (Klipp et al., 2005). Increase in glycerol is regulated at different layers: transcriptional control, glycerol transport, and metabolic adjustments (Saito and Posas, 2012).

Hog1 regulates the expression of osmolyte-synthesizing enzymes by targeting the Msn2 and Sko1 transcription factors that are responsible for *GPD1* (glycerolphosphate dehydrogenase 1), *GPP2* (glycerol phosphate phosphatase) and *TPS2* (threalose phosphate phosphatase) (Albertyn et al., 1994; Martinez-Pastor et al., 1996; Ruis and Schüller, 1995). Inability to properly induce these genes impairs growth at high osmolarity (Hohmann, 2002a).

Due to the poor permeability of glycerol through the membrane, concentrations can be regulated through specific channels. Closure of the Fps1 aquaglyceroporin in response to stress occurs very rapidly in a Hog1-independent manner (Tamás et al., 1999), and is a key factor for the initial increase in intracellular glycerol. Albeit, in response to arsenite stress Hog1 phosphorylates Fps1 to prevent arsenite influx (Thorsen et al., 2006).

Rearrangement of the central carbon metabolism changes in response to osmostress redirecting carbon sources to increase the production of glycerol (Saito and Posas, 2012). A good example of this switch in carbon flux is the Hog1-dependent activation of 6-phosphofructo-2-kinase (PF2K) that controls fructose-2,6-

biphosphate a key activator of glycolysis (Dihazi et al., 2004). Overall, metabolic adjustments play an important at the initial phase, while *de novo* synthesis of enzymes seems to be involved in the long term response.

3.2 Protein synthesis

Translation efficiency rapidly decreases in response to stress, probably due to a dramatic drop in amino acid uptake and transcriptional downregulation of ribosomal genes. The role of Hog1 in regulating protein synthesis seems to be at the recovery stage since polysome formation in *hog1* strain is delayed in response stress (Norbeck and Blomberg, 1998; Uesono and Toh-e, 2002). Rck2, a member of the calmodulin kinase family, directly regulates translation elongation by phosphorylating and inhibiting the elongation factor (EF-2) and is targeted by Hog1 (Bilsland-Marchesan et al., 2000; Teige et al., 2001).

Analysis of mRNA associated with polysomes by microarray identified a class of genes whose translation was favored in response to stress ("translation-activated osmostress genes"). These transcripts are recruited to polysomes, without an increase in total mRNA pool, in a Hog1 dependent manner. Preservation of 3' and 5'UTR region seems to be important for translational regulation. Although the mechanism is not yet understood, it is clear that Hog1 preferentially targets certain mRNAs for translation (Warringer et al., 2010).

3.3 Cell cycle regulation

Coordinated progression through the cell cycle is tightly regulated by nutrient availability and stress stimuli. In response to stress, Hog1 mediates cell cycle delays to allow cell adaptation (Figure 5).

The haploid yeast cell cycle is divided into four phases: S (synthesis), M (mitosis), G1 (Gap1), between M and S-phase and G2 (Gap2) between S and next M-phase. Progression through phases of the cell cycle is driven by a single cyclin dependent kinase (CDK) Cdc28. Regulation of Cdc28 is mainly dependent on the synthesis and degradation of cyclins, association with cyclin inhibitors (Sic1, Far1) and phosphorylation/dephosphorylation of Cdc28 by Swe1 kinase and Mih1 (Clotet and Posas, 2007).

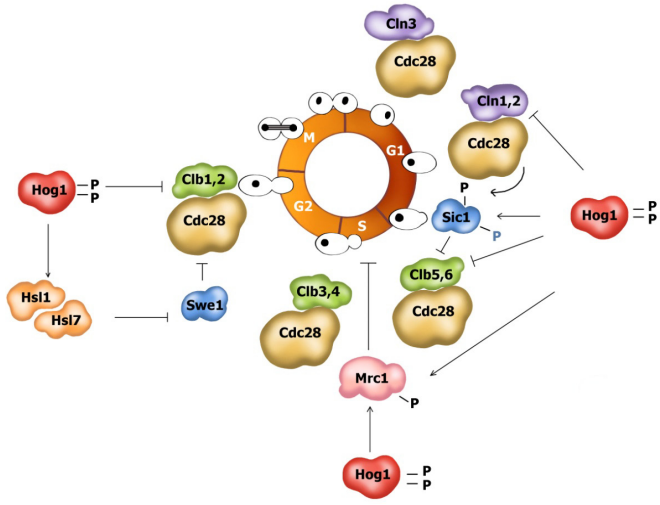

Figure 5. Control of cell cycle progression by Hog1. The CDK Cdc28 associates to phase-specific cyclins (shown around the circle) to regulate passage through the cell cycle (G1 S G2 M). Upon stress, Hog1 modulates progression at several phases of the cell cycle through different mechanisms.

Stress can occur at any given time during the cell cycle. Cells have to be able to modulate cell cycle progression to allow proper adaptation (Alexander et al., 2001) and ensure successful division. Hog1 activation regulates cell cycle progression at several phases of the cell cycle (Duch et al., 2012). G1 phase is transiently delayed by the activation of Hog1 by a dual mechanism that involves direct regulation of the CDK inhibitor Sic1 and downregulation of G1 cyclin expression (Escote et al., 2004; Zapater et al., 2005). Hog1 physically interacts with and phosphorylates Sic1 at Thr173. Unphosphorylated Sic1 is ubiqutinated by Cdc4 ubiquitin ligase and degraded by the proteosome (Nash et al., 2001). Hog1-phosphorylated Sic1 is stabilized and causes a G1/S delay by inhibiting Clb5/Clb6-Cdc28 complexes. Mechanisms that lead to the downregulation of cyclin genes (MBF and SBF) remain unclear (Adrover et al., 2011; Bellí et al., 2001).

When osmostress occurs in cells in S-phase, cells need to prevent collision between replication and transcription machineries in order to prevent transcription-associated recombination (Aguilera, 2002; Aguilera, 2005). To coordinate these two ongoing processes, Hog1 phosphorylates a component of the replication machinery (Mrc1). Phosphorylated Mrc1 by Hog1 delays early and late origin firing and slows down the progression of the replication complex, defining a novel checkpoint pathway that is independent from the DNA-damage (Duch et al., 2013).

Progression through G2 in response to stress is also controlled by a dual mechanism: downregulation of Clb2 expression

as well as the downregulation of Clb2/Cdc28 activity (Alexander et al., 2001; Clotet et al., 2006). As in G1, mechanisms of transcriptional downregulation are not yet understood, but regulation of Clb2/Cdc28 activity is mediated by the Hog1-dependent phosphorylation of Hsl1. Phosphorylation of Hsl1 promotes delocalization of Hsl7 and Swe1 from the bud neck. This results in stabilization of the CDK inhibitor Swe1 and therefore cells arrest in G2.

Hog1 has been linked to exit from mitosis through FEAR (Cdc14 early anaphase release) pathway by promoting activity of Cdc14, although the mechanism underlying this effect remains unclear (Reiser et al., 2006).

3.4 Control of transcription regulation

Exposure to high osmolarity causes a sudden change in water activity and ionic strength, which impacts protein interactions. Most of DNA binding proteins rapidly and transiently disassociate from chromatin upon stress (Proft and Struhl, 2004); restored association of proteins to chromatin occurs faster in cells carrying Hog1.

Genome-wide transcription studies have shown that 5-7% of the protein coding genes show significant changes in their expression levels after mild osmotic shock (e.g. 0.4M NaCl). Osmostress regulated genes are implicated in carbohydrate metabolism, protein biosynthesis, general stress protection and signal transduction. Hog1 plays a key role in transcriptional reprogramming (Causton et al., 2001; Gasch et al., 2000; Pokholok et al., 2006; Posas et al., 2000; Rep et al., 2000). This PhD thesis

intends to give insights into the regulation of transcription by Hog1 and the identification of novel factors that contribute to the gene expression upon stress.

4. Regulation of transcription by hog1

4.1 The transcription cycle in eukaryotes

Transcription is a complex process that generates a mature RNA molecule starting from the DNA template. Like other eukaryotes, yeast has three different RNA Polymerases specialized in the transcription of a subset of RNAs. RNA Polymerase I and RNA Polymerase III are exclusively dedicated to the transcription of infrastructural RNAs, such as ribosomal genes and tRNAs respectively. Transcription of these infrastructural RNAs is required for normal function of the cell (Eddy, 2001). RNA Polymerase II is used to transcribe most protein-coding genes and is the best characterized process of the three types of RNA Polymerases.

RNA Pol II is a large multisubunit enzyme (0.5 MDa) composed by 12 essential proteins from *RPB1* to *RPB12* except for *RPB4* and *RBP7* (Woychik and Young, 1989). A distinct feature of RNA Pol II is the C-terminal domain (CTD) of its largest subunit (Rbp1) formed by multiple repeats of a heptapeptide sequence (YSPTSPS) that is heavily phosphorylated during the transcription cycle. These combinations of phosphorylation/dephosphorylation states of the CTD are important to regulate the different phases of the transcription (Buratowski, 2003; Egloff and Murphy, 2008).

Loading of RNA Pol II onto promoters occurs in its unphosphorylated state through the binding of general transcription factors (GTF). Initiating RNA Pol II is predominantly phosphorylated at Ser 5 and 7 at the 5' region of the gene, while elongating polymerase is phosphorylated at Ser2 and increases towards the 3'-end (Chapman et al., 2007). Recently, phosphorylation of Tyr1 residue has been shown to raise after transcription start site (TSS) and decrease before polyadenylation site. This causes the recruitment of the elongation factor (Spt6) and excludes termination factors from gene bodies (Mayer et al., 2012). Transcription cycle is composed of regulatory stages (Fuda et al., 2009): chromatin opening, PIC (Pre-initiation complex) formation, initiation, elongation, and termination.

1- Chromatin opening: RNA Pol II has to gain access to promoter regions usually covered by nucleosomes that act as natural barriers for transcription. The role of chromatin remodeler and modifying enzymes is to modulate nucleosome positioning and allow access to DNA (Narlikar et al., 2002). In active genes, nucleosome sliding and eviction occurs through the promoters and open reading frames (ORFs) (Cairns, 2009; Jiang and Pugh, 2009), facilitating passage of the RNA Pol II machinery (Workman, 2006). As the gene turns off, nucleosomes reassemble to prevent cryptic transcription (Akey and Luger, 2003).

In yeast, ATP-dependent chromatin remodeling is mediated by protein complexes classified by homology: SWI/SNF family (SWI/SNF and RSC), ISWI family (ISW1 and ISW2), CHD family (Chd1) and the INO80 family (INO80 and SWR1) (Gangaraju and

Bartholomew, 2007; Smith and Peterson, 2004). Recruitment of these complexes to chromatin can be mediated by posttranslational histone modifications and interaction with general specific transcription factors.

Core histones H2A, H2B, H3 and H4 are globular proteins with unstructured N-terminal tails. Posttranslational modifications occur mainly at the tail region but also in the globular domain of histones. Covalent modifications include acetylation, methylation (which can be mono-, di- or tri-methylation), ubiquitylation, sumoylation, ADP-riboslation and phosphorylation (Kouzarides, 2007; Vaquero et al., 2003). There is a dynamic interplay between histone modifications and transcription. Appropriate balance of histone marks can determine the transcriptional state of a gene. Histone modifications are reversible, for example histone acetylation and deacteylation are mediated by histone acetyl transferases (HATs) and histone deacetylases (HDACs) respectively.

The number of residues that can be modified and the enzymes responsible for such modification are currently increasing as detection methods improve, for instance, by the use of new generation mass spectometry. These novel approached have lead to the identification of several new histone marks such as crotonylation (Tan et al., 2011), therefore increasing the complexity and variety of the “histone code”.

2- PIC formation: PIC includes the GTF (TFIID, TFIIB, TFIIE, TFIIF and TFIIH) together with co-factors that can be co-activators and corepressors (Orphanides et al., 1996). Many of them can modulate the activity of GTF, RNA Pol II and induce changes in

chromatin structure. Among the most important activators are the Mediator and Spt-ADA-Gcn5-acetyltransferase complex (SAGA). Mediator is perhaps the most important target of activator proteins in the basic Pol II machinery since it delivers the polymerase and bridges interactions with its activators (Kelleher III et al., 1990; Kim et al., 1994; Malik and Roeder, 2000; Myers and Kornberg, 2000; Yudkovsky et al., 2000). About 13% of yeast promoters contain TATA elements, SAGA tightly regulates the majority of these promoters that usually correspond to stress regulated genes (Hahn and Young, 2011).

3- Transcription initiation begins with the formation of the PIC at the promoter region (closed promoter complex). Gene specific activators recruit general transcription factors that complex with DNA to recruit RNA Pol II and co-activators (e.g. Mediator). RNA Pol II scans the DNA sequence for convenient transcription start sites (TSS). This process involves DNA unwinding and DNA translocation (Hahn and Young, 2011). Isomerization from closed to open promoter complex requires separation of DNA strands around the TSS allowing the entry of single stranded DNA into the active site of RNA Pol II and the formation of the first RNA phosphodiester bond (Orphanides et al., 1996). At this point the complex is unstable and often results in abortive transcription (Shandilya and Roberts, 2012). When the nascent RNA molecule reaches 6 nucleotides long, then the complex is considered to be stable (Cheung and Cramer, 2012).

4- Elongation: Once stable, elongation complex escapes the promoter region and it engages transcript elongation across the

entire coding region. As RNA Pol II moves towards 3' of the gene it may encounter DNA lesions or incorporate noncomplementary nucleotides, then RNA Pol II moves backwards (backtracking) causing transcriptional arrest (Palangat et al., 2005; Petesch and Lis, 2012).

The elongation process is influenced by several factors: The positive elongation factor (P-TEFb), which phosphorylates Ser2 on the CTD facilitates elongation upon pausing (Marshall et al., 1996; Ni et al., 2004). DSIF (DRB-sensitivity-inducing factor) in *S. cerevisiae* is a heterodimeric complex containing Spt4 and Spt5 (Wada et al., 1998). Spt4 antagonizes RNA Pol II pausing effects imposed by the remodeling factor Isw1 (Morillon et al., 2003), while Spt5 is involved in mRNA maturation and surveillance (Sims III et al., 2004). Mutations in DSIF in yeast have been shown to have both positive and negative effects in elongation (Swanson and Winston 1992; Hartzog et al., 1998). Negative elongation factor (NELF) promotes RNA Pol II pausing; this inhibition is reversed by the phosphorylation mediated by P-TEFb.

Stability of components of the initiation and elongation machinery can be modulated posttranslation modifications such as ubiquitylation. For example the ubiquitin protease Ubp3 removes polyubiquitin chains from TBP (Spt15) and RNA Pol II preventing protein degradation (Chew et al., 2010; Kvint et al., 2008).

Chromatin modifications ahead and behind the passage of RNA Pol II are essential to maintain efficient passage of the complex and to prevent cryptic transcription (Akey and Luger, 2003; Workman, 2006). Chromatin remodeling complexes require

an ATP-dependent DNA translocase that allows either changing the location or altering histone-DNA interaction of the nucleosome (Saha et al., 2002). As mentioned above, known chromatin remodelers include RSC, SWI/SNF, ISW, SWR1, INO80 and the related histone chaperones (FACT) (Rando and Winston, 2012).

5- Termination occurs 1kb downstream of the poly(A) site and involves the dissociation of the template DNA and preparation of RNA Pol II for re-initiation. Punctual termination in *S. cerevisiae* is crucial to prevent transcriptional interference with neighboring promoters since intergenic distance between commonly expressed genes is short (Proudfoot et al., 2002). DNA loop conformation juxtaposes promoter-terminator regions and facilitates the maintenance of transcriptional directionality and memory (Lainé et al., 2009; O'Sullivan et al., 2004). In yeast, depletion of Ssu72 (a phosphatase that targets Ser5 on CTD of RNA Pol II) is lethal and its inactivation prevents looping conformation. As a result, massive non-coding RNA transcription emerges from regions that would normally be occluded (Tan-Wong et al., 2012).

4.2 Hog1-dependent control of gene expression

With similar kinetics than Hog1 activation and coinciding with the nuclear localization of the MAPK, a transcriptional burst of osmoresponsive genes occurs within minutes after stress. Transcriptional reprogramming is not essential for the short-term adaptive response but is crucial for long term adaptation since mutants that display impaired transcription are unable to grow under osmostress (de Nadal et al., 2004; Mas et al., 2009; Zapater et al., 2007). Depending on the severity of the stress, up to 80% of the

induced transcripts depend on Hog1 (Capaldi et al., 2008; O'Rourke and Herskowitz, 2004; Posas et al., 2000; Rep et al., 2000). Hog1 coordinates the induction of osmoresponsive genes by controlling the entire process of mRNA biogenesis; transcription initiation, elongation, chromatin remodeling and mRNA export (Figure 6) (de Nadal et al., 2011).

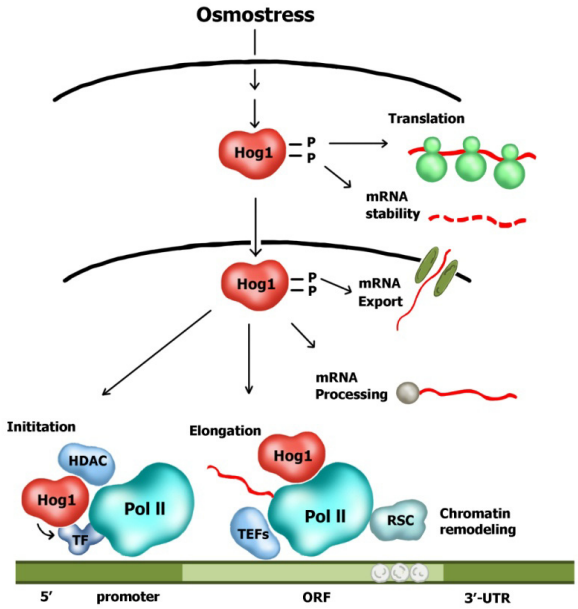

Figure 6. Control of mRNA biogenesis by the Hog1 MAPK. Once activated, Hog1 controls mRNA biogenesis at both nucleus and cytoplasm. Hog1 associates to stress-responsive loci to modulate transcription initiation and elongation.

4.2.1 Transcription initiation

Hog1 directly controls several unrelated transcription factors: Msn2/4, Smp1, Rtg1/Rtg3, Hot1 and Sko1 have been identified so far. This suggests that proper transcription upon stress is not through a single transcription factor, but rather a collaborative

effort of many transcription factors that results in a gene regulatory network (Capaldi et al., 2008; Ni et al., 2009).

The best characterized mechanism by which Hog1 controls transcription initiation is direct phosphorylation of promoter-specific transcription factors. Examples of this are the Smp1 (MEF-2 like activator), Rtg1-3 and Sko1 (ATF/CREB family member). Both are directly phosphorylated and physically interact with Hog1 as determined by co-immunoprecipitation analysis (de Nadal et al., 2003; Nehlin et al., 1992; Proft et al., 2001; Ruiz-Roig et al., 2012; Vincent and Struhl, 1992). The case of Sko1 deserves special attention, since in the absence of stress it is found in a repressor complex (together with Ssn6 and Tup1) at the promoter of stress-inducible genes (Garcia-Gimeno and Struhl, 2000; Pascual-Ahuir et al., 2001). Upon stress, Hog1 phosphorylates Sko1 modifying its association with Ssn6-Tup1 and changing it from a repressor to an activator (Proft et al., 2001). This leads to the recruitment of SAGA and SWI/SNF (Guha et al., 2007; Kobayashi et al., 2008; Proft and Struhl, 2002; Rep et al., 2001).

Hot1 controls the transcription of a small subset of genes: *STL1* a glycerol proton symporter, and the already mentioned *GPD1* and *GPP2*. Interestingly, Hot1 is phosphorylated by Hog1, although this phosphorylation is not crucial for transcription. However, recruitment of Hog1 and Hot1 is interdependent (Alepuz et al., 2003). In addition, recruitment of Hog1 to *CTT1* is mediated through the Msn2/4 transcription factor. Nuclear localization of Hog1 is not sufficient for association to chromatin, but its catalytic activity is indispensable (Alepuz et al., 2001).

Remarkably, nuclear retention of Hog1 depends on the presence downstream transcription factors. Indeed, Hog1 itself binds to chromatin through physical interaction with the transcription factors (Alepuz et al., 2001) that serve as anchoring platform for the MAPK (Alepuz et al., 2001; Pascual-Ahuir et al., 2006; Pokholok et al., 2006; Proft et al., 2006; Reiser et al., 1999; Rep et al., 1999).

The observation that artificial tethering of Hog1 to promoter is sufficient to drive transcription in response to stress together with the tight association of Hog1 and RNA Pol II suggests that Hog1 itself can recruit the basic transcription machinery to promote transcription (Alepuz et al., 2003). Binding of Hog1, stimulates binding of Mediator, SAGA and SWI/SNF. Presence of Mediator is important regardless of the severity of the stimulus, while dependence on SAGA increases proportionally with the degree of stress (Zapater et al., 2007)

Epigenetic marks are also key regulators of transcription initiation. Traditionally histone acetylation has been associated to active transcription. Acetylation is mediated by a histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs) (Bernstein et al., 2000). The histone deaceytlase Rpd3 belongs to Class I HDACs and can be found in two different complexes: a larger Rpd3L and a smaller Rpd3S, regulating transcription of a quite large number of genes (Yang and Seto, 2008). In response to stress, Hog1 binds and targets Rpd3 to osmoresponsive promoters leading to histone deacetylase and

proper recruitment of RNA Pol II, thus allowing transcription to proceed (de Nadal et al., 2004).

4.2.2 Transcription elongation

Recruitment of Hog1 to chromatin is not restricted to promoter regions, chromatin immunoprecipitation shows binding of Hog1 at the coding regions where it travels with elongating RNA Pol II (Pokholok et al., 2006; Proft et al., 2006). Worth noting is that this binding is independent from the promoter region and only depends on the 3'UTR. At this time, the mechanism of Hog1 recruitment at the 3'UTR remains unknown.

Evidence that Hog1 serves as a selective elongation factor for stress-responsive genes comes from uncoupling transcription initiation from elongation by fusing a constitutive promoter to a Hog1-dependent gene. These experiments resulted in Hog1-dependent increase of RNA Pol II. Likewise, interaction between Hog1 and RNA Pol II is stronger upon stress when CTD of RNA Pol II is phosphorylated (Proft et al., 2006).

4.2.3 Chromatin remodeling

Chromatin structure and transcription rate are tightly linked. Nucleosome positioning is dynamic and represents another layer of transcriptional control during the transcription cycle (Cairns, 2009; Jiang and Pugh, 2009).

Osmoresponsive transcription is characterized by a strong induction of gene expression from almost no expression in basal conditions to maximal activation (fold changes range from 5 to 200) in just 10 minutes (Posas et al., 2000). This requires major changes

in chromatin structure that in osmoresponsive genes require the presence of Hog1 (Mas et al., 2009). To achieve efficient nucleosome eviction, Hog1 targets the RSC complex to coding regions by directly binding to it. RSC mutants display impaired induction of osmoresponsive genes and there is no nucleosome eviction despite that Hog1 recruitment level is similar to wild type strain (Mas et al., 2009).

Downregulation of transcription occurs with the same kinetics as Hog1 dephosphorylation and nuclear export. Repositioning of nucleosomes at stress responsive genes is mediated by the INO80 complex (Klopf et al., 2009). Deletion of *ARP8* does not affect kinetics of histone eviction; it rather shows a delay in nucleosome reassembly and a prolonged expression of osmoresponsive genes (Klopf et al., 2009).

4.2.4 mRNA processing, stability and export

Nascent transcripts undergo several processes such as splicing (in the case of intron containing genes), 3' cleavage, polyadenylated, stabilized and exported from the nucleus (Maniatis and Reed, 2002; Orphanides and Reinberg, 2002).

Regulation of osmoresponsive RNAs respect to the pool of total RNAs has been demonstrated at a genome-wide scale (Miller et al., 2011; Molin et al., 2009; Romero-Santacreu et al., 2009). Following osmostress, synthesis and half-life of osmoresponsive mRNAs increase while a broad range of RNAs are being destabilized. In a *hog1* mutant stress induces the formation of P-bodies (Romero-Santacreu et al., 2009).

Interaction with the nuclear pore complex (NPC) facilitates mRNA export and transcriptional induction (Tan-Wong et al., 2009). In response to osmostress, Hog1 phosphorylates components of the inner nuclear basket (Nup1, Nup2 and Nup60), which associate to osmoresponsive promoters depending on Hog1 activity. Consistently, Hog1 is required to maintain *STL1* at the nuclear periphery. Non-phosphorylatable NPC mutants display reduced gene expression and integrity of the NPC is essential to maintain viability under high osmotic conditions (Regot et al., 2013).

4.3 Genome-wide strategies to profile gene expression

Over the last 15-20 years, transcriptional response to a wide variety of stresses has been studied mostly using microarrays. These studies resulted in the identification of a subset of genes that respond indiscriminately of the stress, known as environmental stress response (ESR). ESR represent 5-10% of the annotated coding genes that are upregulated or downregulated in response to DNA damage, amino acid starvation, heat shock, oxidative stress and osmostress (Capaldi et al., 2008; Causton et al., 2001; Gasch et al., 2000). Transcription of ESR has been proposed to protect cells against other unrelated stresses (Berry and Gasch, 2008).

Biochemical studies of the molecular mechanisms by which Hog1 regulates gene expression are usually restricted to a subset of genes. Although single case studies have shown to be very useful, genome-wide approaches provide a broader picture of the role of Hog1 as a master regulator of the massive transcription reprogramming in response to osmostress.

4.3.1 Microarrays and Tiling arrays

The use of microarrays has become a ubiquitous method to interrogate transcriptome of interest under the desired conditions. First reports on global transcription in *S. cerevisiae* in response to osmostress were performed in wild type and *hog1* mutant strains exposed to different strength of osmostress (0,4 M and 0,8 M NaCl) (Posas et al., 2000). Results from this study confirmed the global role of Hog1 in regulating transcription, and suggested a relationship between level of induction and dependence on the HOG pathway.

Further studies complemented and deepened the initial description by using different kinds of osmostress such as sorbitol or KCl and by following the kinetics of transcription over time (Causton et al., 2001; Gasch et al., 2000; O'Rourke and Herskowitz, 2004; Rep et al., 2000). Other systematic analyses have been carried out to characterize the contribution of each transcription factor to the total transcriptional response. Transcription profiling of individual or multiple mutants of transcription factors used by Hog1 shed light into the complexity of transcription factor network (Capaldi et al., 2008). Expression profiling with microarray has been highly efficient in obtaining quantitative measurements of total mRNA (Young, 2000). However, this method cannot fully unravel the complexity of the transcriptome. To overcome these limitations, several new methods with increased sensitivity and coverage have been developed. As a result, the traditional view of transcription has been challenged.

Tiling arrays are high density oligonucleotide arrays in which overlapping probes are placed at a fixed distance over the entire genome independent of annotations. This unbiased strategy of interrogation represents complete non-repetitive tile path over the genome that covers both strands and has allowed the detection of new transcripts and complex transcriptional architectures (Bertone et al., 2004; Kapranov et al., 2003).

The first tiling array was developed for yeast and resulted in an unprecedented resolution of mRNA abundance in exponentially growing cells (David et al., 2006). As much as 85% of the genome is expressed under basal conditions, including a large number of noncoding RNA (ncRNA) transcripts. Further studies using tiling arrays have identified and characterized new noncoding RNA families: CUTs (Cryptic Unstable Transcripts) are noncoding RNA that only appear in mutants of the nuclear exosome (*rrp6*) and SUTs (Stable Unannotated Transcripts) represent a group of stable non-coding RNAs (Xu et al., 2009). More recent studies that also used Tiling arrays reported the appearance of other ncRNA transcripts in the absence of gene looping named STR (Ssu72 Repressed Transcripts) (Tan-Wong et al., 2012), and ncRNAs repressed by the deacetylase Set3 (Kim et al., 2012). RNA-seq technology has also been used for the detection of ncRNAs. As it happens with CUTs, *xrn1* mutant cells show a strong accumulation of antisense transcripts (Xrn1-sensitive Unstable Transcripts) (Van Dijk et al., 2011).In this thesis we have used tiling array to characterize the role of Hog1 in regulating expression coding and noncoding transcriptome/genome in response to osmostress.

Transcription rate in response to osmostress has been measured using two different techniques: genomic run-on (GRO) and dynamic transcription analysis (DTA) (Miller et al., 2011; Romero-Santacreu et al., 2009). DTA analysis uses a non-perturbing RNA labeling using nucleoside analogues, while GRO requires cell permeabilization, a process that stops all physiological processes. Despite the technical differences among protocols there is a high correlation of upregulated genes among both studies and with what had been described with microarray data (Miller et al., 2011). Both studies identified changes in mRNA synthesis and decay in response to osmostress and identified three phases of the stress response: shock, induction and recovery phase (Miller et al., 2011). During the initial shock, synthesis and decay rates globally decrease causing storage of mRNA in P-bodies (Romero-Santacreu et al., 2009). Later, in the induction stage, synthesis rates of osmoresponsive genes increases together with decay rates to ensure high production and removal of stress mRNAs. In the subsequent recovery phase, mRNA decay is restored to prestimulation levels (Miller et al., 2011).

4.3.2 ChIP on chip and ChIP-seq

As mentioned, expression profiling under several osmostress conditions and mutants has been extensively studied at the mRNA level. However, genome-wide positioning of the proteins involved in transcriptional reprogramming is far from complete. The first genome-wide localization of Hog1 was assessed by combining chromatin immunoprecipitation coupled with microarray technology (ChIP on chip). A group of 72 genes showed enrichment

of Hog1 at their promoter and strikingly at 3'UTR region. Binding of Hog1 correlated with Hog1-dependent induction upon osmostress (Proft et al., 2006). Capaldi and colleagues (Capaldi et al., 2008) also used ChIP on chip of Hog1, Sko1 and Hot1 together with microarrays of single, double and triple mutants built a quantitative model of transcriptional regulation. Monitoring nuclear localization of Msn2 transcription factor together with the chromatin association of the MAPK and transcription factors allowed dissecting the mechanisms by which Hog1 controls gene expression. Signal from Hog1 is not linear to downstream targets; it spreads to several transcription factors and recombines at specific promoters resulting in a complex regulatory network.

Interestingly, while quantitative measurements of mRNA levels and localization of some key factors (like Hog1 and transcription factors) has been studied, levels of RNA Pol II could only be inferred by expression patterns or by ChIPs at specific genes. In response to osmostress there is very rapid and transient disassociation of proteins, such as RNA Pol II and transcription factors, from chromatin (Proft and Struhl, 2004) while hundreds of genes are being upregulated (Posas et al., 2000). A recent report using ChIP followed by deep sequencing (ChIP-seq) attempted to determine the role of Hog1 in RNA Pol II distribution genome-wide. Colocalization of Hog1 and RNA Pol II bypasses the global transcription repression (Cook and O'Shea, 2012) confirming with single case studies. Surprisingly, despite the fact that ChIP-seq technology is much sensitive than any of the approaches mentioned above, only a few number of genes (28 as compared to the 70

previously identified) showed enrichment of both Hog1 and RNA Pol II in response to stress (Cook and O'Shea, 2012). In this thesis, we have exploited this method to characterize the genome-wide association of Hog1 and RNA Pol II obtaining much better sensitivity.

4.4 Single cell transcription

Conventional methods used to describe transcription output and signaling properties of the HOG pathway rely in population studies that can only reflect the average response of a population. The extensive knowledge and tools available to study the HOG pathway together with microfluidic devices and modeling approaches represent a new way to challenge the current views of cellular processes involved in osmoadaptation by analyzing behavior of single cells.

Measuring signaling and transcriptional output of the HOG pathway at the single cell level has resulted in the observation that signaling (as measured by nuclear localization of Hog1) increases linearly while transcription is bimodal (determined by the expression of fluorescent reporter) (Pelet et al., 2011). Transcriptional outcome at low osmostress conditions is not continuously distributed while at higher stress concentrations populations respond homogeneously. Chromatin structure seems to be the cause for stochastic expression since histone eviction is partial at concentrations where transcription is bimodal, suggesting that only a fraction of the population undergoes efficient chromatin remodeling. In addition, mutants defective in chromatin remodeling activities (such as *gcn5* or *rsc9ts*) also display incomplete histone

eviction and display bimodal transcription even at concentrations where transcriptional output of wild type cells is uniform. Interestingly, bimodal expression is not specific to osmostress since oxidative and heat stress also display bimodal expression of the transcriptional reporter, suggesting that bimodal behavior may be a general feature of stress-induced genes (Pelet et al., 2011). Based on these observations several models have been developed to identify and predict transcriptional output of osmostress stochastic gene regulation (Neuert et al., 2013; Zechner et al., 2012).

Dynamics of the Msn2 transcription factor localization has been also characterized by single cell studies in response to several environmental stresses. In response to glucose starvation and osmostress, a relatively uniform burst of nuclear Msn2 occurs within the first minutes of stress, while subsequent waves of Msn2 occur under glucose limited conditions. This phenomenon is less frequently observed under osmostress. On the other hand, oxidative stress caused a sustained nuclear retention of Msn2. This different patterns in transcription factor localization can be due to differences in upstream signaling pathways, and can help to explain how different environmental stresses can trigger different dynamics of gene expression (Hao et al., 2013).

OBJECTIVES

Our group is interested in understanding the mechanisms by which eukaryotic cells sense osmostress and how the Hog1/p38 SAPK regulates osmoadaptive responses. Because one of the most important responses controlled by the Hog1 SAPK is the regulation of gene expression, the aims of this thesis project were to give insight into the mechanisms by which Hog1 regulates gene expression.

Specifically, the main objectives of the PhD were:

1- The identification of novel targets for Hog1 in the transcriptional process of transcription of osmoresponsive genes.
2- Characterization of the transcriptome of yeast in response to osmostress using genome-wide analyses.
3- Study of the role of Hog1 in the transcription of osmoresponsive long noncoding RNAs upon stress.

RESULTS AND DISCUSSION

Control of Ubp3 ubiquitin protease activity by the Hog1 SAPK modulates transcription upon osmostress

Carme Solé*, Mariona Nadal-Ribelles*, Claudine Kraft, Matthias Peter, Francesc Posas and Eulàlia de Nadal

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Control of Ubp3 ubiquitin protease activity by the Hog1 SAPK modulates transcription upon osmostress

Carme Solé*, Mariona Nadal-Ribelles*, Claudine Kraft, Matthias Peter, Francesc Posas and Eulàlia de Nadal

*These authors contributed equally to the work

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Ubiquitination is a reversible post-translational modification that regulates many cellular processes. Ubiquitin ligases and ubiquitin-specific proteases (UBPs) determine protein stability and have essential roles in signal transduction cascades (Amerik and Hochstrasser, 2004; Komander et al., 2009; Turcu et al., 2009).

The role of UBPs has been shown to be important to regulate transcription, for example the Msn2 transcription factor is regulated by ubiquitination (Chi et al., 2001). Recently it has been shown that the ubiquitin protease Ubp3 and its cofactor Bre5 act as transcriptional activator by preventing degradation of specific transcription initiation factors (Tbp1/Spt15) (Chew et al., 2010). There are striking evidences for the role of Ubp3 in transcription elongation in response to DNA damage when RNA Pol II is stalled due to DNA lesions. First, Ubp3 copurifies with RNA Pol II and other proteins involved in transcription elongation. Second, the largest subunit of RNA Pol II (Rbp1) can be deubiquitinated by Ubp3 *in vitro*. Third, in response to UV radiation *ubp3* cells show hyperubiqutinated RNA Pol II and results in faster polymerase degradation. Taken together these results suggest that Ubp3 deubiquitnates damage-stalled RNA Pol II to rescue it from

proteosomal degradation and allowing recycling of the polymerase. Accordingly, cells with compromised repair activity show reduced UV sensitivity in the absence of Ubp3 (Kvint et al., 2008).

A genome-wide genetic screen, using the yeast KO collection, was performed in our laboratory to identify genes important for transcription of an osmoresponsive reporter in response to stress. The screening lead to the identification of several proteins such as SAGA, Mediator and Rpd3 as important proteins for gene induction (de Nadal et al., 2004; Zapater et al., 2007). Among the candidates identified, there was the ubiquitin protease Ubp3 which, as mentioned above, had been previously linked to transcription regulation.

Here we studied how the crosstalk between phosphorylation and ubiquitination pathways converge at Ubp3 to determine the extent of transcriptional output of stress responsive genes. Thus, we have defined a novel target for the Hog1 SAPK that represents an extra layer of modulation of osmoresponsive transcription. These data, together with previous studies, suggest that regulation of a deubiquitinase activity is crucial for proper response to environmental stresses.

Ubp3 is required for full transcriptional response of osmoresponsive genes

Despite our knowledge on some of the components involved in stress responsive transcription, the totality of the parts involved and their role remains unclear.

We performed a genetic screen searching by mutants with defective expression of a stress-responsive gene reporter (p*STL1::*LacZ). Scoring expression of β-galactosidase led to the identification of impaired reporter transcription in *ubp3* deficient cells when compared to wild type strain (Figure 1A). The genome of *S. cerevisiae* contains 16 genes that encode for proteins with ubiquitin protease activity. This large number of enzymes suggests that they may have evolved to perform different roles. Accordingly, when we assessed the effect of specific deletions of other ubiquitin proteases only Ubp3, and its cofactor Bre5, had an essential role for the transcription of osmoresponsive genes (Figure 1B,C), suggesting that only Ubp3 activity is required for osmostress induced transcription.

Ubp3 interacts with Hog1 in an osmostress dependent manner.

Hog1 interacts with several of its substrates to control gene expression. We performed *in vivo* coimmunoprecipitation analysis of endogenously tagged Ubp3 (Ubp3-Myc) and Hog1 (Hog1-HA) of cells before and after the addition of NaCl. Ubp3 and Hog1 interacted in a stress-dependent manner regardless of the direction of the immunoprecipitation experiments (Figure 2A,B). The limitation of *in vivo* coimmunoprecipitation studies is that we could not determine if the interaction between the SAPK and Ubp3 was direct or indirect. To tackle this question, we used *in vitro* experiments using recombinant proteins expressed in *Escherichia coli* and determined that Ubp3 can directly interact with Hog1

(Figure S7A). Thus, providing biochemical evidence of the relationship between Ubp3 and Hog1.

We then assessed association of RNA Pol II and Ubp3. Interestingly, interaction of Ubp3 with RNA Pol II is stress and Hog1 independent, suggesting that Ubp3 may be part of the basal transcription machinery required for osmostress gene expression.

Ubp3 associates with osmoresponsive genes upon stress

Association of Hog1 to chromatin and the Hog1 mediated recruitment of the transcriptional machinery is a key step to induce gene expression. We assessed binding of endogenously tagged Ubp3 to stress-responsive genes by chromatin immunoprecipitation experiments (ChIP). In response to stress, Ubp3 associated to stress-responsive promoters and coding regions and this binding was fully dependent on the presence of Hog1 (Figure 3A). These results are in accordance with previously described Hog1 transcriptional targets such as RNA Pol II, SAGA and Mediator (Proft et al., 2006; Zapater et al., 2007).

Ubp3 binding to chromatin coincided with entry of Hog1 to chromatin. This could indicate that Ubp3 is targeting some of the factors being recruited by Hog1, thus protecting the stability of the newly assembled transcription complex. Indeed, when we measured binding of RNA Pol II to *STL1*, we could observe how in *ubp3* cells there was a reduced association at both promoter and coding regions (Figure 3B). Importantly, kinetics of RNA Pol II was the same, but the total amount of RNA Pol II was reduced, which may explain the impairment of gene expression of *ubp3* described above.

Phosphorylation of Ubp3 by the Hog1 SAPK is required for the transcriptional response upon osmostress

Phosphorylation is one of the most studied mechanisms by which MAPKs control transcription. Ubp3 is a 100 KDa protein that contains 12 putative Hog1 phosphorylation sites (S/TP sites). We mutated all phosphorylatable sites to alanine and assessed transcription upon stress. A non-phosphorylatable mutant (Ubp3-12m) displayed the same transcriptional defect than *ubp3*, confirming that phosphorylation of Ubp3 is important for transcription (Figure 4A).

To specifically map the phosphorylation sites targeted by Hog1, we reverted individual mutations and assessed transcription. Cells carrying a mutant with 11 mutations (Ubp3-11m), containing only an intact Ser695, responded to the same extend as the wild type strain, while cells expressing Ubp3-12m showed defective transcription (Figure 4A). Therefore, phosphorylation at Ser695 residue seems to be the site that integrates signaling from the HOG pathway. To further characterize the role of this single phosphosite, we expressed Ubp3 with the single mutation at Ser695A and observed the same transcriptional defect than *ubp3* (Figure 4B). Correspondingly, Upb3^{S695A} strain displayed reduced binding of RNA Pol II upon stress at both promoter and coding regions (Figure 4C). On the other hand, Upb3^{S695A} was still able to associate chromatin albeit to a lesser extent, which can probably be explained by the reduced amount of RNA Pol II present on these promoters (Figure S3).

A catalytically inactive mutant of Ubp3 (Ubp3^{C469A}) renders cells sensitive to the elongation inhibitor 6-azauracil (6-AU) (Kvint et al., 2008). Remarkably, a non-phosphorylatable Upb3^{S695A} did not cause any growth impairment in cells growing with 6-AU. These results indicate that Upb3^{S695A} is fully functional and that this mutation specifically serves to allow proper transcriptional response to stress (Figure S5).

Interestingly, a recent report has shown how Fus3 in response to pheromone stimulation specifically phosphorylates Ubp3 at Ser695 to modulate its activity and thus, limiting cross-talk with the cell wall integrity pathway (Hurst and Dohlman, 2013). Altogether, it may be that phosphorylation of Ser695 is specific to integrate signaling from MAPK pathways.

Ubp3 is phosphorylated upon osmostress in a Hog1-dependent manner

By using *in vitro* kinase assays, in which Hog1 is activated by a hyperactive form of the MAPKK (Pbs2^{EE}) (Proft et al., 2001), we assayed phosphorylation of purified GST-Ubp3 or GST-Ubp3^{S695A} from *E. coli*. Phosphorylation was completely abolished in the mutant compared to wild type Ubp3 (Figure 5A). This confirmed that Hog1, at least *in vitro*, specifically phosphorylates this residue. We performed gel mobility shifts assays to look at migration patterns *in vivo* of wild type Ubp3 or Ubp3^{S695A} in response to stress (Figure 5B). It is worth noting that Ubp3 showed different phosphorylation states already under basal conditions as seen when compared with the alkaline phosphatase treated sample.

Importantly, in response to stress a hyper phosphorylated form of Ubp3 appeared in cells carrying wild type form and completely abolished in the absence of Hog1 or in the single mutant Upb3^{S695A} . Thus, confirming that Ser695 is the specific phosphorylation site for Hog1 upon osmostress.

Ubp3 regulates transcription initiation and elongation in stress-responsive genes

The fact Ubp3 is targeted to promoter and coding regions of osmoresponsive genes suggested that Ubp3 could control transcription initiation and elongation. We performed ChIP experiments against total ubiquitination levels by using tagged ubiquitin (Myc-Ub) (Figure 6A). In response to stress total ubiquitination levels decreased approximately 40% respect to untreated cells, this decrease was fully dependent on the presence of Ubp3. This result indicated that, in response to stress deubiquitination is required for proper transcription and Ubp3 is important to control ubiquitination levels. The Msn2 transcription factor and Tbp1/Spt15 have been shown previously to be controlled by ubiquitination (Chew et al., 2010; Chi et al., 2001). We took advantage of the fact that osmoresponsive genes are controlled by several transcription factors and followed binding of Tbp1 and Msn2 to the *CTT1* (an Msn2 dependent gene) (Figure 6B,C). Recruitment of both proteins was severely impaired in the absence of Ubp3, demonstrating that Ubp3 modulates the association of key elements required for transcription initiation.

To dissect the role of Ubp3 in transcription elongation we uncoupled transcription initiation and elongation by using the LexA-*STL1* system, in which LexA-VP16 drives the expression of the osmoresponsive gene *STL1* (Proft et al., 2006) in cells lacking the endogenous *STL1* locus (Figure 6D). As expected, there was a basal transcription of *STL1* but, more importantly, in response to stress only the strains carrying the wild type Ubp3 could induce transcription while *ubp3* and Ubp3^{S695A} were unable to do so.

Taken together, these results confirmed for the first time that Ubp3 controlled simultaneously transcription initiation and transcription elongation in response to stress.

Ubp3 activity is regulated by phosphorylation by Hog1 SAPK

The requirement of the phosphorylatable residue in Ubp3 to control transcription initiation and elongation, suggested that the catalytic activity of Ubp3 may be controlled by phosphorylation. Interestingly, expression of a catalytically inactive Ubp3 (Ubp3^{C469A}) (Cohen et al., 2003) showed the same transcriptional impairment than *ubp3* cells. Hence, deubiquitinase activity is necessary for induction upon stress (Figure 7A).

To confirm that Hog1 modulates activity of Ubp3, we assayed *in vitro* its capability to deubiquitinate a well known substrate of Ubp3, the largest subunit of RNA Pol II (Rpb1) (Figure 7B, C). Indeed, when Ubp3 was purified from cells that had been exposed to osmostress, we observed an increased deubiquitinase activity when compared to Ubp3 purified from unstressed cells. Moreover, a

non-phosphorylatable allele of Ubp3^{S695A} did not change its activity in response to stress.

Taken together, we were able to demonstrate that Hog1 interacts with and phosphorylates Ubp3 at Ser695. This phosphorylation leads to an increased deubiquitinase activity towards components of the transcription initiation and elongation machinery.

Perspectives

The identification and characterization of the control of the deubiquitinase activity by Hog1 has been the main focus of the article presented here. Although we have provided the molecular mechanism by which Hog1 directly controls the activity of Ubp3 and, highlighted the relevance of the ubiquitination balance to regulate transcription of osmoresponsive genes, there are some questions that remain unsolved.

Hog1 upregulates deubiquitinase activity by direct phosphorylation of Ser695, how this increase on activity is accomplished is unclear. Our results suggest that there is no change in affinity for the co-factor Bre5 or towards its substrates. Further research needs to be done in order to determine how this increase in activity is achieved.

Another key open question is to complete, if necessary, the targets of Ubp3 in the process of transcription since we cannot exclude the possibility that other proteins may be targeted by Ubp3 especially in transcription initiation. A recent report in mammalian cells has shown that USP10, the human homologue of Ubp3, is

required to deubiquitinate the histone variant H2A.Z. USP10 can *in vivo* and *in vitro* deubiquitinate H2A.Z resulting in increased mono-ubiquitination. Interestingly, knock down of USP10 results in impaired transcription of androgen receptor (AR) regulated genes, linking deubiquitination to transcriptional activation (Draker et al., 2011).

It seems clear that RNA Pol II is targeted for deubiquitination under osmostress by Ubp3. The molecular events that set motion of RNA Pol II transcriptional arrest are not fully understood (Kvint et al., 2008) and the obstacles that it could encounter during elongation under osmostress are unknown. Our results suggest that upon osmotic stress, Ubp3 would be recruited to survey the ubiquitination levels of elongating RNA Pol II and prevent undesired degradation. The observation that Ubp3 interacts with other well-known elongation components such as Spt5, TFIIF and Def1 (Kvint et al., 2008) raises the question whether there could be other elongation targets other than RNA Pol II under osmostress.

Recently, it has been shown that activity of Ubp3 is modulated by the Fus3 MAPK in response to pheromone stimuli through the same phosphorylation site than Hog1 (Ser695). Phosphorylation of Ubp3 is important to maintain signaling specificity from the mating pathway by limiting the activation of the Kss1 MAPK (Hurst and Dohlman, 2013). These results pose an interesting scenario in which, phosphorylation at a single residue by SAPKs target and regulate the activity of Ubp3 to alter its function. Thus, modulation of Ubp3 activity functions to integrate signaling

from different pathways in response to changes in the extracellular environment.

Personal contribution to this work: Except for the deubiquitinase assays that were performed by Claudine Kraft (Dr. Matthias Peter lab, ETH), I have been fully involved in the design, execution and discussion of the experiments and results described in this article.

Hog1 bypasses stress-mediated down-regulation of transcription by RNA polymerase II redistribution and chromatin remodeling

Mariona Nadal-Ribelles*, Núria Conde*, Oscar Flores, Juan González-Vallinas, Eduardo Eyras, Modesto Orozco, Eulàlia de Nadal and Francesc Posas

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Hog1 bypasses stress-mediated down-regulation of transcription by RNA polymerase II redistribution and chromatin remodeling

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*These authors contributed equally to this work

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Cells respond to changes in their environment by triggering a massive change in gene expression. Transcription profiling of yeast cells under stress conditions has been studied in detail and the genome can be divided in three types of genes: genes whose expression is induced (upregulated), repressed (downregulated) or non-stress responsive. In response to stress, the HOG-p38 related kinase pathway orchestrates transcription reprogramming through regulation of initiation, elongation and chromatin remodeling (see introduction).

Localization by ChIP on chip of several components had been assessed as well, but resolution of these experiments was limited and probably underestimated the role of Hog1 as suggested by DNA microarray analysis. With the aim of characterizing how the changes in gene expression in response to stress are accomplished, we assessed genome-wide localization of Hog1 and RNA Pol II by ChIP-seq and assessed the changes in chromatin structure by micrococcal nuclease followed by deep sequencing (MNase-seq) in response to osmostress.

A general phenomena that occurs in response to osmostress is a major downregulation of transcription, probably due to the stress-

dependent dissociation of DNA bound proteins from chromatin (Proft and Struhl, 2004). Our results suggest that Hog1 serves to redistribute RNA Pol II to bypass this general downregulation of gene expression caused by osmostress by targeting RNA Pol II machinery to stress-responsive genes. Colocalization of Hog1 and RNA Pol II showed that the stronger binding of both proteins positively correlated with maximal expression. In addition, we have identified non RNA Pol II targets of Hog1 such as tRNAs which are transcribed by RNA Pol III.

Stress induces a rapid recruitment of RNA Pol II at stress-responsive loci

Induction of osmoresponsive genes is very fast, especially when compared to expression of genes that respond to other stresses that tend to be slower but prolonged over time. There are examples in which the same locus is being induced by different stresses that have different mechanisms of regulation and accordingly, kinetics of induction differs among stresses.

DNA microarray profiling of wild type cells lead us to identify 662 osmoresponsive genes (FC>1.75) with an average fold change in gene expression of greater than six (FC≥6), while the rest of the genome had a tendency to downregulate its expression (Figure 1A). Coinciding with this observation, RNA Pol II disassociated from the entire genome in response to stress, except for those genes whose expression was induced. This indicates that there must exist a specialized mechanism to specifically promote

the binding of RNA Pol II to the stress responsive loci (Figure 1B,C).

Genes whose expression increases (FC>2) in response to osmostress can be further classified into Hog1-dependent or Hog1 independent depending on the degree of expression in *hog1* cells when compared to wild type cells (Posas et al., 2000). Analysis of a group of the top 100 Hog1-dependent genes showed higher induction than top 100 Hog1-independent genes (Figure S2). Taken together, these results suggest that the presence of Hog1 is required to achieve maximal transcription efficiency.

Hog1 associates with chromatin of RNA Pol II and Pol III genes

When Hog1 association was monitored by ChIP on chip (Capaldi et al., 2008), the number of genes with Hog1 associated never outnumbered the 70 genes. Although, microarray data suggested that the role of Hog1 probably was underestimated since expression of several hundreds of genes was dependent on the presence of the MAPK. We performed ChIP-seq analysis of Hog1 association and found Hog1 recruited in at least 300 genes, in contrast to previous reports (Cook and O'Shea, 2012).

Hog1 strongly associated to approximately 80% of the Hog1 dependent genes while only at 25% of Hog1 independent (Figure 2A). Notably, association of Hog1 was specific along the genome, since other groups such as non-stress responsive genes did not show Hog1 binding. Hog1-dependent genes showed a biased association of the MAPK towards coding regions while Hog1-independent genes recruitment was mainly at promoters (Figure 2B). Suggesting

that may be the binding pattern of Hog1 can help to make transcription more efficient. These results corroborate, at the genome wide scale, the role of Hog1 as a selective elongation factor.

Interestingly, Hog1 was not restricted to class II genes; we also detected enrichment at RNA Pol III dependent genes and long terminal repeat (LTR). Association of Hog1 to at least 16 tRNA loci and two reference genes (*SCR1* and *RPR1*) showed similar kinetics than the observed for osmoresponsive genes (Figure 2C). To support these results, we experimentally validated the recruitment of Hog1 at *SCR1*, *RPR1* and tF(GAA)D by ChIP in response to 0.4 M NaCl stress (Figure 2D). As Hog1 physically interacts with RNA Pol II, we assessed if Hog1 could interact with RNA Pol III transcriptional machinery. We performed coimmunoprecipitation experiments of Hog1 with a specific subunit of RNA Pol III (Rpc82) (Figure 2D). As it does with RNA Pol II, Hog1 interacted with RNA Pol III in a stress-dependent manner. Therefore, Hog1 targets a new subset of genes whose expression tends to be regulated in response to stress (Willis and Moir, 2007). This poses an interesting question of how Hog1 is recruited to a set of RNAs that are transcribed by a different transcription machinery that has its own transcription factors.

Efficient recruitment of RNA Pol II and maximal gene expression requires Hog1

Overlaying NaCl upregulated genes with Hog1 and RNA Pol II binding allowed to classify osmoresponsive genes depending

expression and the presence of one or both proteins (Figure 3A). We identified a group of upregulated genes with neither Hog1 nor RNA Pol II; expression of these genes correlates with mRNAs that were stabilized upon stress identified in previous studies (Miller et al., 2011; Romero-Santacreu et al., 2009). Genes with increased RNA Pol II but no significant Hog1 binding mostly contained genes classified as Hog1 independent. Remarkably, a large number of genes overlapped with increased expression and binding of both Hog1 and RNA Pol II (Figure 3B). RNA Pol II binding at genes with Hog1 was more pronounced than genes without Hog1 enrichment. Therefore, presence of Hog1 strengthens RNA Pol II binding.

To test whether the presence of Hog1 stimulated stronger Pol II recruitment leading to more effective transcription, we looked at the correlation with these three parameters (Figure 3C). Indeed, we found a positive correlation; stronger binding of Hog1 coincides with robust association of RNA Pol II and higher induction upon stress. In conclusion, efficient upregulation of stress-responsive requires binding of Hog1 which leads to a stronger RNA Pol II recruitment.

It has been reported that cells challenged with low NaCl concentrations (0.1 M NaCl) already show maximal amplitude of Hog1 activation, but the length of Hog1 activation increases proportionally with stress (Macia et al., 2009; Pelet et al., 2011). If presence of Hog1 determines transcriptional output of a gene by improving RNA Pol II recruitment, it was logical to expect that longer residence time of Hog1 causes stronger gene induction. We

exposed cells to increasing amounts of NaCl (0.1 M, 0.2 M and 0.4 M NaCl) and followed Hog1 and RNA Pol II recruitment by ChIP and expression by Northern blot. Interestingly, initial kinetics of RNA Pol II binding was identical at 0.2 M and 0.4 M NaCl, but occupancy was prolonged at 0.4 M NaCl coinciding with a prolonged binding of Hog1. These results suggest that residence time of Hog1 at a target promoter determines the degree of induction. Hence, imposing a "dose-dependent" transcriptional response. It is relevant to note that these results are in accordance with recent data obtained by measuring transcription at the single cell level (Pelet et al., 2011). In response to different concentrations of osmostress Hog1 activation is linear but transcriptional output is bimodal. This transcriptional threshold is strongly influenced by transcription factors and components of chromatin remodelers (SAGA and RSC). In this scenario, stronger binding of Hog1 would be the driving event for efficient binding of the transcriptional machinery and associated complexes causing a massive chromatin remodeling and hence, determining the degree of gene induction.

Hog1 mediates chromatin changes at stress-responsive loci

Previous results from our group have shown that Hog1 stimulates histone eviction (Mas et al., 2009). This change in chromatin structure is an absolute requirement for appropriate gene induction (Mas et al., 2009). We investigated the role of Hog1 in remodeling chromatin at all stress-responsive loci using genome-wide MNase digestion and deep sequencing (MNase-seq) in wild type and *hog1* cells before and after stress. We analyzed and

quantified +1 nucleosome occupancy in three gene clusters: non stress-responsive genes, Hog1 independent and Hog1-dependent. As expected, nucleosome profile around the TSS of non stress-responsive genes did not change in response to stress. Hog1 independent genes showed a moderate nucleosome eviction (around 30%) around the TSS which was similar in wild type and *hog1* cells. In contrast, Hog1-dependent genes displayed massive nucleosome eviction at promoter and coding regions that was completely dependent on the presence of Hog1 (Figure 4A,B).

Hog1 bypasses stress-mediated down-regulation of transcription via RNA polymerase II redistribution and chromatin remodeling

Taken together, our results showed that Hog1 specifically targets genes for induction while the rest of the genome is in a repressive state. Genome-wide distribution of Hog1 positively correlated with binding of RNA Pol II to many more genes than previously described. This tight association of Hog1 and RNA Pol II led us to unravel a dose-dependent transcriptional response determined by the strength and residence time of the SAPK at the target genes. Highly efficient transcription requires Hog1 that facilitates transcription by stimulating RNA Pol II binding and massive chromatin remodeling.

A recent report also used ChIP-seq to determine Hog1, RNA Pol II, Sko1 and Hot1 localization (Cook and O'Shea, 2012). We obtained qualitatively similar results in RNA Pol II dynamics, but quantitatively the number of genes targeted by Hog1 is significantly

different. Cook and colleagues identified 28 genes colocalization of Hog1 and RNA Pol II; these genes were within the top 100 genes of Hog1-RNA Pol II bound genes in our analysis.

Perspectives

The work in the article presented here opens many questions. As discussed above, Hog1 targets RSC to promote nucleosome eviction. We have shown that the presence of Hog1, to the target genes, induces a more pronounced chromatin remodeling but the role of RSC as the major chromatin remodeler of osmostress transcription has not been assessed at the genome-wide level.

Interestingly, epigenetic marks in response to stress challenge all the dogmas of "normal" transcription. It is well known that full induction in response to stress requires a decrease in acetylation, a mark that traditionally has been linked to active transcription (de Nadal et al., 2004). Also, methylation of histone 3 lysine 4 (H3K4) seems to be a repressive mark for stress-induced genes since deletion of the methyltransferase Set1 restores transcription in cells deficient for RSC (*rsc9*^{ts}) (unpublished results). This observation has been recently corroborated by studies that profiled expression of stress regulated genes using histone and deletion mutants (Weiner et al., 2012). Understanding localization and dynamics of chromatin remodelers together with the identification of the histone marks present at a given locus will provide a complete snapshot of the requirements for gene expression upon stress.

The identification of novel regulatory targets for Hog1 poses an interesting question. Hog1 is usually associated to target genes to

move from a repressed to a highly transcribed state. Interestingly, RNA Pol III dissociates from chromatin in response to stress in a Hog1 independent manner. RNA Pol III machinery is composed by specialized transcription factors, and the molecular mechanisms and the physiological relevance by which Hog1 targets RNA Pol III loci remains yet to be understood. Binding at LTRs together with targeting of RNA Pol III suggests that Hog1 may have alternative functions in chromatin other than activating transcription of protein coding genes.

The role of Hog1 in gene induction has been extensively studied by our group and others but Hog1-downregulated genes have received little attention. Genes belonging to the mating pathway or encoding cyclins have been shown to be repressed in response to stress; this repression is completely Hog1 dependent. One interesting example is the G1 cycling *CLB5* whose downregulation correlates with the presence of Hog1 at the promoter region and regulation of cell cycle progression (Adrover et al., 2011). This suggests a specific role for Hog1 as transcriptional repressor and point out the existence of a specific mechanism that has not been characterized.

In general, our results demonstrate that there is a dedicated mechanism controlled by the Hog1 MAPK that specifically targets gene induction under globally repressive condition.

Personal contribution to this work: Except for the initial bioinformatic analyses which were performed by NC, OF, MO, JG and EE, I have been fully involved in the design, execution and discussion of the experiments and results described in this article.

Control of Cdc28 CDK1 by a stress-induced lncRNA

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Control of Cdc28 CDK1 by a stress-induced lncRNA

Mariona Nadal-Ribelles[1,3], Carme Solé[1,3], Zhenyu Xu[2], Lars M. Steinmetz[2], Eulàlia de Nadal[1,4] and Francesc Posas[1,4]

[1]Cell Signaling Unit, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra (UPF), E-08003 Barcelona, Spain
[2]EMBL Heidelberg, Meyerhofstraße 1, 69117 Heidelberg, Germany
[3]These authors contributed equally to this manuscript.
[4]Corresponding authors

Summary

Genomic analysis has revealed the existence of a large number of long non-coding RNAs (lncRNAs) with different functions in a variety of organisms, including yeast. Cells display dramatic changes of gene expression in response to environmental changes. Upon osmostress, hundreds of stress-responsive genes are induced by the stress-activated protein kinase (SAPK) p38/Hog1. Using whole-genome tiling arrays, we found that a novel set of lncRNAs are induced specifically by Hog1 upon stress. One of the genes expressing a stress-induced lncRNAs in antisense orientation is *CDC28,* the CDK1 kinase that controls the cell cycle in yeast. Cdc28 lncRNA mediates the establishment of gene looping and the relocalization of Hog1 and RSC from the 3'UTR to the +1 nucleosome to induce *CDC28* expression. The increase on the levels of Cdc28 results in cells able to re-entry more efficiently cell cycle after stress. Therefore, a stress-induced lncRNA in *CDC28* controls cell cycle progression.

Introduction

The existence of long non-coding RNAs (lncRNAs) is widespread in eukaryotes from yeast to mammals (Guttman and Rinn, 2012; Jacquier, 2009). There are long non-coding transcripts that are expressed in yeast during vegetative growth and meiosis that influence the expression of protein-coding genes, revealing a new layer of transcriptional regulation (Wei et al., 2011; Wu et al., 2012). Recent evidence indicates that lncRNAs might regulate transcription at multiple levels. Sense-oriented lncRNAs of *IMD2* and *URA2* alter expression of central nucleotide synthesis pathway components by transcriptional interference and transcription start site selection (Kuehner and Brow, 2008; Thiebaut et al., 2008). Expression of non-coding transcripts can also trigger changes in chromatin epigenetic state. Induction of 3-end antisense transcription in the *GAL1-10* locus under repressed conditions causes H3 K4me2/me3 through Set1 and H3 K36me3 through Set2 across the coding region. These events trigger a decrease in H3 acetylation by Rpd3S HDAC that leads to glucose repression (Houseley et al., 2008; Kim et al., 2012; Margaritis et al., 2012; Pinskaya et al., 2009; van Werven et al., 2012). Nucleosome occupancy is another level of transcriptional regulation by non-coding transcripts. Some examples of this regulation is the down-regulation of *SER2* by an increase in nucleosome occupancy at *SRG1* or chromatin remodeling at the *PHO5* promoter by a non-coding antisense (Hainer et al., 2011; Uhler et al., 2007). Thus, albeit in a few cases expression of specific lncRNAs alters normal mRNA biogenesis, the general biological relevance and functionality of lncRNAs remains elusive. Remarkably, changes in nutrient availability result in changes in lncRNA expression (Xu et al., 2009; Xu et al., 2011), indicating that environmental insults and signal transduction pathways might affect lncRNA transcription.

Exposure of cells to stress requires immediate and specific cellular responses for proper adaptation and to maximize cell survival to environmental changes (Chen and Thorner, 2007; Gehart et al., 2010; Hohmann et al., 2007). Thus, increases of oxidative stress, a change of external pH, nutrient

supply, temperature changes or osmolarity imbalances require adaptive responses for maximal cell survival (Weake and Workman, 2010; de Nadal et al., 2011). Eukaryotic cells have evolved sophisticated sensing mechanisms and signal transduction systems that induce adaptive responses to stress. Nadal-Ribelles et al., 2012; Pelechano et al.,

Stress-activated protein kinases (SAPKs) are prototypical elements in intracellular signaling networks that serve to respond and adapt to extracellular changes. Exposure of yeast to high osmolarity results in activation of the p38-related Hog1 SAPK (Saito and Posas, 2012), which is essential for the control of two main adaptive responses; the control of cell cycle (Clotet and Posas, 2007; Duch et al., 2012) and gene expression (Martinez-Montanes et al., 2010; de Nadal and Posas, 2010).

Cells activate checkpoint surveillance mechanisms in response to extracellular stimuli to modulate cell cycle progression to permit adaptation to changes in environmental conditions. The Hog1 SAPK acts in multiple stages of the cell cycle by targeting several core components of the cell cycle machinery. For instance, Hog1 controls G1/S transition by the down-regulation of cyclin expression and the stabilization of the Sic1 cyclin dependent kinase inhibitor (CDKi) (Adrover et al., 2011; Escote et al., 2004). Hog1 also modulates other phases of the cell cycle such as G2/M by acting on Hsl1 or S-phase targeting Mrc1 (Duch et al., 2013). Cells unable of delaying cell cycle progression upon osmostress display reduced viability upon those conditions (Duch et al., 2013; Escote et al., 2004), suggesting that in the presence of stress, cells need to delay cell cycle to permit the generation of adaptive responses before progressing into the next phase of the cell cycle.

The p38-related Hog1 SAPK is a key element for reprogramming gene expression in response to osmostress by acting on hundreds of stress-responsive genes. Hog1 is recruited to the osmoresponsive genes by specific transcription factors (Capaldi et al., 2008). Once bound to chromatin, Hog1 serves as a platform to recruit RNA polymerase II (Alepuz et al., 2003; Nadal-Ribelles et al., 2012) and associated transcription factors (de Nadal et al., 2004; Sole et al., 2011; Zapater et al., 2007). Hog1 is present also at the coding regions of stress-responsive genes (Cook and O'Shea, 2012; Nadal-Ribelles et al., 2012; Pelechano et al., 2006; Proft et al., 2006) where it stimu strong chromatin remodeling mediated b interplay of the INO80 and the chromatin remodeling complexes (Klo al., 2009; Mas et al., 2009). The chron dynamics set a threshold for gene indu upon Hog1 activation (Pelet et al., 2011 addition to its impact in gene induction, H controls mRNA stability (Miller et al., 2 Molin et al., 2009; Romero-Santacreu e 2009), mRNA export (Regot et al., 2013) mRNA translation (Warringer et al., 2(Thus, the Hog1 SAPK plays a key role i regulation of mRNA biogenesis controlling several steps in the transcri process (de Nadal et al., 2011; de Nadal Posas, 2010; Martinez-Montanes et al., 2 Weake and Workman, 2010)

Here, we show that, in additio mRNA biogenesis, the Hog1 SAPK associates and controls the induction novel set of lncRNAs induced in respon osmostress. One of the genes expressi stress-induced lncRNAs in antis orientation is *CDC28,* the CDK1 kinase controls the cell cycle in yeast. Inductio the *CDC28* lncRNA permits the increas the levels of Cdc28 allowing cells to re- more efficiently cell cycle after st Therefore, Hog1 directly coordinates regulation of transcription and cell progression by controlling expression stress-induced lncRNA in *CDC28* regulates cell cycle progression.

Results

Hog1 mediates the expression of a nev of stress-inducible lncRNAs

Most gene transcriptome stu performed to define the landscape of g that respond to stress have analyzed expression of coding genes. To cover

expression of the whole-genome upon stress, we monitored genome-wide transcription using strand-specific tiling arrays that enable investigation of the whole transcriptome on both strands, including non-coding RNAs (David et al., 2006). The number of coding genes induced upon stress were 343 at 0.4 M NaCl (15 min) and 294 at 1.2 M NaCl (100 minutes) using a stringent threshold (see methods). The dependence of the Hog1 SAPK in gene expression was assessed in a *hog1* strain and showed that expression of 56% and 84% of the stress-induced genes depended on Hog1 at 0.4 M and 1.2 M NaCl, respectively. Overall, the number of coding genes induced upon osmostress detected in the tiling arrays was similar to previous reports (Capaldi et al., 2008; Gasch et al., 2000; Nadal-Ribelles et al., 2012; Posas et al., 2000).

Remarkably, in addition to coding genes, up to 173 lncRNAs were strongly induced upon treatment with 0.4 M NaCl and up to 216 with 1.2 M NaCl (Fig. 1). Almost a hundred of them were shared between the two stress conditions (Fig. S1). The average length of these stress-induced lncRNAs is of 843 nucleotides (Fig. S1). We assessed the relevance of Hog1 in stress-mediated lncRNA induction and found that 50% and 91% of the genes induced by treatment with 0.4 M and 1.2 M NaCl, respectively, depended on the presence of Hog1 (http://steinmetzlab.embl.de/francescData/arrayProfile/index.html) (Fig. 1B). Some of the stress-inducible lncRNAs overlapped with previously annotated CUTs or SUTs (Wu et al., 2012; Xu et al., 2009). However, most of them are not expressed in the absence of *RRP6*, *TRF4* or *XRN1* and are present only upon stress (Fig. 1C and S1). Thus, Hog1 mediates the expression of a new set of stress-inducible lncRNAs.

Hog1 associates with the promoters of stress-induced lncRNAs, stimulates RNA Pol II recruitment and gene expression

Hog1 associates with chromatin of stress-responsive genes in response to osmostress (Alepuz et al., 2001; Alepuz et al., 2003; Cook and O'Shea, 2012; Pokholok et al., 2006; Proft et al., 2006). Actually, Hog1 is present in at least 80% of the Hog1-induced genes upon stress when genome-wide association of Hog1 was assessed by ChIP and deep-sequencing (Nadal-Ribelles et al., 2012). Then, we analyzed the ChIP-Seq data to assess the association of Hog1 to the 5' regions of the stress-induced lncRNAs. We found that Hog1 is present in ~63% of Hog1-dependent lncRNAs promoters, whereas it is recruited to <30% of Hog1-independent lncRNAs (Fig. 2A). Association of Hog1 to stress-genes serves to stimulate the recruitment of RNA Pol II (Alepuz et al., 2003). Genome-wide association of RNA Pol II experiments showed that RNA Pol II strongly associates with stress-responsive loci in response to osmostress (Cook and O'Shea, 2012; Nadal-Ribelles et al., 2012). Then, we asked whether RNA Pol II was recruited at the stress-induced lncRNAs and found that there was a significant increase on RNA Pol II (2.3 fold increase) at those regions upon stress as it was observed for stress-responsive genes (Nadal-Ribelles et al., 2012). In contrast, RNA Pol II was not recruited at the lncRNA promoters in a *hog1* strain (Fig. 2B). Therefore, Hog1 associates to and stimulates the recruitment of RNA Pol II at the promoters of stress-induced lncRNAs.

Once recruited to stress-responsive genes, Hog1 mediates chromatin remodeling upon stress (Mas et al., 2009; Pelet et al., 2011). Genome-wide MNase (Micrococcal Nuclease) digestion of chromatin and deep sequencing (MNase-Seq) showed that upon osmostress a dramatic change of nucleosome occupancy occurred at both the promoter and ORF regions of stress-responsive genes that was completely abolished in *hog1* cells (Nadal-Ribelles et al., 2012). When we analyzed the chromatin organization at the genes containing Hog1-induced lncRNAs, we found that the regions beyond the transcription termination site (TTS), which corresponded at the 5'UTR regions of the stress-induced lncRNAs, also suffered strong chromatin remodeling upon stress that was dependent on Hog1 (Fig. 2C). In contrast, no

changes in chromatin structure were observed in promoters of lncRNAs that do not respond to stress (Fig. S2). Therefore, as observed in stress-responsive genes, the promoter regions of Hog1-induced lncRNAs also undergo strong chromatin remodeling.

Expression from stress-responsive promoters can be quantitatively measured by fusion to quadruple Venus (qV) fluorescent protein (Pelet et al., 2011; Regot et al., 2013). To characterize one of these lncRNAs promoters further, we fused the 3′ untranslated region (3′UTR) of *CDC28* in both the sense and the antisense orientation to qV-YFP and assessed gene expression by flow-cytometry in wild type and *hog1* strains. Expression of qV-YFP was induced upon stress depending on the presence of Hog1 and only when placed in the antisense orientation (Fig. 2D). Taken together, in response to stress, Hog1 and RNA Pol II are recruited at the 5' regions of the stress-induced lncRNAs that are associated to strong chromatin remodeling and induction of gene expression, which suggest the 5'region of the stress-induced lncRNAs as *bona fide* stress-responsive promoters.

Induction of *CDC28* lncRNA expression promotes the induction of *CDC28* gene expression upon stress

To functionally characterize the role of stress-induced lncRNAs, we asked whether there is a correlation between expression of the sense and antisense induced transcription from the tiling array data. We found a correlation for only a few relevant cases (8 out of 91; Hog1-dependent lncRNAs at 0.4 M NaCl) in which an increase of the antisense in response to stress was associated with an increase in the sense transcript (Fig. 3A). The number of genes that showed correlation increased to 41 out of 91 when more sensitive transcription analyses were considered (Miller et al., 2011; Romero-Santacreu et al., 2009).

One of the genes with a clear correlation of the lncRNA expression and sense expression was *CDC28* (Fig. 3B). *CDC28* encodes the main CDK kinase that drives progression of the cell cycle in yeast (Nasmyth, 1993). Albeit the transcription of *CDC28* was assumed to be constant throughout the cell cycle (Spellman et al., 1998), we found that upon osmostress, there is an increase on *CDC28* expression that correlates positively with induction of an antisense lncRNA in *CDC28*. In a *hog1* strain, neither the lncRNA nor the sense of *CDC28* was induced (Fig. 3B). Systematic insertion analysis at the 3'UTR of *CDC28* showed that insertion of a *KanR* marker 180 nt downstream of the transcription termination site (*lncRNAΔ*) did not alter the normal expression of *CDC28* but abolished expression of the lncRNA. In this strain, the induction of *CDC28* upon osmostress was impaired (Fig. 3B and S3). Thus, the presence of the stress-inducible *CDC28* lncRNA correlates with induction of the *CDC28* gene expression.

Hog1 associates with the 3'UTR and the +1 nucleosome regions in *CDC28* to promote chromatin remodeling

To characterize the mechanism by which Hog1 induces sense and antisense transcription, we monitored Hog1 association with the *CDC28* locus by a high-coverage chromatin immunoprecipitation (ChIP) assay in the presence or absence of stress (0.4 M NaCl). Hog1 associated with the 3′UTR of *CDC28* (the promoter region of the *CDC28* lncRNA) upon stress. Strikingly, Hog1 also associated with a region close to the transcription start site (TSS) (region +41 to +125) corresponding to the *CDC28* +1 nucleosome. By contrast, in cells unable to induce *CDC28* lncRNA, Hog1 association with the TSS was abolished completely but was only reduced at the 3′UTR (Fig. 4A).

The presence of Hog1 at the region corresponding to the +1 nucleosome around the TSS led us to analyze the chromatin architecture before and after stress since Hog1 stimulates chromatin remodeling at specific stress-responsive loci (Mas et al., 2009; Nadal-Ribelles et al., 2012). To assess chromatin structure, we analysed the nucleosome pattern in the *CDC28* gene by

performing Micrococcal Nuclease (MNase) digestion of chromatin from wild type, *hog1* and *CDC28 lncRNAΔ* strains subjected or not to osmostress. We found that the chromatin at the 3′UTR of *CDC28* changed upon osmostress, as expected for a stress-responsive promoter. Strikingly, the region corresponding to the +1 nucleosome at the 5′ region of *CDC28* was also remodeled strongly upon stress (Fig. 4B). The eviction of the +1 nucleosome was not observed in *hog1*-deficient cells or in cells deficient for the *CDC28* lncRNA (Fig. 4B). Therefore, Hog1 association and remodeling at the +1 nucleosome region of *CDC28* occurs in response to osmostress and in the presence of *CDC28* lncRNA.

The RSC chromatin remodeling complex mediates chromatin remodeling at the *CDC28* +1 nucleosome region upon stress

Hog1 stimulates chromatin remodeling at specific stress-responsive loci by recruiting the RSC chromatin remodeler (Mas et al., 2009). To decipher whether the RSC complex was also important to mediate chromatin remodeling in *CDC28* upon stress, we analyzed expression of *CDC28* sense and lncRNA in cells deficient in the RSC complex (*rsc9^{ts}*). Induction of the *CDC28* lncRNA in cells deficient in the RSC complex (*rsc9^{ts}*) under non-permissive temperature (37ºC) was similar to that seen in the wild type (Fig. 4C). Thus, RSC is not necessary for lncRNA expression. In clear contrast, *rsc9^{ts}* mutant cells did not induce *CDC28* expression upon stress. This suggests a key role of RSC for the increase of *CDC28* sense upon stress.

Then, we assessed the recruitment of RSC and Hog1 to *CDC28* upon stress. We performed ChIP assays to follow the binding of Rsc1 of the RSC complex and Hog1 to various regions of *CDC28* before and after the addition of NaCl. We found that RSC associates with 3'UTR and +1 nucleosome regions of *CDC28* in response to stress only in the presence of Hog1 (Fig. 4D). In contrast, association of Hog1 was not altered in the *rsc9^{ts}* strain under non-permissive temperature (Fig. S4A), suggesting that Hog1 mediates the recruitment of RSC to *CDC28* to remodel chromatin upon stress. Correspondingly, chromatin remodeling at the +1 nucleosome, assessed by MNase digestion, was impaired in *rsc9^{ts}* mutants under non-permissive temperature (Fig. 4E). Thus, recruitment of RSC by Hog1 is essential to mediate chromatin reorganization at the +1 nucleosome region and *CDC28* gene induction.

Both the expression of the *CDC28* lncRNA and Hog1 are required for *CDC28* induction upon stress

In the absence of *CDC28* lncRNA expression, the presence of Hog1 at the 3'UTR of *CDC28* is not sufficient to increase *CDC28* expression. Then, we asked whether the expression of the *CDC28* lncRNA alone was sufficient for *CDC28* induction. Thus, we inserted at the endogenous 3'UTR of *CDC28* an inducible *GAL1* promoter in the antisense orientation (*CDC28::pGAL1*). Expression from the *GAL1* promoter is driven by the Gal4-ER-VP16 activator in the presence of β-estradiol (Louvion et al., 1993). Transcription was assessed in control or *CDC28::pGAL1* cells expressing the Gal4-ER-VP16 activator and found that albeit the presence of estradiol strongly induced expression of the *CDC28* lncRNA, this was not sufficient to stimulate sense transcription (Fig. 5A). We then monitored Hog1 recruitment and found that the presence of estradiol did not mediate Hog1 recruitment in *CDC28* (Fig. 5B). Correspondingly, chromatin remodeling at the +1 nucleosome did not occur by the sole induction of the *CDC28* lncRNA from the *GAL1* promoter in the presence of the Gal4-ER-VP16 activator (Fig. 5C). Thus, the induction of the *CDC28* lncRNA alone is not sufficient to mediate chromatin remodeling and *CDC28* gene induction.

Then, we assessed whether the recruitment of Hog1 together with expression from the *GAL1* promoter could induce *CDC28* gene expression. *CDC28::pGAL1* cells were transformed with a control plasmid

or a plasmid carrying the Gal4DBD-Msn2 activator. Msn2 is a transcription factor that mediates the recruitment of Hog1 to Msn2 dependent genes (Alepuz et al., 2001). Tethering Msn2 to the Gal4-binding domain stimulated stress-inducible transcription of the lncRNA in the *CDC28::pGAL1* strain upon stress (Fig. 5D). In this strain, Hog1 was recruited at the *GAL1* promoter in the 3′-UTR region of *CDC28* as well as at the +1 region of *CDC28* (Fig. 5E). We then monitored chromatin remodeling at the +1 region. In contrast to the Gal4-ER-VP16 activator, expression of the *CDC28* lncRNA from the *GAL1* promoter by the Gal4DBD-Msn2 activator there was remodeling of the +1 nucleosome upon stress (Fig 5F). Correspondingly, *CDC28* gene expression was induced upon stress (Fig. 5D). Thus, induction of the *CDC28* lncRNA and the recruitment of Hog1 at the +1 region are required for chromatin remodeling at the 5′ region of *CDC28*, leading to an increase of *CDC28* gene expression.

The establishment of gene looping permits the recruitment of Hog1 at the +1 nucleosome region and induction of *CDC28*

The lack of Hog1 recruitment and remodeling at the 5′ region of *CDC28* in cells deficient in lncRNA induction prompted us to assess whether the presence of Hog1 at this region was mediated by gene looping formation (O'Sullivan et al., 2004; Tan-Wong et al., 2012). Gene loop formation depends on the protein Ssu72 that serves to link promoters with terminators (Ansari and Hampsey, 2005). Since *SSU72* is an essential gene, we used a strain expressing *SSU72* under the *GAL1* promoter that is repressed in the presence of glucose (YPD). Cells were grown in the presence of galactose and then shifted to glucose for 5 hours and subjected to osmostress. Depletion of Ssu72 did not alter induction of the *CDC28* lncRNA but prevented induction of the *CDC28* (Fig. 6A). Then, we assessed the recruitment of Ssu72 at the terminator and promoter regions of *CDC28* by ChIP in a wild type and *hog1* strains. We found that there is a clear increase on Ssu72 in response to osmostress at both the 3UTR and +1 nucleosome regions of *CDC28* depending on Hog1 (Fig. S4B). Thus, the enhanced recruitment of Ssu72 in response to stress does not alter *CDC28* lncRNA expression but it is essential for the increase of *CDC28* expression.

The lack of *CDC28* induction in the absence of Ssu72 suggested that gene looping might mediate the transfer of activities from the 3'UTR to +1 nucleosome regions in *CDC28*. Then, we assessed association of Hog1 in these regions by ChIP in the presence or absence of Ssu72. Association of Hog1 at the 3'UTR region of *CDC28* upon stress was not altered by the absence of Ssu72. In clear contrast, the absence of Ssu72 (YPD) completely abrogated the association of Hog1 at the +1 nucleosome region of *CDC28* (Fig. 6B). We then assessed chromatin remodeling at the +1 nucleosome region in the presence or absence of Ssu72. Correspondingly, chromatin remodeling at the +1 region did not occur upon stress in cells depleted for Ssu72 (YPD) (Fig 6C). Thus, gene looping mediates the recruitment of Hog1 at the 5 region of *CDC28* to induce chromatin remodeling.

To further confirm that the establishment of gene looping between the 3'UTR and promoter regions of *CDC28* occurred upon stress, we applied the 3C assay (see methods) to the *CDC28* gene. We found that there was a clear increase on gene looping formation upon stress between the 3'UTR and the +1 nucleosome regions as detected by the presence of O1-T PCR products. The O1-T PCR product is ligation dependent (D) and it is not detected when an alternative region (O2) was assessed (Fig. 6D). Of note, the increase on gene looping formation upon stress is dependent on Hog1 (Fig. 6D). Therefore, gene looping is critical for the recruitment of Hog1 from 3'UTR to the +1 nucleosome region of *CDC28* to promote chromatin remodeling and induce *CDC28* gene expression.

Stress-induced *CDC28* lncRNA results in an increase of Cdc28 that permits cells to re-enter the cell cycle more efficiently in response to stress

We asked whether an increase of *CDC28* mRNA results in an increase of Cdc28 protein production upon stress. Endogenous Cdc28 protein was immunoprecipitated after labeling with [^{35}S]methionine (see Methods). Synthesis of Cdc28 protein in the wild type increased ~twofold in response to stress, whereas no increase was observed in a *CDC28*-lncRNA deficient strain (Fig. 7A). Thus, stress-induced Cdc28 lncRNA expression leads to increased levels of Cdc28 kinase.

Hog1 mediates an immediate but transient cell cycle delay by different mechanisms to permit stress-adaptation in response to osmostress (Clotet and Posas, 2007; Duch et al., 2012). We observed that the increase in Cdc28 levels occurred when cells were already recovering from the initial arrest caused by stress. Thus, we hypothesize that this increase of Cdc28 can serve to accelerate cell cycle re-entry after stress. To test this hypothesis, we assessed the exit from the arrest caused by osmostress in a phase of cell cycle in which the levels of Cdc28 are maximal. Thus, we synchronize cells using a temperature sensitive allele of *cdc15* (*cdc15^{ts}*) (see Methods). Cells deficient in *CDC28* lncRNA were able to arrest and exit cell cycle from *cdc15^{ts}* synchronization as efficiently as the wild type in the absence of stress. In contrast, cells deficient in *CDC28* lncRNA production delayed cell cycle re-entry upon stress compared to the wild type (Fig. 7B). Thus, stress-induced *CDC28* lncRNA results in an increase of Cdc28 that permits cells to re-enter the cell cycle more efficiently in response to stress.

Discussion

Stress-activated protein kinases regulate gene expression to maximize cellular adaptation to environmental stress (de Nadal et al., 2011; Weake and Workman, 2010). In yeast, activation of the prototypical SAPK Hog1 leads to major changes in the gene expression pattern of the cell by acting on several mechanisms that control mRNA biogenesis (de Nadal and Posas, 2010; Martinez-Montanes et al., 2010; Weake and Workman, 2010). Here, we provide evidence that, in addition to control expression of coding genes, Hog1 also induces a dedicated set of stress-responsive lncRNAs. Thus, we define an intrinsic regulation of lncRNAs by signaling pathways in response to environmental insults.

A specific set of Hog1-dependent lncRNAs is induced in response to osmostress

Expression of lncRNAs is widely observed, from yeast to human, but its biological relevance is not well understood (Wei et al., 2011; Wu et al., 2012). In response to osmostress, about 200 lncRNAs are rapidly induced. Of those, approximately 90 are present in both stress conditions (0.4 and 1.2 M NaCl), indicating that there might be a general stress response of lncRNAs as it happens with protein-coding stress responsive genes. The induction of these stress-induced lncRNAs depends mostly on the presence of Hog1. Correspondingly, Hog1 associates to the promoters of lncRNAs upon stress and stimulates RNA Pol II recruitment and chromatin remodeling as it happens in osmoresponsive genes. Actually, the fusion of the promoter of one of these lncRNAs (*CDC28*) to a GFP reporter showed that expression occurred only upon stress, in antisense orientation and depending on Hog1. This observation is remarkable, since most of the described antisense transcripts have been described to arise from bidirectional promoters (Tan-Wong et al., 2012; Xu et al., 2009). The fact that this terminator can function as a heterologous promoter suggests that there must exist a different transcriptional unit recruited to this region that is independent of the neighboring regions. Accordingly, a recent study by ChIP-exo precisely positioned distinct transcriptional machineries at bidirectional promoters, supporting the idea of unique transcription units (Rhee and Pugh, 2012).

Most of the stress-induced lncRNAs only appear to be transcribed in response to osmostress. Except for SUTs, which are stable transcripts, the rest of lncRNA are only detectable in strains deleted for components of the nuclear o cytosolic exosome (CUTs and XUTs) (Xu et al., 2009), when gene looping is impaired (Ssu72-restricted transcripts) or by deletion of the histone deacetylase *SET3* (Kim et al., 2012; Tan-Wong et al., 2012). Expression analysis of some representative Hog1-dependent lncRNAs showed that they were not expressed under basal conditions in the absence of *RRP6*, *XRN1* or *TRF4*. Transcription was only induced upon stress, but stability was altered in these mutants. Thus, Hog1 regulates the transcription of a novel class of stress-induced lncRNAs whose induction might have relevant implications for proper cellular adaptation to environmental insults.

The *CDC28* lncRNA and Hog1 induce chromatin remodeling and *CDC28* expression via gene looping

To unravel the biological function of the stress-induced lncRNAs, we investigated whether there was correlation between the expression of sense and lncRNAs. Overall, there was no clear correlation except for some genes that showed either negative or positive correlation. Remarkably, one of the most striking observations was the positive correlation between the induction of *CDC28* and a lncRNA in *CDC28* expressed in antisense orientation. *CDC28* gene induction was completely dependent on the presence of Hog1 since in *hog1* cells there was neither induction of the lncRNA nor the *CDC28* sense. This posed the question on how the SAPKs and the induction of a lncRNA lead to gene induction. Remarkably, Hog1 associated at the 3'region of *CDC28*, which corresponds to the promoter region of the *CDC28* lncRNA, and at a region surrounding the +1 nucleosome of *CDC28*. Transcription of *CDC28* is not controlled by any of the transcription factors targeted by Hog1, thus opening the possibility that Hog1 uses the 3'UTR region to mediate its association to the +1 nucleosome region to promote gene expression. This interesting Hog1-binding pattern resembles some of the features of osmoresponsive genes, in which Hog1 recruitment at the ORFs depends on the 3'UTR region (Proft et al., 2006).

Targeting of the *CDC28* 3'UTR region with a marker abolishes lncRNA expression. Nevertheless those cells still recruit Hog1 at the 3'UTR but not at the +1 nucleosome region in *CDC28.* Remarkably, these cells neither induce chromatin remodeling nor *CDC28* gene induction, suggesting that the lncRNA induction is required for sense induction. On the other hand, when *CDC28* lncRNA was induced by a heterologous activator that does not promote recruitment of Hog1 at the 3'UTR, the SAPK does not bind at the +1 nucleosome region and cells can neither induce chromatin remodeling nor gene expression. Therefore, the combination of the induction of *CDC28* lncRNA transcription and the recruitment of Hog1 is necessary for gene induction. Correspondingly, the combination of artificial thethering of Hog1 to a strain containing the *GAL1* at the *CDC28* 3'UTR together with the expression of the lncRNA from the *GAL1* promoter allow chromatin remodeling and induction of *CDC28* sense expression. RSC mediates chromatin remodeling at stress-responsive loci (Mas et al., 2009). The depletion of RSC does not alter the induction of *CDC28* lncRNA, however it prevents chromatin remodeling at the +1 nucleosome region and gene induction. Thus, the targeting of RSC by Hog1 at the +1 nucleosome region is required for gene induction.

Unlike osmoresponsive genes in which Hog1 travels with elongating polymerase (Proft et al., 2006), Hog1-binding pattern at the *CDC28* loci suggested that Hog1 could reach 5' end of the gene without traveling through the coding region. In yeast, gene looping has been shown to juxtapose promoter-terminator regions during active transcription (O'Sullivan et al., 2004). Indeed, osmostress stimulates gene looping in

CDC28 that is dependent on Hog1. Generation of looping can be prevented by impairing the function of an essential gene *SSU72* (Ansari and Hampsey, 2005). Depletion of Ssu72 did not alter induction of *CDC28* lncRNA but completely abolished *CDC28* gene induction most likely because Hog1 cannot be transferred from the 3'UTR to the +1 nucleosome position.

All together suggest the following tentative model for the induction of *CDC28* by Hog1 (Fig. S4C). In response to osmostress Hog1 associates at the 3'UTR region of *CDC28* and induces lncRNA transcription. Once antisense transcription is induced, gene looping is established and Hog1 is transferred to the +1 nucleosome region in *CDC28*. The recruitment of Hog1 serves to target the RSC chromatin remodeler which remodels the +1 region thus permitting an increase of the transcription of the *CDC28* gene. Taken together, the regulation of *CDC28* transcription by the induction of a stress-responsive lncRNA provides a novel paradigm by which a lncRNA mediates gene induction through changes of chromatin architecture.

Induction of the *CDC28* lncRNA controls cell cycle re-entry upon stress

Which is the biological relevance of the expression for most lncRNAs remains unclear. Here, we have shown that *CDC28* expression is regulated by a lncRNA. The *CDC28* gene encodes the main CDK kinase (CDK1) that drives progression of the cell cycle in yeast (Nasmyth, 1993). Cdc28 is regulated by several mechanisms, including cyclin association and CDK inhibitors (Bloom and Cross, 2007). However, the increase in transcription of *CDC28* observed upon stress was unexpected since transcription of *CDC28* was assumed to be constant (Spellman et al., 1998). The increase on *CDC28* transcription resulted in an increase of *de novo* synthesis of Cdc28 indicating that, indeed, the increase in mRNA leads to an increase on the cellular pool of Cdc28. In response to osmostress, Hog1 mediates an rapid but transient arrest of cell cycle progression to allow adaptation through several mechanisms (Clotet and Posas, 2007; Duch et al., 2012; Saito and Posas, 2012). One of the mechanisms under the control of Hog1 consists in the down-regulation of Cdc28 activity, which seems to be contradictory with an increase of Cdc28 protein. Nevertheless, the increase on Cdc28 protein levels occurred when cells started to recover from stress; thus we postulated that this increase in Cdc28 protein level should have an effect at the recovery phase. Indeed, cells deficient in *CDC28* lncRNA arrested upon stress similar to wild type upon stress but re-entered cell cycle less efficiently, suggesting that the increase on Cdc28 permits a faster recovery of the cell cycle delay caused by stress. Therefore, Hog1 is able to induce a cell cycle delay and promote later on recovery from it by controlling immediate targets by direct phosphorylation and late responses by transcriptional modulation, thus achieving a different temporal outcome.

In summary, we present here a new mechanism of Cdc28 regulation through a stress-inducible lncRNA production that is able to alter cell cycle progression in response to environmental challenges. Cdc28 regulation provides a novel paradigm by which a lncRNA together with a SAPK mediate gene induction through changes of chromatin architecture. Moreover, this study provides insights into how lncRNAs might affect the regulation of gene expression through chromatin changes in eukaryotic cells.

Experimental Procedures

Website

The following link http://steinmetzlab.embl.de/francescData/arrayProfile/index.html directs to an interface to visualize array expression data. Raw array data are available from ArrayExpress under accession number E-MTAB-1686.

Yeast strains and plasmids

Saccharomyces cerevisiae strain BY4741 (MATa *his3*-Δ1 *leu2*-Δ0 *met15*-Δ0 *ura3*-Δ0) and its derivatives YGM61 (*HOG1::KanMX4*) (Mas et al., 2009), YCS36 (*RRP6::KanMX4*), YCS50 (*TRF4::KanMX4*), YCS54 (*XRN1::KanMX4*) were obtained by long flanking-homology PCR-based gene disruption. lncRNA

disruptions were obtained with the same strategy than deletion strains, inserting the *KanMX4* marker at 20, 100, 180, 300 or 400 bp from *CDC28* STOP codon (YCS73, 76, 79, 103, or 105, respectively). Chromosomally integrated HA-tagged strains of genomic ORFs were done by PCR-based strategy: YMZ33 (*HOG1-6HA::HIS3*) (Zapater et al., 2007), YMN38 (*lncRNA::KanMX4 HOG1-6HA::HIS3*), YMN117 (*CDC28-pGAL1::KanMX4 pADH1-GAL4DBD-hER-VP16::URA3 HOG1-6HA::HIS3*). YMN149 (*NAT::pGAL1-3HA-SSU72*), YMN151 (*NAT::pGAL1-3HA-SSU72 HOG1::KanMX4*) and YMN165 (*NAT::pGAL1-SSU72 HOG1-6HA::HIS3*) were obtained by replacing endogenous promoter of *SSU72* (pYMN-24 and 22, respectively from PCR-TOOLBOX). PHO85-TAP strain from the yeast TAP collection (Open Biosystems) was transformed with lncRNA disruption cassette (YCS266: *PHO85-TAP::HIS3 CDC28-term(180bp)-KanMX4 (lncRNA*Δ). YGM68 (*RSC1-6HA::HIS*), YGM79 (*RSC1-6HA::HIS3 HOG1::KanMX4*) and YGM248 (*rsc9*^{ts} *HOG1-6HA::HIS3*) were derivatives from W303-1A (Mas et al., 2009). Inducible expression of lncRNA was obtained in two steps: first, by PCR-based strategy integrating *GAL1* promoter (pYMN-22 from PCR-TOOLBOX (Janke et al., 2004) at 96 bp from *CDC*28 STOP codon (YMN90, *CDC28::pGAL1);* second, the plasmid ADGEV that induces the GAL4DBD-hER-VP16 (Louvion et al., 1993) was linearized with *NdeI* and integrated to a wild-type (YMN94) or *CDC28::pGAL1* to regulate *GAL* promoters with 100nM β-estradiol (Sigma-Aldrich, St Louis, MO) in YPD (YMN95, *CDC28::pGAL1 Gal4DBD-hER-VP16*). Termosensible derivatives from W303-1A were *rsc9*^{ts} (gift from Gustav Ammerer, Vienna), *cdc15*^{ts} (*cdc15-2*) strain (gift from Jeremy Thorner, Berkeley) and *cdc15*^{ts} *lncRNA*Δ (YCS192). YCPLac111 empty vector or p*ADH1*-Msn2ZFΔ-Gal4DBD (gift from Christoph Schüller, Vienna); ADGEV construct encoding the hybrid transcription factor "GEV" (Gal4DBD-hER-VP16 fusion protein) under the control of the *ADH1* promoter (Louvion et al., 1993); *CDC28* sense or lncRNA expression reporter were obtained by cloning 300 bp immediately downstream of *CDC28* STOP codon into a pRS305-quadrupleVenus (YFP) reporter plasmid (Pelet et al., 2011). These regions were amplified from genomic DNA in "terminator sense" or "promoter lncRNA" orientation. Plasmids were linearized with *KasI* and integrated in wild type (YMN50 and YMN53) and *hog1*Δ (YMN56 and YMN54).

Tiling array

Wild type (BY4741) and *hog*Δ cells were grown to mid-log phase (A_{660} 0.6-0.8) in YPD before being subjected (or not) to mild osmostress (0.4M NaCl for 15 minutes) or hyper osmostress (1.2M NaCl for 100 minutes). Hybridization of tiling array was performed as described (David et al., 2006).

Definition of stress-induced lncRNAs

Stress-induced lncRNAs were defined as up-regulated with a minimum of two fold change in response to stress (at the indicated osmolarity) in the wild type (BY4741) strain. Hog1 dependence was determined by the percentage of expression in a *hog1*Δ mutant respect to the wild type strain. Supplemental Table S1 provides the entire list of the osmoresponsive lncRNA and their Hog1 dependence.

ChIP-seq and Mnase-seq

Wild type and *hog1*Δ mutant *S. cerevisiae* strains were grown to mid-log phase and exposed to 5 minutes of osmostress (0.4 M NaCl) for Hog1 immunoprecipitation (anti-HA 12CA5) and 10 minutes for RNA Pol II immunoprecipitation (anti-RNA Pol II 8WG16, Covance) and nucleosome positioning. ChIP and MNase protocols were performed and purified DNA was sequenced as described. Enrichment of Hog1 and RNA Pol II was done by running the Pyicos enrichment protocol comparing untreated to treated samples (Nadal-Ribelles et al., 2012).

MNase nucleosome mapping

Spheroplasts and digestion with MNase were done essentially as described but with some modifications (Mas et al., 2009). Indicated strains were grown in specific medium, then subjected (or not) to osmostress (0.4M NaCl for 10 minutes) or treated with β-estradiol (100nM, 10 minutes). For the analysis of *CDC28*, DNA was used in a real-time PCR with specific oligonucleotides included in Supplementary Experimental Procedures.

ChIP assays

Chromatin immunoprecipitation was done as described previously (Zapater et al., 2007). Briefly, indicated yeast cultures were grown to mid-log phase before were exposed (or not) to osmotic stress (0.4M NaCl, 5 minutes) or treated with β-estradiol (100nM, 5 minutes). Real-time PCR of the indicated regions were performed using specific oligonucleotides included in Supplementary Experimental Procedures.

Northern Blot

Yeast cultures were grown to mid-log phase in the indicated medium and subjected to osmostress (0.4M NaCl) or treated with β-estradiol (100nM) for the length of time indicated. Total RNA was extracted, resolved in 1% agarose gels and transferred to Nylon membranes. Sense and lncRNA riboprobes were prepared using *MAXIscript® SP6 In Vitro Transcription Kit* (Ambion). As loading control, expression of *ENO1* was probed using radiolabeled PCR fragments (Rediprime II DNA Labeling System, GE Healthcare Life Sciences). Primers used to produce PCR products are described in Supplementary Experimental Procedures.

3C analysis

Cells were grown to mid-log phase before being subjected or not to osmostress (10 min, 0.4 M NaCl). 3C analysis was performed as described previously with minor modifications indicated in Supplementary Experimental Procedures.

Metabolic labeling
Briefly, indicated PHO85-TAP strains were grown in YPD and shifted to MET media for 2 hours before being stressed. Mixture of ^{35}S-methionine and 0.4M NaCl were added simultaneously for the indicated times. Fold induction is represented respect to 10 minutes of treatment.

Flow cytometry
Flow cytometry experiments were performed as described (Pelet et al., 2011). Cells were grown in YPD before being stressed for 45 minutes at 0.4M NaCl. To study cell cycle progression, overnight cultures were diluted to OD_{660} of 0.3 and grown for 3 hours at 25ºC in YPD. *cdc15^{ts}* (*cdc15-2)* cells were synchronized at 37ºC (incubated for 2 hours) and released at 25ºC to allow cell cycle progression, in YPD supplemented or not with 0.4M NaCl.

SUPPLEMENTAL INFORMATION
Detailed experimental procedures can be found in the Supplemental Experimental Procedures.

ACKNOWLEDGMENTS
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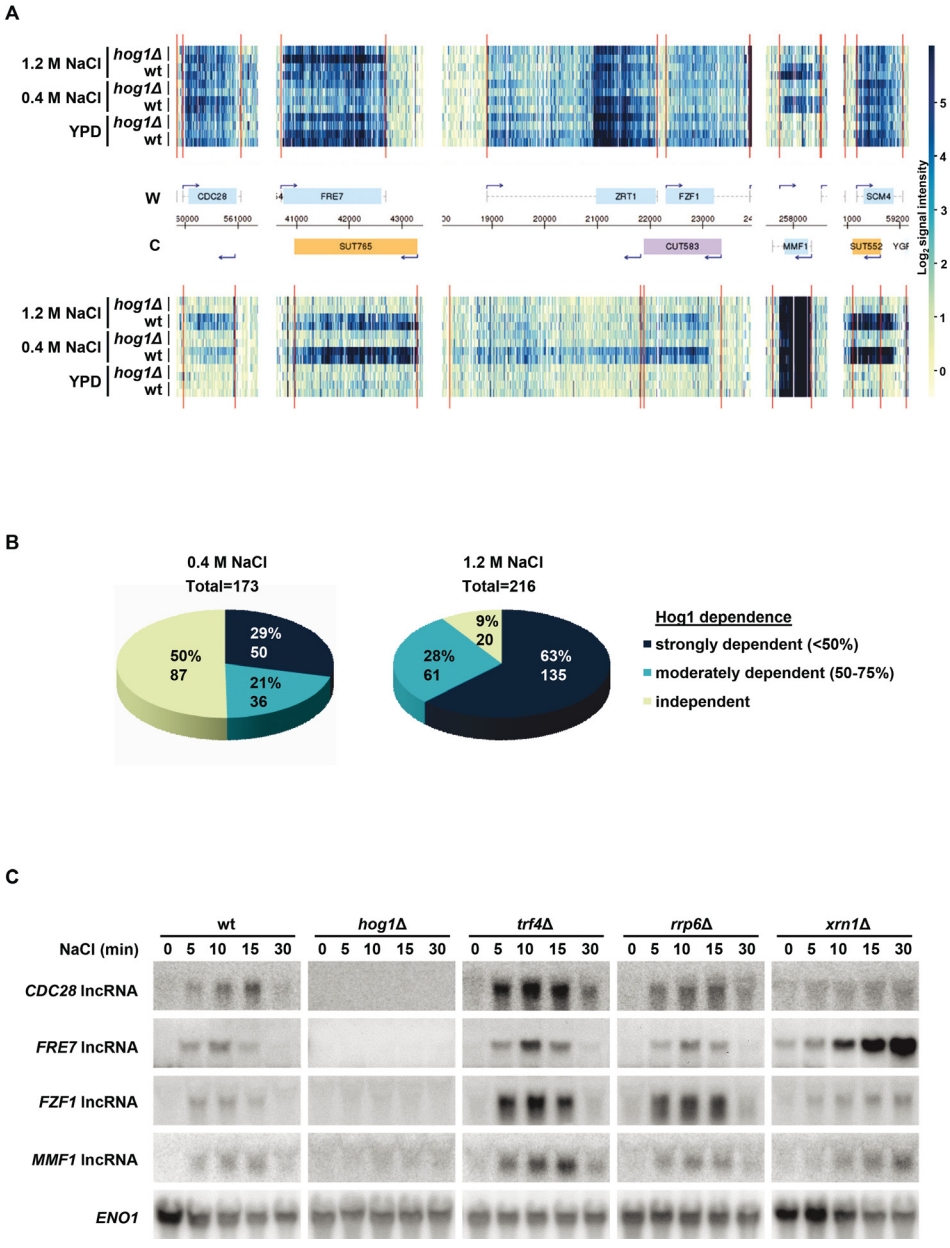
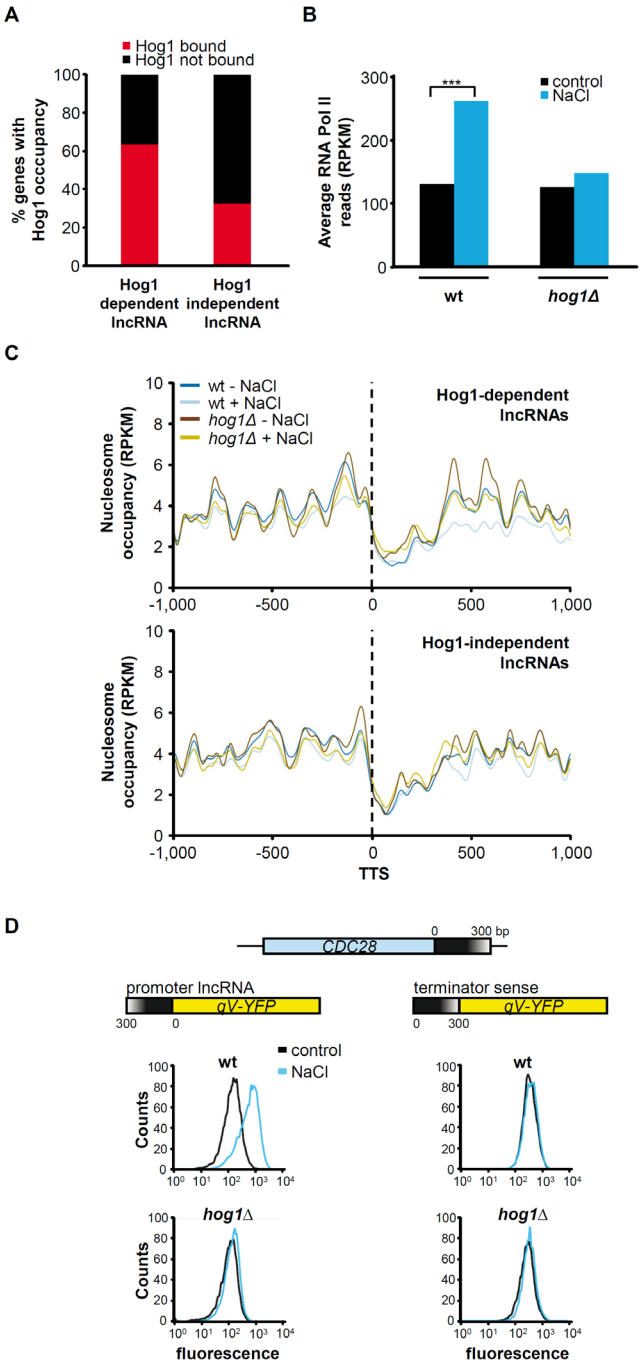
Figure 1

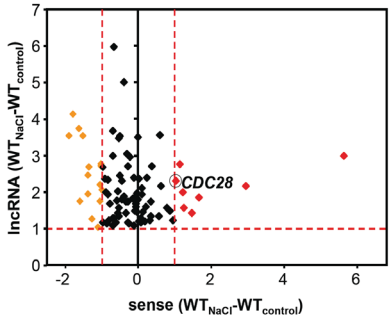


Nadal-Ribelles, Solé *et al.*
Figure 2



Nadal-Ribelles, Solé *et al*.
Figure 3

A



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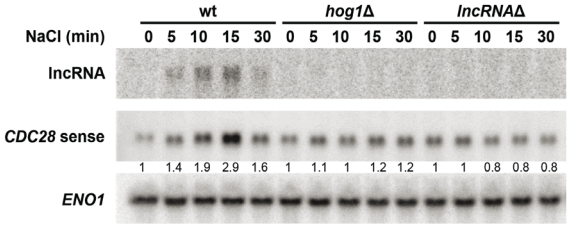

Nadal-Ribelles, Solé *et al*.
Figure 4

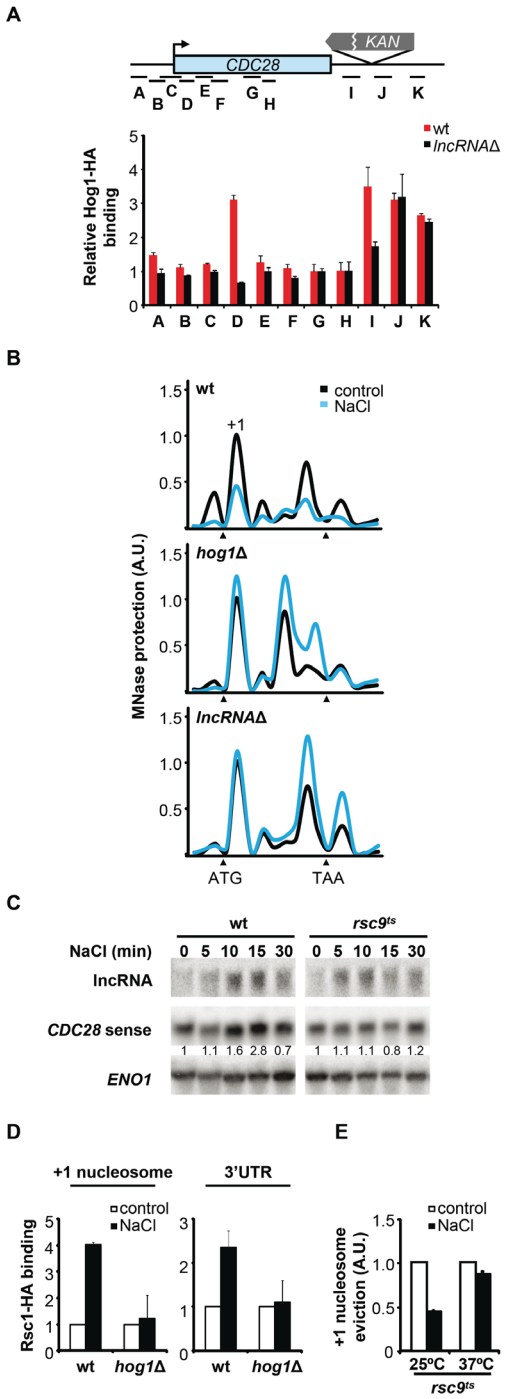

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Figure 5

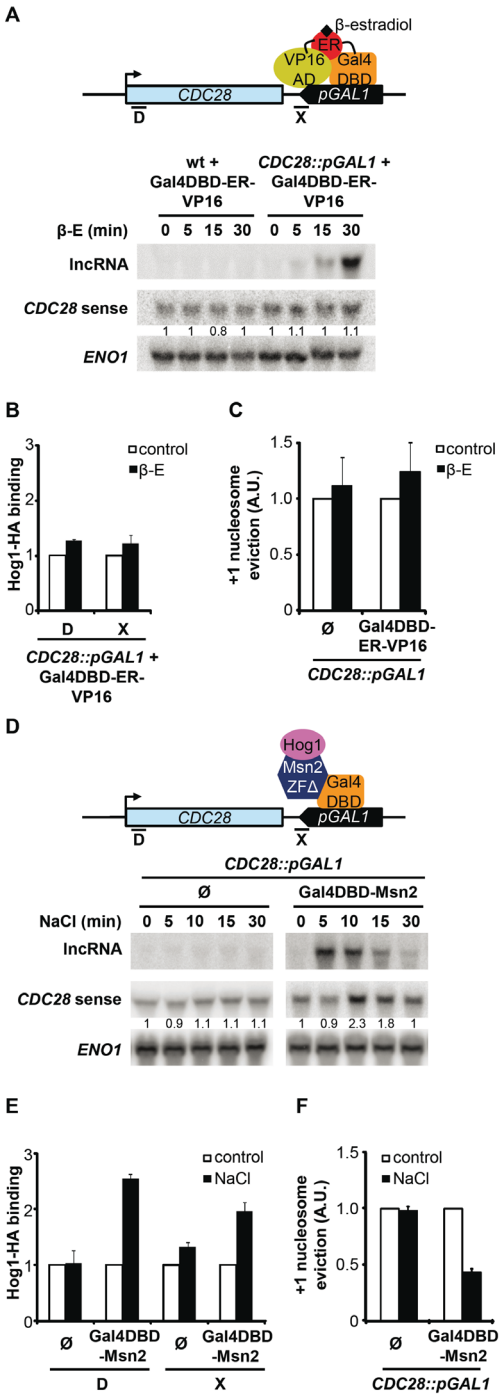

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Figure 6

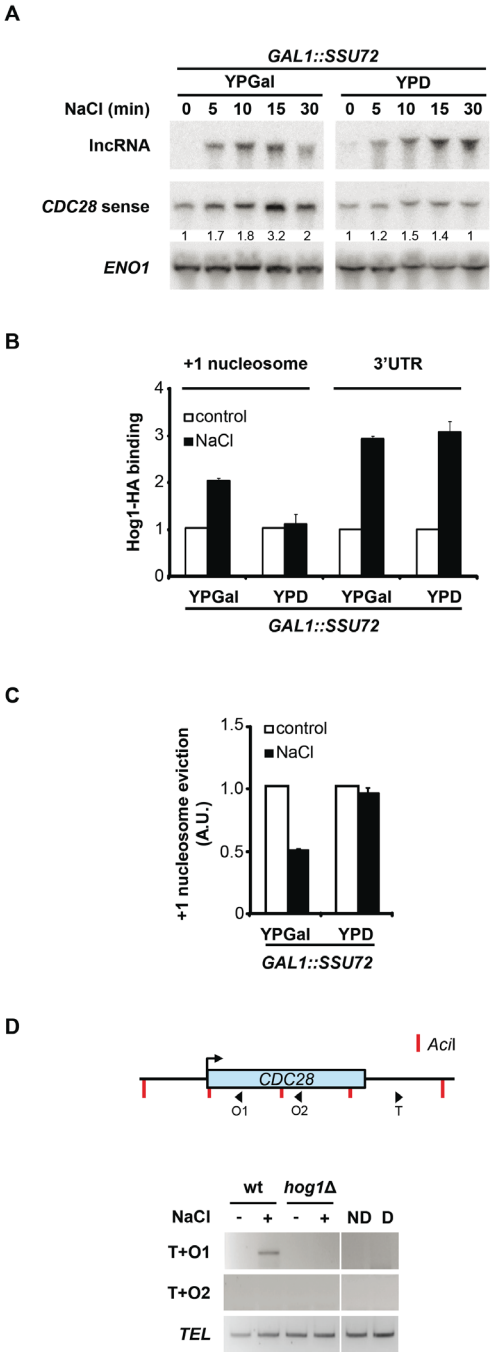

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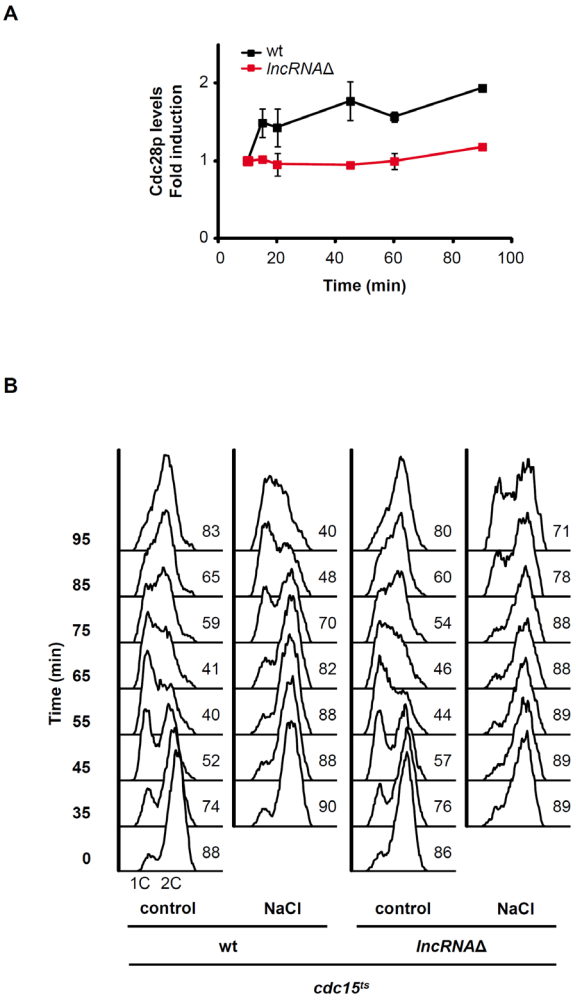
Figure 7



Supplemental Information

Control of Cdc28 CDK1 by a stress-induced lncRNA

Mariona Nadal-Ribelles[1,*], Carme Solé[1,*], Zhenyu Xu[2], Lars Steinmetz[2], Eulàlia de Nadal[1,†] and Francesc Posas[1,†]

Supplemental Figures and legends (Figures S1 to S4)

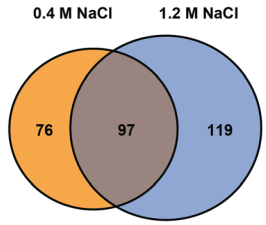
Supplemental table (Table S1)

Supplemental Experimental Procedures

Supplemental References

Nadal-Ribelles, Solé *et al.*
Figure S1

A



B

	SUTs	CUTs	Novel lncRNAs	Total
Upregulated lncRNA 0.4 M NaCl	37	68	68	173
Hog1-dependent 0.4 M NaCl	18	34	34	86
Upregulated lncRNA 1.2 M NaCl	50	94	72	216
Hog1-dependent 1.2 M NaCl	46	88	62	196

C

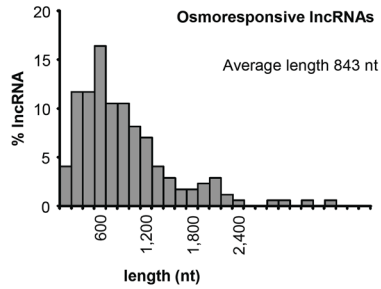


Figure S1, related to Figure 1. Stress induces a new set of lncRNAs.
(A) Venn diagram showing overlap between induced lncRNAs at 0.4M and 1.2M NaCl (genes from figure 1B). **(B)** Hog1-dependence and overlap of lncRNAs with previously annotated antisense transcripts or manually curated (novel lncRNAs). **(C)** Distribution of lncRNA lengths. Histogram represents length distribution (nt) of upregulated lncRNAs (0.4M NaCl) grouped in bins of 150 nt.

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Figure S2

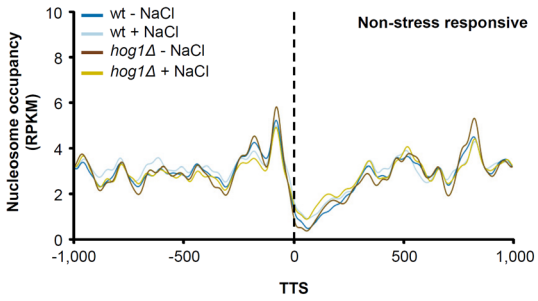

Figure S2, related to Figure 2. Hog1 mediates changes in chromatin architecture at lncRNA promoters.
Distribution of nucleosome hits (RPKMs; Reads per Kilobase per Million) expanding 1Kb up and downstream from transcription termination site (TTS) of wild type and *hog1*Δ mutant strains under basal (dark blue and red) and 0.4M NaCl (light blue and yellow). Plot represents coverage of reads of approximately 90 non-stress responsive genes. Dotted black line marks TTS.

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Figure S3

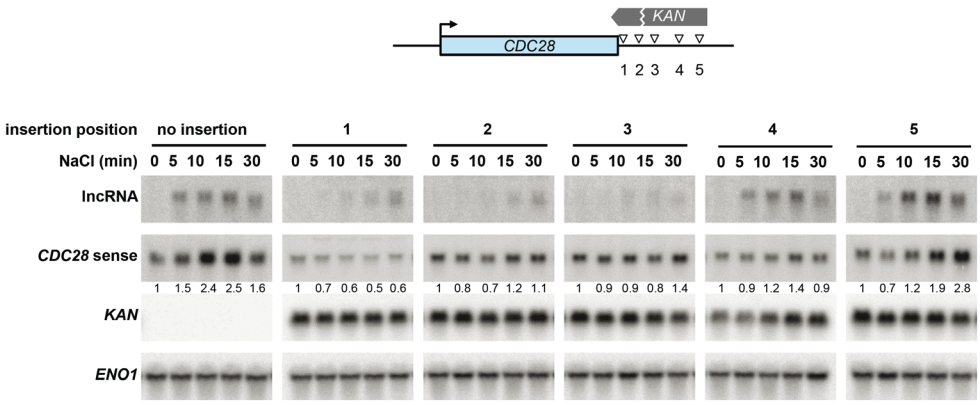

Figure S3, related to Figure 3. Induction of *CDC28* sense correlates with antisense expression.
Systematic insertion of a *KanR* marker at the 3'UTR region of *CDC28* respective to STOP codon: position 1 (20 nt after STOP), 2 (100 nt), 3 (180 nt), 4 (300 nt) or 5 (400 nt) are indicated in the scheme with an arrow. *CDC28* sense and lncRNA transcripts were assayed by Northern blot in the indicated strains after osmostress. *ENO1* and *KAN* were probed as control. Normalized quantification of *CDC28* sense is shown.

Nadal-Ribelles, Solé *et al.*
Figure S4

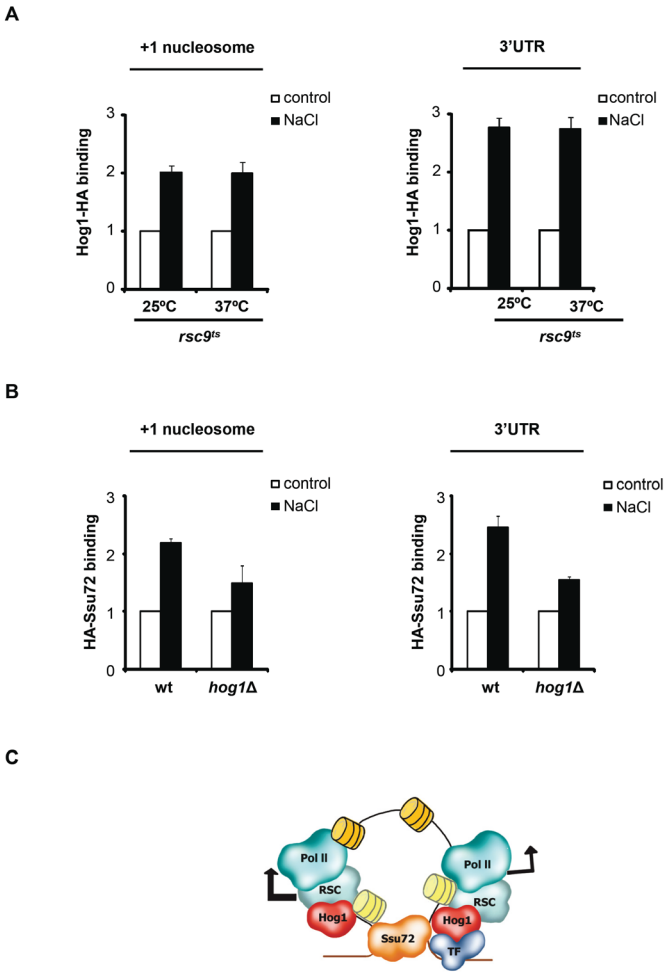

Figure S4, related to Figure 6. In response to stress, RSC-independent binding of Hog1 mediates recruitment of Ssu72 to +1 nucleosome region and 3'UTR of *CDC28*.
(A) Hog1 recruitment was assessed by ChIP at +1 nucleosome region (amplicon D) and 3'UTR (amplicon J) of *CDC28* in a *rsc9^{ts}* strain grown 2 hours at permissive (25ºC) or non-permissive (37ºC) temperature. Then, cells were or not subjected to osmostress (0.4M NaCl, 5 min). **(B)** Ssu72 binding was assessed as in (A) in a wild-type and *hog1*Δ strains. **(C)** Schematic representation of *CDC28* genomic locus in response to osmostress with the Hog1 SAPK and its transcriptional partners. Initially, Hog1 is recruited at the 3'UTR region of *CDC28*. Then, gene looping is established and Hog1 and RSC are transferred to the +1 nucleosome region to remodel and induce gene expression.

Supplemental Table S1: Lists of stress-induced lncRNAs, related to Figure 1

0,4M NaCl (173)			
Hog1-dependent (86)		Hog1-independent (87)	
Strongly dependent (50)	Moderately dependent (36)		
SUT-FS0088	CUT016	SUT-FS0008	SUT-FS0143
SUT648	SUT-FS0108	CUT200	CUT640
SUT-FS0033	SUT-FS0052	SUT409	SUT-FS0080
CUT796	SUT-FS0030	SUT-FS0112	SUT-FS0066
SUT-FS0090	CUT905	CUT571	SUT-FS0110
SUT-FS0010	SUT-FS0103	CUT138	SUT060
CUT471	CUT254	SUT-FS0141	SUT072
SUT552	CUT776	SUT-FS0135	SUT-FS0047
SUT765	CUT022	CUT550	SUT829
SUT-FS0083	SUT605	SUT-FS0084	SUT462
CUT583	CUT364	CUT644	CUT697
SUT-FS0060	SUT797	CUT010	SUT-FS0128
SUT-FS0032	SUT-FS0022	SUT-FS0059	CUT838
CUT898	CUT176	SUT608	SUT232
SUT-FS0042	SUT472	SUT360	SUT-FS0111
SUT164	CUT500	CUT256	CUT605
CUT850	SUT-FS0144	SUT-FS0073	CUT882
SUT-FS0017	SUT-FS0058	CUT799	SUT-FS0126
SUT-FS0074	CUT533	CUT918	SUT792
SUT-FS0096	SUT-FS0051	CUT093	CUT696
SUT-FS0004	CUT403	CUT175	SUT-FS0027
SUT-FS0123	SUT-FS0139	SUT-FS0070	SUT752
SUT203	CUT515	CUT309	CUT265
SUT-FS0024	SUT-FS0037	SUT-FS0092	SUT-FS0036
CUT875	SUT416	SUT-FS0072	SUT738
SUT-FS0019	SUT-FS0020	SUT-FS0039	CUT790
CUT561	SUT198	CUT415	SUT268
SUT-FS0097	SUT230	SUT-FS0012	CUT064
CUT602	SUT009	CUT501	CUT836
CUT734	CUT158	CUT592	SUT272
CUT647	SUT-FS0055	SUT647	CUT801
SUT322	CUT291	CUT499	SUT676
SUT-FS0125	CUT832	SUT427	SUT-FS0109
SUT-FS0034	CUT220	SUT814	CUT557
CUT720	CUT160	CUT891	SUT184
CUT872	CUT475	SUT-FS0007	SUT-FS0009
SUT373		SUT266	SUT-FS0151
SUT-FS0100		SUT-FS0101	
SUT-FS0026		SUT-FS0102	
CUT239		CUT627	
SUT062		SUT-FS0131	
SUT-FS0137		CUT312	
SUT344		CUT861	
CUT904		SUT-FS0005	
CUT842		CUT529	
CUT726		SUT-FS0113	
CUT321		SUT-FS0122	
SUT817		CUT714	
SUT-FS0081		SUT-FS0057	
SUT538		SUT-FS0075	

1,2M NaCl (216)					
Hog1-dependent (196)					Hog1-independent (20)
Strongly dependent (135)			Moderately dependent (61)		
CUT174	SUT-FS0038	CUT343	SUT-FS0037	SUT-FS0109	CUT705
CUT291	SUT-FS0016	SUT833	CUT529	SUT-FS0007	CUT801
SUT706	CUT621	SUT-FS0103	CUT473	CUT891	SUT281
CUT138	CUT175	CUT839	CUT223	SUT159	SUT-FS0005
CUT555	CUT917	SUT134	CUT010	SUT161	SUT-FS0142
SUT-FS0144	CUT176	CUT319	CUT890	SUT109	SUT624
CUT762	CUT389	SUT596	SUT462	SUT-FS0006	SUT323
CUT062	CUT417	SUT-FS0095	SUT322	CUT784	CUT064
CUT505	SUT-FS0072	SUT648	SUT252	CUT154	SUT605
CUT726	CUT160	SUT145	CUT605	CUT309	CUT483
CUT777	CUT814	CUT220	CUT470	SUT-FS0116	CUT841
CUT079	CUT602	CUT246	SUT-FS0143		CUT531
SUT-FS0073	CUT020	CUT592	SUT-FS0141		SUT-FS0147
SUT-FS0065	SUT-FS0132	SUT-FS0099	SUT158		SUT576
CUT848	CUT842	SUT-FS0029	CUT861		SUT395
SUT062	CUT832	SUT009	CUT681		SUT268
CUT057	SUT-FS0066	CUT262	SUT-FS0063		SUT003
CUT697	SUT-FS0120	SUT-FS0140	CUT316		SUT690
SUT251	SUT814	SUT586	SUT-FS0019		SUT676
SUT-FS0053	CUT364	SUT774	SUT671		SUT-FS0048
SUT-FS0010	SUT472	CUT872	CUT312		
CUT370	SUT797	SUT788	CUT819		
SUT-FS0017	CUT815	SUT150	CUT854		
SUT-FS0020	SUT-FS0071	SUT-FS0079	SUT-FS0068		
SUT214	CUT500	SUT203	SUT127		
CUT016	SUT-FS0123	CUT882	SUT213		
SUT360	SUT-FS0152	SUT-FS0046	CUT499		
CUT375	SUT-FS0024	SUT470	SUT585		
CUT231	SUT-FS0113	CUT790	SUT778		
SUT765	SUT-FS0114	SUT-FS0023	SUT232		
CUT265	CUT908	SUT700	CUT584		
CUT898	SUT-FS0128	SUT-FS0108	CUT779		
SUT-FS0075	SUT154	CUT923	CUT880		
SUT-FS0033	CUT640	CUT463	SUT-FS0098		
SUT-FS0090	SUT-FS0022	CUT763	CUT012		
CUT346	CUT836		SUT-FS0102		
SUT-FS0080	SUT-FS0136		SUT-FS0070		
CUT471	SUT-FS0061		CUT850		
SUT552	SUT-FS0137		SUT124		
SUT-FS0083	CUT835		CUT355		
SUT-FS0042	SUT-FS0130		SUT439		
SUT-FS0074	SUT565		CUT714		
CUT734	CUT233		SUT300		
SUT-FS0088	CUT239		CUT515		
CUT583	SUT-FS0129		SUT839		
CUT647	CUT699		SUT-FS0131		
SUT-FS0008	SUT-FS0030		SUT155		
CUT904	SUT102		SUT-FS0110		
CUT918	SUT559		CUT776		
SUT-FS0119	SUT-FS0125		SUT738		

Supplemental Experimental Procedures

Tiling array

Total RNA was extracted with standard hot-phenol protocol and treated with RNase-free DNaseI using Turbo DNA-free kit (Ambion). For first-strand cDNA synthesis, 20 µg of total RNA was mixed with 1.72 µg of random hexamers, 0.034 µg of oligo(dT) primer and incubated at 70°C for 10 min followed by 10 min at 25°C, then transferred on ice. The synthesis included 2,000 units of SuperScript II Reverse Transcriptase, 50 mM TrisHCl, 75 mM KCl, 3 mM MgCl2, 0.01 M DTT, dNTP + dUTP mix (0.5 mM for dCTP, dATP and dGTP; 0.4 mM for dTTP and 0.1 mM for dUTP, Invitrogen), 20 µg/mL actinomycin D in a total volume of 105 µL. The reaction was carried out in 0.2 mL tubes in a thermal cycler with the following thermal profile: 25°C for 10 min, 37°C for 30 min, 42°C for 30 min followed by 10 min at 70° for heat inactivation and 4°C on hold. Samples were then subjected to RNase treatment of 20 min at 37°C (30 units RNase H, Epicentre, 60 units of RNase Cocktail, Ambion). First-strand cDNA was purified using the MinElute PCR purification kit (Qiagen) and 5 µg were fragmented and labeled using the GeneChip WT Terminal labeling kit (Affymetrix) according to manufacturer's protocol. The labelled cDNA samples were denatured in a volume of 300 µl containing 50 pM control oligonucleotide B2 (Affymetrix) and Hybridization mix (GeneChip Hybridization, Wash and Stain kit, Affymetrix) of which 250 µl were hybridized per array (*S. cerevisiae* yeast tiling array, Affymetrix, PN 520055). Hybridizations were carried out at 45°C for 16 h with 60 rpm rotation. The staining was carried out using the GeneChip Hybridization, Wash and Stain kit with fluidics protocol FS450_0001 in an Affymetrix Fluidics station.

Manual annotation of transcription boundaries

Transcription boundaries throughout the genome were determined by automated segmentation by (Xu et al., 2009). Manually curated segmentation was necessary to accurately detect osmoresponsive lncRNA.

A webserver was created for interactive manual curation. Data were stored in a MySQL database and a web interface based on the Scalable Vector Graphics (SVG) technology allowed the display and edition of the segment boundaries by the curators.

Definition of stress-induced lncRNAs

Stress-induced lncRNAs were defined as up-regulated with a minimum of two fold change in response to stress (at the indicated osmolarity) in the wild type (BY4741) strain. Hog1 dependence was determined by the percentage of expression in a *hog1*Δ mutant respect to the wild type strain. Accordingly, "Hog1-independent" lncRNAs were defined as those whose expression in a *hog1*Δ mutant was at least 75% of wild type strain. Hog1-dependent were further classified into "moderately dependent" if expression was between 75-50% and "strongly dependent" defined as lncRNA whose expression in a *hog1*Δ mutant represents less than 50% in comparison to its expression in the wild type strain. Supplementary Table S1 provides the entire list of the osmoresponsive lncRNA and their Hog1 dependence.

ChIP-seq and Mnase-seq

Wild type and *hog1*Δ mutant *S. cerevisiae* strains were grown to mid-log phase and exposed to 5 minutes of osmostress (0.4 M NaCl) for Hog1 immunoprecipitation (anti-HA 12CA5) and 10 minutes for RNA Pol II immunoprecipitation (anti-RNA Pol II 8WG16, Covance) and nucleosome positioning. ChIP and MNase protocols were performed and purified DNA was sequenced as described (Nadal-Ribelles et al., 2012).

Enrichment of Hog1 and RNA Pol II was done by running the Pyicos enrichment protocol (Althammer et al., 2011) comparing untreated to treated samples. Binding of Hog1 to stress induced lncRNA was considered positive if enrichment in response to stress was above the *z-score*>2.2 (*p*-value=0.02) across the overlapping gene (entire ORF, promoter (500 bp upstream from TSS) and terminator (500 bp from TTS)) as annotated in *S. cerevisiae* genome (sacCer2). Occupancy of RNA Pol II to stress induced lncRNA is represented as the average of normalized reads (RPKM reads per kilobase per million) in the presence or absence of stress across the overlapping ORF (sacCer2). The statistical significance of the difference was assessed by a paired Student *t*-test of acceptance of equality at (*p*-value < 0.001) comparing read density in response to stress.

MNase profiles of nucleosome occupancy were performed as described (Nadal-Ribelles et al., 2012) and averaged nucleosome occupancy is represented by alignment of genes to their annotated TTS (sacCer2).

MNase nucleosome mapping

Indicated strains were grown in specific medium, then subjected (or not) to osmostress (0.4M NaCl for 10 minutes) or treated with β-estradiol (St. Louis, MO, USA, 100nM, 10 minutes). Spheroplasts and digestion with MNase were done essentially as described but with some modifications (Mas et al., 2009). Spheroplasts were prepared from mid-log phase cultures, following crosslinking with 1% (v/v) formaldehyde for 20 minutes, treated with 125mM glycine for 15 minutes and washed four times with Tris-buffered saline (20mM Tris-HCl pH, NaCl 150mM). Cells were then lysed and immediately digested with 7.5 to 125mU of MNase (Worthington Biochemical Corporation, Lakewood; NJ., USA). DNA was subjected to electrophoresis in a 1.5% (w/v) agarose gel and the band corresponding to the mononucleosome was cut and purified using a QIAquick gel

extraction kit (Qiagen). For the analysis of *CDC28*, DNA was used in a real-time PCR with specific oligonucleotides included in the Table provided. PCR quantification was referred to an internal loading control and nucleosome occupancy was normalized to 1 at the +1 nucleosome region.

ChIP assays

Chromatin immunoprecipitation was done as described previously (Zapater et al., 2007). Briefly, indicated yeast cultures were grown to mid-log phase before were exposed (or not) to osmotic stress (0.4M NaCl, 5 minutes) or treated with β-estradiol (100nM, 5 minutes). For crosslinking, cells were treated with 1% formaldehyde for 30 minutes at room temperature. Antibody used in this study was mouse monoclonal anti-HA 12CA5. Phenol-chlorophorm purified DNA was subjected to a quantitative PCR analysis of *CDC28* with a sequence detector (Applied Biosystems 7700) using described oligonucleotides in the Table provided. Immunoprecipitation efficiency was calculated in triplicate by dividing the amount of PCR product in the immunoprecipitated sample by that in the *TEL* (telomeric region on the right arm of chromosome VI) sequence control. The binding data are presented as fold induction with respect to the non-treated condition.

Northern Blot

Yeast cultures were grown to mid-log phase in the indicated medium and subjected to osmostress (0.4M NaCl) or treated with β-estradiol (100nM) for the length of time indicated. Total RNA was extracted, resolved in 1% agarose gels and transferred to Nylon membranes. Sense and lncRNA riboprobes were prepared using *MAXIscript® SP6 In Vitro Transcription Kit* (Ambion). As loading control, expression of *ENO1* was probed using radiolabeled PCR fragments (Rediprime II DNA Labeling System, GE Healthcare Life

Sciences). Primers used to produce PCR products are described in the Table provided. Signals were quantified with a Fujifilm BAS-5000 phosphorimager and ImageQuantTL software. Autoradiographs were obtained on Kodak Biomax XAR films (Sigma-Aldrich).

3C analysis

3C analysis was performed as described previously (Miele *et al.*, 2006)) with minor modifications. Cells were grown to mid-log phase before being subjected or not to osmostress (10 min, 0.4 M NaCl). Chromatin crosslinking was achieved by treating the cells with formaldehyde (1% v/v) for 15 minutes. The DNA was digested with *Aci*I restriction enzyme (New England Biolabs, Ipswich, MA, 200 U) for 3 hour at 37°C and ligated with T4 Ligase (New England Biolabs) for 2 hours at 16ºC. PCR conditions were standardized to 33 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 7 min. PCR primers used for the 3C analysis of *CDC28* were cdc28Rv_188 (O1), cdc28Rv_531 (O2) and normalized to internal control (*TEL*). PCR products were resolved in 2% agarose gels.

Metabolic labeling

Metabolic labeling was performed as described previously (Graham, 2001). Briefly, indicated PHO85-TAP strains were grown in YPD and shifted to MET- media for 2 hours before being stressed. Mixture of 35S-methionine and 0.4M NaCl were added simultaneously for the indicated times. 35S-Methionine incorporation was stopped (buffer stop) and cells were lysed (IP buffer). Clarified extracts were immunoprecipitated with anti-pSTAIR antibody (Millipore). Radiolabeled immunoprecipitated Cdc28 and total protein (input) were resolved in SDS-PAGE, transferred to PVDF membrane and exposed to a Fujifilm BAS-5000 phosphorimager. Quantification was performed using ImageQuantTL

software and newly synthesized Cdc28 levels were normalized by total radiolabeled protein (input). Fold induction is represented respect to 10 minutes of treatment.

Flow cytometry

Flow cytometry experiments were performed as described (Pelet et al., 2011). Cells were grown in YPD before being stressed for 45 minutes at 0.4M NaCl. Protein translation was stopped by addition of cyclohexamide (CHX, 0.1 mg/ml). Cells were sonicated and fluorescence was measured by flow cytometry (FACSCalibur, Becton Dickinson).

To study cell cycle progression, overnight cultures were diluted to OD_{660} of 0.3 and grown for 3 hours at 25ºC in YPD. *cdc15*^{ts} cells were synchronized at 37ºC (incubated for 2 hours) and released at 25ºC to allow cell cycle progression, in YPD supplemented or not with 0.4M NaCl. For flow cytometry analyses (FACS), cells were fixed in ethanol, treated overnight with RNAse A at 37ºC in 50mM Sodium Citrate, stained with propidium iodide and analyzed in FACSCalibur (Becton Dickinson). A total of 10000 cells were analyzed and the percentage of G2-M population quantified for each time point using WinMDI 2.9 software.

Table: Oligonucleotides used in this study

Amplicon	Oligo name	Sequence (5' to 3')
A	cdc28Fw-188	ATGGCCTTATTAGGGCAACA
	cdc28Rv-188	TTTTTGAGTTCTTGTTGTTTTCCA
B	cdc28Fw-86	TGGAAGGACCAAGTCCTCTT
	cdc28Rv-86	GTAGCTGATGTGCTGGCTTG
C	cdc28Fw-1	AAGCCAGCACATCAGCTACA
	cdc28Rv-1	ACACCGTATGTACCTTCACCG
D	cdc28Fw_83	CGGTGAAGGTACATACGGTGT
	cdc28Rv_83	TTTTCTTCAATGCGACTACTCTT
E	cdc28Fw_188	AGACGAGGGTGTTCCCAGTA
	cdc28Rv_188	GAGTGAACAATATCGTATAATCTGACA
F	cdc28Fw_278	TTCACTCTGATGCACACAAGC
	cdc28Rv_278	TCCTAACGGTTGGTCCTTTG
G	cdc28Fw_356	CAAAGGACCAACCGTTAGGA
	cdc28Rv_356	CACGATGCAGAATACGGTGT
	cdc28Fw_449	CGTGATTTAAAACCGCAGAA
	cdc28Rv_449	CTCAACGGAACACCAAAAGC
H	cdc28Fw_531	TGGTGTTCCGTTGAGAGCTT
	cdc28Rv_531	TCGACACCTGTACTATATTGTTTTCC
	cdc28Fw_617	ATACATGGTCCATCGGCTGT
	cdc28Rv_617	CGATCTCACTATCGCCACTG
	cdc28Fw_694	TGAGATCGATCAGATTTTCAAGA
	cdc28Rv_694	TGGCTTGAAATCAGGCAAGT
	cdc28Fw_781	ACTTGCCTGATTTCAAGCCA
	cdc28Rv_781	TAGGGTCATACGCGAGGAGT
	cdc28Fw_884	CTCCTCGCGTATGACCCTA
	cdc28 Rv_884	TGACAGTGCAGTAGCATTTGT
I	cdc28Fw_979	TGCTACTGCACTGTCATTATAGCC
	cdc28Rv_979	TGAATATATTTTCTGATGGAGTTACCC

J	cdc28Fw_1066	CATCGTCATATACGGAACATTCA
	cdc28Rv_1066	GAACACGCCCAGCTTTTAAC
K	cdc28Fw_1160	GTTAAAAGCTGGGCGTGTTC
	cdc28Rv_1160	AATTGAGGCCCCAGCATAC
L	cdc28Fw_1328	ATCTGACTCGCCCTTGGTTT
	cdc28Rv_1328	TCAACGGTGACAAGTGAAACTC
X	cdc28Fw_979	TGCTACTGCACTGTCATTATAGCC
	5'GAL1core	ATAGGATGATAATGCGATTAGTTTTTTAGCCTT
TEL	TEL RTa	ACCACTCAAAGAGAAATTTACTGGAAGA
	TEL RTb	CTCGTTAGGATCACGTTCGAATC
CDC28 lncRNA	CDC28 SP6asFw	CGGATTTAGGTGACACTATAGAATACGTTCCTCGATT TGGACCTGA
	CDC28 SP6asRv	TAGGTCTTTTCTGCGCCATT
CDC28 sense	CDC28 SP6seFw	GTTCCTCGATTTGGACCTGA
	CDC28 SP6seRv	CGGATTTAGGTGACACTATAGAATACTAGGTCTTTTC TGCGCCATT
FRE7 lncRNA	FRE7 SP6asFw	CGGATTTAGGTGACACTATAGAATACGCCTTATGGTG GAATCGAGA
	FRE7 SP6asRv	CTGTAAACCTGCCACGGAAT
FZF1 lncRNA	FZF1 SP6antisenseFw	CGGATTTAGGTGACACTATAGAATACGTTTGCTTCAG CAACACCAA
	FZF1 SP6antisenseRv	GCTGCTGGATTTCTCAGAGG
MMF1 lncRNA	MMF1 SP6antisenseFw	CGGATTTAGGTGACACTATAGAATACTCCAGTCTTGA GGAGGGGTA
	MMF1 SP6antisenseRv	GAAGCAACACCAACACAGGA
ENO1	5'ENO1sondaORF	ATGGCTGTCTCTAAAGTTTA
	3'ENO1sondaORF	AATTTGTCACCGTGGTGGAA
KAN	FwKanRsonda	CCGCGATTAAATTCCAACAT
	RvKanRsonda	ATTCCGACTCGTCCAACATC

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Control of Cdc28 CDK1 by a stress-induced lncRNA

Mariona Nadal-Ribelles,*, Carme Solé,*, Zhenyu Xu, Lars M. Steinmetz, Eulàlia de Nadal, Francesc Posas

* These authors contributed equally to this work

(Submitted)

Development of new approaches to measure abundance of mRNA species has challenged the simplistic textbook view of transcription. Previous studies on gene expression were limited to protein coding genes. Exposure of cells to adverse conditions causes a dramatic change in gene expression.

Here we used tiling arrays, which together with RNA-seq provide a true genome-wide coverage. The use of tiling arrays in yeast has provided an unprecedented resolution of the complexity of transcriptome. Probably one of the most striking observations is the wide presence of antisense transcripts (David et al., 2006; Xu et al., 2009).

Existence of lncRNA is ubiquitous in eukaryotes. In yeast four out of five classes of lncRNAs have been identified by tiling arrays. Functionality and biological relevance of these lncRNAs remains elusive especially in yeast since the RNAi pathway is absent.

Here, we showed that Hog1 regulates the transcription of a novel class of lncRNAs that are induced upon osmostress and whose induction may have relevant implications for proper cellular adaptation to environmental changes. We have specifically focused our efforts in the characterization of a lncRNA in antisense

orientation at the coding region of the *CDC28* (cyclin dependent kinase 1, CDK1).

Hog1 controls transcription of a new set of lncRNA in response to stress.

To assess gene expression at high resolution we made use of tiling arrays that cover both strands of the entire yeast genome (David et al., 2006). We have shown that in response to mild (0.4 M NaCl) and hyper osmostress (1.2 M NaCl) a group of approximately 200 lncRNAs were strongly induced (FC>2). Of these, 97 lncRNA were common between both osmolarities (Figure 1A and Figure S1A). Recruitment of Hog1 at the 97 lncRNA common at both osmolarities was as high as 85%. This overlap of genes induced at both osmolarities suggests a specialized mechanism to induce lncRNA expression.

We compared the transcription of these lncRNA in wild type and *hog1* cells and found that, as it happens with osmoresponsive genes, dependence on Hog1 increased with the strength of the stress (Figure 1B). Thus, Hog1 plays a major role in the induction of this novel class of stress responsive lncRNA.

We then assessed whether these stress induced lncRNAs had similar properties than previously described lncRNAs. Except for SUTs which are stable transcripts, the rest of lncRNAs are only detectable in strains deleted for components of the nuclear o cytosolic exosome (CUTs and XUTs) (Xu et al., 2009). Transcription and degradation of these lncRNAs is believed to be constant. On the other hand, two other families of lncRNAs only

appear in strains where gene looping is impaired (Ssu72-restricted transcripts) or the deletion of the histone deacetylase Set3 (Kim et al., 2012; Tan-Wong et al., 2012). We validated by Northern blot analysis of Hog1-dependent lncRNAs (Figure 1C), and found that they were not expressed under basal conditions in the absence of *RRP6*, *XRN1*, and *TRF4*. Transcription of lncRNAs only existed in response to stress, and their stability was regulated upon stress by the same machineries that control basal lncRNA transcription. It is worth noting that these stress induced lncRNAs differ in properties with any class of lncRNA described so far. This observation confirms a new regulatory role for Hog1 at the transcription level, and further research needs to be done to fully understand the features of stress-induced lncRNAs.

Hog1 binds and recruits RNA Pol II at genes expressing lncRNAs.

Kinetics of induction of stress induced lncRNAs was identical to osmostress responsive genes, a strong induction within the first minutes and down-regulation at the later times of stress. Analysis of the ChIP-seq data of Hog1 showed that Hog1 was present approximately at 65% of the lncRNA with a Hog1-dependent induction, while only in 30% of Hog1 independent showed Hog1 recruitment (Figure 2A). This ratio of Hog1 binding is very similar to what we had previously reported for osmoresponsive genes (Nadal-Ribelles et al., 2012).

As it happens with osmoresponsive genes, there was an increased association of RNA Pol II at the Hog1-dependent

lncRNAs, which was completely dependent on the presence of Hog1 (Figure 2B). A key difference of these lncRNAs when compared to osmoresponsive genes is that these genes already have the normal transcriptional machinery loaded into their coding regions since they are being expressed under non stress conditions.

Binding of RNA Pol II and Hog1 leads to major changes in chromatin remodeling. Accordingly, Hog1 induces profound nucleosome eviction at the promoter region of the lncRNAs (corresponding to the 3'UTR of the sense transcript). This massive change in chromatin structure is strongly dependent on the presence of Hog1 (Figure 2C). It is worth noting that nucleosome eviction was specific to stress responsive loci and more pronounced for Hog1-dependent lncRNAs since we did not observe significant changes in Hog1-independent nor non stress responsive genes (Figure 2C, S2). Therefore, the 3'UTR regions of these genes undergoes similar chromatin remodeling process similar to *bona fide* stress-responsive promoters, with the added difficulty of having already transcription occurring at the same loci on the opposite direction.

We created a reporter in which we fused GFP to 300 bp from the 3'UTR of *CDC28* in its natural orientation or in antisense orientation (Figure 2D). Basal transcription was observed regardless of the direction of the 3'UTR indicating the intrinsic promoter capacity of this region. Most importantly, induction was only seen in the antisense orientation in response to osmostress and completely dependent in the presence of Hog1. This observation is really interesting, since most of the described antisense transcripts

have been shown to arise from bidirectional promoters (Tan-Wong et al., 2012; Xu et al., 2009). The fact that this terminator can function as a heterologous promoter suggests that there must exist a different transcriptional unit recruited to this region that is independent of the neighboring/surrounding region. Accordingly, a recent study by ChIP-exo, a modification of ChIP, precisely positioned distinct PIC complexes at nucleosome free regions (NFR) with divergent transcription, supporting the idea of unique transcription units (Rhee and Pugh, 2012). These results support the idea that lncRNA transcription is a tightly regulated process rather than random transcription noise arising from bidirectional promoters.

As mentioned above, genes that express Hog1-dependent lncRNAs tend to be expressed in the absence of stress. We investigated whether there was a correlation between the expression of sense and lncRNA expression (Figure 3A). Overall, there was no clear correlation except for a restricted group of genes that showed either negative or positive correlation. One of the most important observations was the positive correlation between the induction of *CDC28* and its lncRNA.

To decipher the role of the CDC28 lncRNA we abolished lncRNA expression by introducing a KanMx marker at different distances from the STOP codon at the 3'UTR of CDC28 (Figure S3). Disruption of the *CDC28* lncRNA (by introducing the marker 180bp after STOP codon) completely impaired induction of the sense (Figure 2E). Accordingly, in *hog1* cells there was neither induction of the lncRNA nor induction of the sense (Figure 3B).

Thus, the presence of the stress-inducible CDC28 lncRNA correlates with induction of the CDC28 sense. This increase in transcription was unexpected since transcription of *CDC28* is assumed to be constant throughout the cell cycle and in response to other environmental stresses (Gasch et al., 2000; Spellman et al., 1998).

Hog1 associates with the 3'UTR and the +1 nucleosome regions to promote chromatin remodeling through RSC

To further characterize the mechanism of responsible for lncRNA transcription, we monitored the presence of Hog1 across the *CDC28* locus by high-coverage ChIP (Figure 3A). As expected, Hog1 was recruited at the 3'UTR (lncRNA promoter) but surprisingly, it also specifically associated with the 5' region corresponding to the +1 nucleosome (Albert et al., 2007). By contrast, in cells disrupted for *CDC28* lncRNA, recruitment of Hog1 was partially abolished at the terminator but inexistent at the +1 nucleosome.

This is an interesting observation that resembles some of the features found in osmoresponsive genes, in which Hog1 recruitment at the ORFs of osmoresponsive genes depends on the 3'UTR region (Proft et al., 2006). Transcription of *CDC28* is not controlled by any of the transcription factors used by Hog1. It may be that Hog1 uses the 3'UTR region to mediate binding at the ORF and to induce expression of a gene that cannot be controlled from the promoter region.

When we followed nucleosome positioning by MNase digestion at the *CDC28* loci in response to stress in wild type cells, we found strong histone eviction at both the 3' UTR and the +1 region which correlates with Hog1-bound regions that is not present in *lncRNAΔ* and *hog1* cells (Figure 4B). At osmoresponsive genes, Hog1 remodels chromatin by targeting RSC to the coding region (Mas et al., 2009). Therefore, we measured expression of sense and lncRNA in the absence of RSC. Impaired RSC function ($rsc9^{ts}$) resulted in similar levels of *CDC28* lncRNA but completely abolished the increase of *CDC28* sense (Figure 4C). Accordingly, a $rsc9^{ts}$ mutant did not show remodeling of the +1 nucleosome (Figure 4E). Indeed, we found that recruitment of RSC at the +1 and 3'UTR was completely dependent on Hog1. Interestingly, this binding was stronger at the +1 nucleosome region, suggesting that RSC is preferentially recruited to remodel the +1 region. Therefore, RSC is not required to for lncRNA transcription while it is essential to mediate the remodeling of the +1 nucleosome.

Antisense transcription and Hog1 recruitment are required to induce *CDC28*

To dissect the role of the lncRNA and Hog1, we designed two strategies. First, we controlled the expression of *CDC28* lncRNA in *cis* by integrating *GAL1* promoter at the *CDC28* terminator and expressing a GAL4-ER-VP16 that allows expression of GAL1 promoters in the presence of β-estradiol (Louvion et al., 1993). Second, using the strategy just mentioned we tethered Hog1 to the *GAL1* promoter by expressing Msn2 fused to the Gal4 DNA binding

domain (GAL4DBD-Msn2). Using the first strategy the sole induction of the *CDC28* lncRNA did not induce sense expression neither it caused any detectable change at the +1 nucleosome (Figure 5A, B). Interestingly, when we expressed the GAL4DBD-Msn2, we restored the presence of Hog1 at the both ends of *CDC28*, the remodeling of the +1 and hence, the stress-dependent induction of the sense (Figure 5D). In conclusion, these results indicate that the presence of both Hog1 and the lncRNA mediate the increase in sense transcription.

Up to date, cases in which sense and antisense expression anticorrelate, such as the case of *PHO84*, have received most of the attention (Camblong et al., 2007; Camblong et al., 2009). Instead, a case where the sense/lncRNA pair is induced simultaneously presents an interesting paradigm of regulation and much less is known. Co-expression may be a challenging situation for the cells since there is the potential collision between the two transcriptional machineries. Mechanisms that prevent or solve this encounter are beginning to be studied, but are far from being understood (Hobson et al., 2012).

The establishment of gene looping permits the recruitment of Hog1 at the +1 nucleosome region and induction of CDC28

Unlike osmoresponsive genes in which Hog1 travels with elongating polymerase, the distal binding pattern of Hog1 at the *CDC28* loci suggested that Hog1 could reach the 5' end of the gene without traveling through the coding region. In yeast, gene looping has been shown to juxtapose promoter-terminator regions during

active transcription. Generation of looping can be prevented by impairing the function of an essential gene *SSU72* (Ansari and Hampsey, 2005). When we abolished the expression of *SSU72* by using the *GAL1* promoter, under repressing condition (glucose), we observed that the induction of *CDC28* lncRNA was similar to wild type, but the increase of sense expression was completely abolished (Figure 6A). Indeed, association of Hog1 at the +1 nucleosome was abrogated in cells with impaired gene looping (Figure 6B). Interestingly, Ssu72 was recruited to the same regions as Hog1, and always in a Hog1-dependent manner (Figure S4B). As expected, we did not detect remodeling at the +1 nucleosome region in cells depleted for Ssu72 (Figure 6C).These results point out the need of a determined chromatin conformation, mediated by gene looping, that permits Hog1 to reach the +1 nucleosome region from the 3'UTR and induction of *CDC28*.

An increase of Cdc28 is important for cell cycle re-entry in response to stress

The levels of Cdc28 are usually considered steady throughout the cell cycle, and thus transcription is assumed to be relatively constant as well (Spellman et al., 1998). We measured *de novo* synthesis of Cdc28 by immunoprecipitating metabolically labeled Cdc28 (S^{35}-methionine) in response to osmostress (Figure 7A). While wild type strains had an increased synthesis rate of Cdc28, a strain deficient in lncRNA transcription showed no increase. Indicating that, indeed, the increase in *CDC28* mRNA mediated by the *CDC28* lncRNA leads to an increase in the total pool of Cdc28.

In response to osmostress, Hog1 mediates an immediate but transient arrest of cell cycle progression to allow adaptation through several mechanisms (Clotet and Posas, 2007), among them a decrease in Cdc28 activity. Interestingly, the increase in Cdc28 protein levels occurred when cells started to recover from stress, therefore we postulated that this increase in protein level should have an effect at the recovery phase. To test this hypothesis we followed cell cycle progression from anaphase synchronized cells (*cdc15*^{ts}) (Figure 7B). In the absence of stress, cells progressed equally, but in the presence of osmostress a wild type strain re-entered cell cycle more efficiently than cells deficient in *CDC28* lncRNA. Cdc28 is the master regulator of cell cycle and it does so by association with phase-specific cyclins that determine substrate affinity (Loog and Morgan, 2005). Here we have shown that increased levels of Cdc28 can drive changes in cell cycle progression.

The presence of lncRNAs is widely observed but its biological relevance is far from being understood. Data presented in this paper demonstrates the existence of a dedicated subset of lncRNAs strongly induced and regulated by signal transduction pathways. A particular example studied is Cdc28, the master regulator of cell cycle, providing a new paradigm in which cell cycle progression can be modulated by the expression of a lncRNA by a SAPK.

Perspectives

Non-coding RNAs are present in most species from yeast to human. The functional relevance of this novel class of non protein coding transcripts is poorly understood, especially in yeast. In the last five years tiling arrays performed in yeast have identified the presence of antisense transcripts in a number of mutants. Some of the identified transcripts do not overlap with protein coding genes while some of them do. This latter class of overlapping lncRNAs stimulates the investigation towards the identification of a functional significance.

Here, we presented evidence that the antisense transcriptome is dynamic and serves to integrate information from signal transduction pathways (HOG pathway), thus identifying a novel class of putative regulatory lncRNAs. Interestingly, we have observed a 50% overlap of lncRNAs between two different NaCl concentrations. The fact that these lncRNAs respond to stress suggests the idea that there might exist general stress-responsive lncRNAs as it happens with protein-coding stress responsive genes. In *S. cerevisiae,* the presence of these transcripts in other types of stresses has not been assessed yet. Further experiments will shed some light on the importance of lncRNA transcription for environmental insults.

LncRNAs do not show a clear correlation with expression of their sense transcript in any of the published studies (David et al., 2006). Stress induced lncRNAs are regulated by the same machineries as described before (Rrp6, Xrn1, Trf4) but stress-responsive lncRNAs do not fit with the canonical description since

they only exist in the presence of stress. Further characterization of the properties of these transcripts will help to understand the pathways that regulate their abundance.

Interestingly, terminators of housekeeping genes such as *CDC28* display promoter capacity. Up to date, bidirectional promoters are believed to be the major source of antisense transcripts (Xu et al., 2009). We have demonstrated that *CDC28* terminator can function independently from its natural context and still maintained the same regulatory behavior. There is probably a mixture of lncRNAs that fire from bidirectional promoters and those that can drive transcription independently. If there exist functional differences among these transcripts has yet to be determined.

Mechanistic studies on antisense functionality are scarce. We have dissected the mechanism of action of a Hog1-induced lncRNA by focusing our attention in the lncRNA that originates from the 3'UTR of the master regulator of cell cycle, *CDC28*. Induction of the lncRNA together with the presence of Hog1 causes a gene looping conformation that allows Hog1 to reach the +1 nucleosome region and remodel chromatin through the recruitment of RSC. This causes an induction of mRNA that leads to an increase in Cdc28 levels that are required to resume cell cycle upon stress.

Recent reports have shown that the transcription of certain lncRNAs, but not the lncRNA itself, can deposit co-transcriptional regulatory histone marks to fine tune gene expression (Berretta et al., 2008; Kim et al., 2012). It is clear that the characterization of these lncRNA transcripts, especially those that overlap with protein

coding genes, can lead to the identification of a novel level of transcriptional regulation that includes changes in chromatin structure, changes in histone marks and recruitment of specific factors to targeted genes.

Taken together, the presence of lncRNA transcription provides an exciting era on the field of transcription in eukaryotes and challenges the classical view of "linear" transcription. The regulated transcription and stability of lncRNAs may provide a new level of transcriptional or post transcriptional control to modulate cell physiology.

Personal contribution to this work: Except for the hybridization and data collection of the Tiling array, which was performed in collaboration with the Dr. Lars Steimentz's laboratory (EMBL).I have been fully involved in all aspects of the design, execution and discussion of the experiments described in this manuscript.

GLOBAL DISCUSSION

Over the last 15 years substantial body of knowledge on the role of Hog1 in transcription regulation has revealed that 5-7% of the genome undergoes dramatic changes in gene expression in response to osmostress in a Hog1-dependent manner (Causton et al., 2001; Gasch et al., 2000; Posas et al., 2000; Rep et al., 2000).. Biochemical evidence from our group and others have contributed to the current understanding of the mechanisms employed by Hog1 to mediate this massive change in the transcription (de Nadal et al., 2004; Mas et al., 2009; Zapater et al., 2007). In depth characterization of the Hog1-mediated regulation transcriptome of the budding yeast (*S. cerevisiae*) under osmostress conditions has been one the main interest of the present thesis. The first article presented in this thesis (Solé et al., 2011) aimed to describe a target for Hog1 whose novel function is required for proper gene induction.

Hog1 tightly binds to chromatin in response to stress and elicits gene expression by various mechanisms. To provide new insights into these mechanisms, we took advantage of a genetic screen designed to detect mutants with defective osmostress gene expression. This strategy has already lead to the identification of key factors by which Hog1 mediates gene expression such as the Rpd3 complex, SAGA and Mediator (de Nadal et al., 2004; Zapater et al., 2007) depicting the complex dynamic interplay between post-translational modification marks and transcription.

Interestingly, from this genetic screen, we were able to identify defective transcription in cells deficient for the ubiquitin protease Ubp3. In response to osmostress, Ubp3 is recruited to

promoter and coding regions of osmoresponsive genes in a Hog1-dependent manner. Hog1 interacts with and phsophorylates Ubp3 at Ser695 and causes a change in deubiquitinase activity that is essential for proper gene induction. Accordingly, cells carrying a catalytically dead ($Ubp3^{C465A}$) or a non phosphorylatable mutant at the Hog1 phosphorylation site (Ser695A) showed impaired transcription to the same extend as *ubp3* null mutants.

Transcription initiation and elongation are controlled by Hog1. It is worth noting that Hog1 specifically targets components of the initiation and elongation machinery. Ubp3 and RNA Pol II are the only enzymes whose presence at both promotes and coding region fully depends on Hog1. Moreover, we have shown that Ubp3 function is required independently at initiation and elongation. Although we could not narrow down the substrates of Ubp3 at initiation, *in vitro* deubiquitinase assays indicated that RNA Pol II could be more efficiently deubiquinated using Ubp3 purified from stressed cells, suggesting RNA Pol II as the likely substrate in elongation. These results are in accordance with previously published data in which Ubp3 deubiquitinates stalled RNA Pol II in response to UV-radiation (Kvint et al., 2008). Thus, Ubp3 could serve to integrate stress signals to change the balance ubiquitinated proteins and establish a different dynamic transcriptional response.

As mentioned above, our laboratory has shown that several chromatin modifying activities are required to induce proper transcription and establishing an osmostress-specific modification network tightly regulated by Hog1. In the second article presented (Nadal-Ribelles et al., 2012), we addressed the mechanism by

which osmostress gene induction is carried out in parallel with major gene repression in yeast. There is a massive dissociation of proteins from chromatin in response to stress (Proft and Struhl, 2004). Accordingly, there is a genome-wide tendency to lose association of RNA Pol II which leads the entire genome into a repressive state. On the other hand, while the entire genome is undergoing RNA Pol II dissociation, Hog1 selectively targets stress-responsive genes for transcription by recruiting RNA Pol II and chromatin remodelers that lead to profound changes in nucleosome eviction. Using ChIP-seq and MNase-seq assays, we characterized the location of Hog1, RNA Pol II and nucleosomes in wild type and *hog1* strains with and without osmostress. This experimental set up has allowed us to uncover several unknown properties of Hog1-dependent transcriptome.

Our results probably represent a more accurate role of Hog1 than previously estimated (Capaldi et al., 2008; Cook and O'Shea, 2012). Higher resolution obtained by ChiP-seq data has allowed an increase of the number of Hog1 targets by almost a factor of five. Having a global snapshot of gene expression, protein localization and nucleosome occupancy revealed a dose dependent correlation of chromatin and chromatin remodeling linked to the presence of Hog1, making transcription of these genes more efficient. One of the most surprising observations was the presence of Hog1 in RNA Pol III genes as well as the interaction with the RNA Pol III machinery, which supports and extends the role of Hog1 in transcription beyond previously anticipated.

Genome-wide expression profiling in response to osmostress has been assessed under several concentrations, timing and types of stresses (glycerol, KCl, NaCl) (Capaldi et al., 2008; Posas et al., 2000; Rep et al., 2000). However, all these studies were limited to protein coding genes and did not fully represent the entire transcriptome. With the aim of characterizing the transcriptional response in response to osmostress and the role of Hog1 in reprogramming gene expression, we profiled transcription of the both stands in the entire genome by using tiling arrays. We analyzed transcription of wild type and *hog1* strains in response to mild (0.4 M NaCl) and hyper osmotic shock (1.2 M NaCl). Surprisingly, transcriptome of yeast in response to osmostress is much more complex than previously anticipated and so is the role of Hog1. Tiling arrays have allowed the identification of a novel class of Hog1 regulated long noncoding RNAs (lncRNAs).

As mentioned before, lncRNAs in yeast have been classified based on their stability properties. Here, we have identified a novel class of lncRNAs that are strongly induced in a Hog1-dependent manner but that do not fit with the current classification of antisense transcripts. Although stress induced lncRNAs may overlap with previously annotated lncRNA transcripts, they are only regulated in response to stress. We did not observe basal transcription of these stress-responsive lncRNAs in *rrp6* or *xrn1* mutant strains but, we observed a clear upregulation of transcription upon stress. Interestingly, stability of these antisense transcripts is also regulated by the nuclear or cytosolic exosome but only once they have been induced.

Moreover, the majority (around 65%) of genes expressing a Hog1-dependent lncRNA showed association of the SAPK, while Hog1-independent genes have a reduced occupancy of Hog1 (30%). This percentage of Hog1 association confirms a direct regulatory role of Hog1 in these genes. Accordingly, recruitment of RNA Pol II increases in response to stress in a Hog1-dependent manner at the promoter regions of stress-induced lncRNAs. Increased RNA Pol II in genes with lncRNA is not as strong as we observed in osmostress responsive genes (Nadal-Ribelles et al., 2012). RNA Pol II density represents an average of the reads in both sense and antisense orientation, and albeit some genes are downregulated when the lncRNA is induced, we could still observe a clear tendency of increased RNA Pol II association in genes containing lncRNAs.

A clear indicator of Hog1-dependent transcription is a major nucleosome eviction that the SAPK imposes to facilitate induction (Mas et al., 2009; Nadal-Ribelles et al., 2012). There was a strong nucleosome eviction at lncRNA containing genes that specifically depended on the presence of Hog1 for the Hog1-dependent lncRNAs. We demonstrated that the 3'UTR region of *CDC28* behaves as a *bona fide* stress promoter even if isolated from its chromatin context, suggesting that mechanisms of induction of lncRNAs may be similar to the ones described for stress induced genes. Some lncRNA promoters seemed to arise from a bidirectional promoter, it remains unclear whether all stress induced lncRNA maintain the capacity to function independent from its neighboring gene.

All in all, we have shown that Hog1 not only targets promoters of osmoresponsive genes, it also induces transcription from terminator regions. Our results suggest that the mechanisms used by Hog1 for induction of lncRNAs share some similarities to those observed for osmoresponsive genes. It would be interesting to further characterize the activities required for stress-induced lncRNA transcription at a genome-wide scale (i.e. transcription factors, chromatin modifiers and remodelers).

Except for some specific cases such as the *PHO84* gene that is downregulated by an antisense transcript (Camblong et al., 2009) no clear correlation (either positive or negative) has been described with their respective genes. At the same time, albeit there is an increasing amount of description of lncRNA types, functionality of these transcripts is far from being understood especially in yeast where the RNAi machinery is absent. We were specifically interested in the lncRNA transcribed at the *CDC28* locus which showed a positive correlation of *CDC28* sense-lncRNA expression. This parallel induction of sense and lncRNA was not observed in *hog1* or in strains with disrupted lncRNA expression.

Traditionally, Hog1 travels with elongating RNA Pol II and therefore binds across the entire coding region of targeted genes. Surprisingly, when we followed by ChIP the association of Hog1 at *CDC28* we found that recruitment was specific at the 5'end and the 3'UTR without recruitment in the regions within.

Hog1 is capable to reach the +1 nucleosome region of *CDC28* and this distal recruitment requires the presence of a protein relevant for the establishment of gene looping protein (Ssu72).

Recruitment of Ssu72 is Hog1-dependent and juxtaposes 5'end and 3'UTR. It is important to notice that impaired gene looping prevented binding of Hog1 at the +1 nucleosome region and thus completely abrogated sense induction without altering lncRNA expression. Regions with Hog1 bound displayed nucleosome eviction that explains sense/lncRNA upregulation. Further experiments demonstrated that the presence of the *CDC28* lncRNA driven by a Hog1-independent promoter is not sufficient to evict the +1 nucleosome, but tethering Hog1 to this promoter automatically restored binding and remodeling to the +1 nucleosome, hence inducing sense again. In sum, we have demonstrated a new mechanism by which the presence of a lncRNA together with a SAPK serves to fine tune regulation of gene expression. The remodeling of chromatin at osmoresponsive and lncRNA genes depends on the Hog1-dependent recruitment of RSC complex. Thus, it seems that RSC is the selected complex to mediate the massive nucleosome eviction that Hog1-dependent transcription requires.

Characterization of the molecular mechanisms by which Hog1 induces transcription of a lncRNA was one of the objectives of the article. But the open question in the transcription field is the functionality, if any, of these lncRNAs. Having dissected the role of Hog1 in inducing at the transcriptional level *CDC28*, we analyzed if there was a change of the total protein levels and if this could have any physiological relevance.

In response to osmostress, Hog1 induces an immediate but transient blockage of cell cycle through several mechanisms

including a decrease/inhibition of Cdc28 activity (Escote et al., 2004). Knowing this scenario, it seemed counter intuitive that in response to stress Hog1 would induce the mRNA Cdc28 at the same time that cyclin transcription is being delayed (Adrover et al., 2011). Subsequently, the assessment *de novo* synthesis of Cdc28, showed an increase in Cdc28 in the wild type strain which was abolished in *hog1* and *lncRNA* strains. In timing, the increase in protein levels occurred when cells had already blocked cell cycle and were starting to recover. Activity of Cdc28 increases as cells advance through the cell cycle (Morgan, 1997). To test the hypothesis that an increase of Cdc28 could serve to promote cell cycle re-entry after stress, we followed cell cycle progression in cells synchronized at anaphase (*cdc15ts*) were CDK activity is maximal. Cells with impaired lncRNA expression had a delayed re-entry in cell cycle.

Altogether, the induced expression of a Hog1 dependent lncRNA can fine tune the expression of the main cell cycle regulator Cdc28 being able to alter cell cycle progression in response to environmental challenges. An interesting observation from these results is that the amount of Cdc28 itself can lead to changes in cell cycle progression, if this as well represents an increase on Cdk1 activity remains to be determined. In response to stress there is a Hog1 dependent downregulation and delay of genes encoding for cyclins. It could be that progression through the following phases of the cycle after stress is achieved with lesser amount of cyclins; therefore it would make sense to have an increase of Cdc28 to recover cell cycle progression.

Historically, control of transcription and cell cycle progression have been analyzed as two independent events but the results described here together with a recent report, suggest that cell cycle and osmostress transcription are tightly linked. Hog1 coordinates a stress specific S-phase check point to allow proper transcription of stress responsive genes (Duch et al., 2013). At the same time, it induces a lncRNA whose function is to increase Cdc28 levels to ensure efficient cell cycle re-entry once adaptation has occurred.

In conclusion, the results obtained in this PhD Thesis provide a comprehensive analysis of the transcriptome of *S. cerevisiae* in response to osmostress. Specifically we have focused our attention on the identification of novel activities required for transcription of osmoresponsive genes. Moreover, we have been interested in globally understanding the role of Hog1 in reprogramming and targeting expression of several protein coding and noncoding RNAs and its impact on cell cycle progression.

CONCLUSIONS

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

- The search for novel activities important for osmostress gene expression yielded Ubp3, a ubiquitin protease, which is required for full transcriptional response upon osmostress.
- Phosphorylation of Hog1 at Ser695 induces changes in Ubp3 activity required for proper transcription initiation and elongation.
- Hog1 phosphorylated-Ubp3 deubiquitinates more efficiently RNA Pol II.

- Osmostress induces a rapid dissociation of RNA Pol II of the entire genome causing genome-wide downregulation.
- Hog1 bypasses the stress-induced downregulation of gene expression by targeting RNA Pol II to stress-responsive loci.
- Hog1 associates to RNA Pol II and RNA Pol III genes.
- Gene expression is dependent on the duration and intensity of Hog1 binding to target genes.
- Hog1 mediates chromatin remodeling to facilitate transcription at stress-responsive genes.

- Hog1 controls the expression of a new set of lncRNAs.
- Hog1 binds and recruits RNA Pol II at genes with lncRNAs.
- Terminators of genes containing lncRNA function as *bona fide* osmoresponsive promoters.

- Induction of *CDC28* lncRNA expression promotes chromatin remodeling the induction of *CDC28* gene expression upon stress.
- The establishment of gene looping permits the recruitment of Hog1 at the +1 nucleosome region and induction of *CDC28.*
- Stress-induced *CDC28* lncRNA results in an increase of Cdc28 that permits cells to re-enter the cell cycle more efficiently in response to stress.

SUPPLEMENTARY ARTICLES

Transient activation of the HOG MAPK pathway regulates bimodal gene expression

Serge Pelet, Fabian Rudolf, Mariona Nadal-Ribelles, Eulàlia de Nadal, Francesc Posas, Matthias Peter

Science 332.6030 (2011): 732-735.

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Transient activation of the HOG MAPK pathway regulates bimodal gene expression

Serge Pelet, Fabian Rudolf, Mariona Nadal-Ribelles, Eulàlia de Nadal, Francesc Posas, Matthias Peter

Science 332.6030 (2011): 732-735.

Osmostress-responsive genes rapidly switch from a repressed (almost silent) state to a maximal induction in a matter of minutes (e.g. expression of *STL1* in 10 minutes reaches a fold change above 200).

Gene activation needs to overcome an intracellular threshold, which is set by the cellular physiology, but little is known at the molecular level how cells set such a threshold. In this study, we developed and applied quantitative single cell measurements to investigate the dynamic interplay between Hog1 activation and the transcriptional output. Interestingly, we found that Hog1 activation increased linearly with osmostress, while the transcriptional output of the HOG-pathway exhibited a bimodal behavior. This bimodality was strongly influenced by specific transcription factors and components of the RSC and SAGA chromatin-remodeling complexes.

Moreover, using microfluidic assays to precisely control stress signals in real time, we demonstrated that a slow stochastic transition from a repressed to an active transcriptional state strongly depended on chromatin structure in conjunction that together with transient Hog1 activation is responsible for this bimodal behavior. The results described in this article showed for the first time a

molecular mechanism on how a cell can impose a transcriptional threshold in response to a linear signaling behavior.

Personal contribution to this work: Although I closely followed the work, my personal contribution was mainly focused on the experimental analysis of the role of chromatin remodeling in the bimodality of stress-responsive genes.

Time-dependent quantitative multicomponent control of the G_1-S network by the stress-activated protein kinase Hog1 upon osmostress

Miquel Àngel Adrover*, Zhike Zi*, Alba Duch, Jörg Schaber, Alberto González-Novo, Javier Jimenez, Mariona Nadal-Ribelles, Josep Clotet, Edda Klipp, and Francesc Posas

Science signaling 4.192 (2011): ra63

Adrover, Miquel Angel, Zhike Zi, Alba Duch, Jorg Schaber, Alberto Gonzalez-Novo, Javier Jimenez, Mariona Nadal-Ribelles, Josep Clotet, Edda Klipp, and Francesc Posas. "Time-dependent quantitative multicomponent control of the G1-S network by the stress-activated protein kinase Hog1 upon osmostress."*Science signaling* 4, no. 192 (2011): ra63. DOI: 10.1126/scisignal.2002204

Time-Dependent Quantitative Multicomponent Control of the G_1-S Network by the Stress-Activated Protein Kinase Hog1 upon Osmostress.

Miquel Àngel Adrover*, Zhike Zi*, Alba Duch, Jörg Schaber, Alberto González-Novo, Javier Jimenez, Mariona Nadal-Ribelles, Josep Clotet, Edda Klipp, and Francesc Posas

* These authors contributed equally to this work

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In this manuscript we performed an in depth analysis of the signaling events mediated by the Hog1 kinase in response to osmostress that lead to a cell cycle arrest in budding yeast. The study combines the use of biochemical and mechanistic studies with mathematical modeling to define the interplay of all the components involved in the G1/S transition.

Cell cycle delay induced by Hog1 in response to osmostress depends on the stage of G1 when the cells are challenged. The cell cycle delay correlates with enhanced activation of Hog1 as expected, along with the concomitant repression of Clb5. This appears to be the dominant molecular event that takes precedence over Cln2 production or Sic1 degradation. We also provided evidence by ChIP that Hog1 binds in response to stress at the *CLB5* promoter, correlating with its downregulation. Overexpression of Clb5 resulted in a less efficient arrest of cell cycle in response to osmostress, indicating that downregulation of *CLB5* is the driving event that blocks cell cycle at the G1/S transition specially at early G1. At late G1, the control of Sic1 becomes highly relevant to prevent Clb5-Cdc28 to fire replication. As mentioned above,

expression patterns of cyclins propose a very interesting function of Hog1 as a transcriptional repressor. Traditionally Hog1 associates to chromatin to recruit the transcriptional machinery and to “switch on” transcription. How and to which proteins target Hog1 to repress transcription is not yet understood.

Personal contribution to this work: Although I closely followed the work, my personal contribution was mainly of technical support performing the chromatin immunoprecipitation experiments.

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