The Role of Histone Modifications in Transcriptional Regulation upon Stress

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A mis padres

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Cris, Septiembre 2014

SUMMARY

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In response to fluctuations in the environment, all living organisms have the ability to sense, respond and adapt to the new conditions. In budding yeast (*Saccharomyces cerevisiae*) there is a massive and rapid reorganization of the transcriptional program in response to a stressful situation, which is governed by different signaling pathways, transcription factors, chromatin remodelers and histone modifiers. Many examples of histone posttranslational modifications (PTM) have been associated with transcriptional activation or repression under normal growth conditions, however little is known about the role of histones in the cellular adaptive response upon stress.

In this study, we systematically analyze by high throughput screens cellular growth and transcription initiation of stress-responsive genes in 569 histone point mutants upon heat and osmostress. These screens provide a novel global map of the histone residues required for cellular survival and transcriptional regulation in response to heat and osmostress. Moreover, we show that the histone residues required in response to stress depend on the type of gene and/or the type of stress, suggesting a “personalized”, rather than general, subset of histone requirements for each chromatin context.

Furthermore we characterized some examples of newly identified histone marks from histones H3 and H4 involved in the transcriptional regulation upon different stress

conditions. The lysine K79 on the surface of histone H4 and a novel regulatory region on histone H4 have been shown to be essential for transcription activation of Msn2/4 dependent genes specifically in response to heat stress. We also found that serine S47 on histone H4 is an example of residue specifically required in response to osmostress and two phosphorylable residues on histone H3: serine S57 and threonine T58 are commonly required for transcription activation upon both stress conditions. In summary, this work reveals new insights into the role of histones in transcriptional regulation under stress conditions.

Todos los organismos vivos tienen la capacidad de adaptarse a condiciones adversas en el medio en el que viven para sobrevivir. En la levadura *Saccharomyces cerevisiae* se produce una rápida reorganización en el patrón transcripcional en respuesta a una situación de estrés. Esta reorganización transcripcional está regulada por diferentes vías de señalización, factores de transcripción, complejos remodeladores de cromatina y enzimas modificadoras de histonas. Numerosos ejemplos de modificaciones postraduccionales en histonas han sido asociados con la activación o la represión génica en condiciones normales de crecimiento. Sin embargo, se conoce muy poco sobre el papel de las histonas en la adaptación celular frente a una situación de estrés.

En este trabajo, hemos analizado de forma sistemática el crecimiento celular y el inicio de la transcripción de genes que se inducen en respuesta a estrés en 569 mutantes puntuales de histonas en condiciones de estrés térmico y osmótico. Nuestros resultados proporcionan un mapa global de los residuos de las histonas esenciales para la supervivencia celular y la activación transcripcional en respuesta a estrés térmico y osmótico. Asimismo, estos análisis revelan que existe una especificidad respecto a los residuos de las histonas que son necesarios en respuesta a estrés dependiendo del tipo de gen y/o del tipo de estrés. Estos resultados sugieren un tipo de regulación

“personalizada”, en lugar de generalizada, en cada contexto de cromatina.

Además, en este trabajo hemos caracterizado algunos ejemplos de residuos en las histonas H3 y H4 que tienen un papel importante en la regulación transcripcional en respuesta a estrés. La lisina K79 en la superficie de la histona H4 y una nueva región reguladora en la histona H4 son esenciales para la transcripción de los genes dependientes de los factores de transcripción Msn2/4 en respuesta a estrés térmico. También hemos caracterizado la serina S47 de la histona H4 como un ejemplo de residuo necesario específicamente en la respuesta transcripcional a estrés osmótico y dos residuos en la histona H3: serina S57 y treonina T58 que son necesarios para la regulación de la transcripción tanto en respuesta a estrés térmico como osmótico. En conjunto, los resultados presentados en esta tesis proporcionan una nueva visión del papel de las histonas en la regulación transcripcional frente a una situación de estrés.

PREFACE

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Only 30 years ago, nucleosomes were considered as a merely way to package DNA into the nucleus, seen by most molecular biologists as static and uninteresting structures. Over the last fifteen years, this view changed completely with the demonstration that chromatin dynamics are essential for transcriptional regulation. New insights in the biochemistry of transcription and the power of yeast genetics demonstrated that histones play a key role in transcription. New techniques led to the identification of chromatin remodeling complexes, histone modifying enzymes and the role of histone posttranslational modifications in gene expression. Genome-wide analysis by DNA microarrays, tiling arrays, ChIP-seq and mass spectrometry had contributed to expand the knowledge on the chromatin and transcription fields by identifying, for instance, a great number of histone posttranslational modifications (PTM).

The first histone PTMs were described in yeast cells and due to the high degree of evolutionary conservation between yeast and human histones, many of them have been found in all eukaryotes, from yeast to humans. Numerous histone PTMs have been described in the histone tails and related to transcriptional activation or repression. However, over the last few years histone PTMs have been described on the globular domain of the four core histones H2A, H2B, H3 and H4 to have direct roles on transcriptional regulation, increasing the knowledge and the importance of the role of

histones in the transcription field. Of note, the histone PTMs required for the transcriptional response to stress are still unknown.

In this thesis, we have performed high throughput screens to identify histone residues that are essential for cell survival and for transcriptional regulation upon heat and osmostress. This work offers a new comprehensive global map of the histone requirements for cellular adaptation in response to heat and osmostress in *S. cerevisiae*. It also characterizes new histone marks required for the transcriptional response upon heat and/or osmostress.

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1. INTRODUCTION

1 INTRODUCTION

1.1 TRANSCRIPTION AND CHROMATIN IN YEAST

Transcription is a complex process responsible for the generation of a mature messenger RNA (mRNA) molecule using a DNA molecule as a template. In eukaryotic cells transcription is performed by three different RNA polymerases (RNA Pol), each of them specialized in a subset of mRNA molecules. RNA Pol I is responsible for transcription of ribosomal RNA genes, RNA Pol II transcribes most of the protein-coding genes and RNA Pol III transcribes tRNA genes, 5S RNA genes and snRNA genes.

RNA Pol II is the best characterized of all of them. It is a large multisubunit enzyme (0.5 MDa) composed of 12 proteins encoded by the genes *RPB1* to *RPB12*, numbered from the largest to the smallest. All RNA Pol II subunits except for Rpb4 and Rpb7 are essential (Woychik & Young 1989) . Rpb1 and Rpb2 are the largest and most evolutionary conserved subunits and, together form the catalytic site, the pore for entering nucleotide triphosphates, and the binding sites for DNA and the DNA-RNA hybrid in the transcription elongation complex. A unique feature of RNA Pol II is a repeated seven-residue motif (YSPTSPS) at the C-terminal domain (CTD) of Rpb1. The unstructured CTD is heavily phosphorylated and dephosphorylated. These different phosphorylation states are important along the transcription cycle.

1.1.1 The eukaryotic transcription cycle

The transcription cycle consist of several stages (Fuda et al. 2009): chromatin opening, Pre-Initiation Complex (PIC) formation, initiation, elongation and termination.

Chromatin opening: Nucleosomes *per se* act as a natural barrier for transcription; therefore the first thing RNA Pol II has to achieve is to gain access to DNA. At this stage histone modifying enzymes, chromatin remodeling complexes and chaperones play an essential role to regulate chromatin structure and allow accessibility to promoter regions (see below).

PIC formation: The PIC includes the general transcription factors (TFIIB, TFIID, TFIIE, TFIIF and TFIIH) and cofactors that act as coactivators or corepresors (Orphanides et al. 1996), which together facilitate the entry of unphosphorylated RNA Pol II to the PIC. At this stage, activators such as Spt-ADA-Gcn5 acetyltransferase complex (SAGA) and Mediator are recruited to promoters. The formation of the PIC at the promoter does not guarantee productive transcription (Shandilya & Roberts 2012).

Transcription initiation: once the PIC is assembled at the promoter region, RNA Pol II scans the DNA sequence for transcription start sites. RNA Pol II scanning involves DNA unwinding and DNA translocation (Hahn & Young 2011) leading to formation of an open complex between RNA Pol II and the DNA template. Then, RNA Pol II initiates transcription with the formation of the first phosphodiester bond

(Orphanides et al. 1996). During initiation, RNA Pol II is phosphorylated at Ser5 and Ser7 of the CTD, which is the signal for the recruitment of mRNA capping enzymes and other factors required for initiation of transcription (Egloff & Murphy 2008). At this stage the complex is still unstable and often results in abortive transcription (Shandilya & Roberts 2012).

Elongation: Once the nascent mRNA reaches six nucleotides long, the complex is considered to be stable (Cheung & Cramer 2012) and enters into productive elongation across the coding region. As the RNA Pol II progresses towards the 3' end of the gene, there is a gradual loss of Ser5 and Ser7 phosphorylation and an increase of Tyr1 and Ser2 phosphorylation of the CTD. Tyr1 phosphorylation increases after the transcription start site (TSS) and decreases before polyadenylation site (Mayer et al. 2012). Recently, it was found that Tyr1 phosphorylation causes the recruitment of the elongation factor Spt6 and excludes termination factors from gene loci (Mayer et al. 2012). Ser2 phosphorylation signals for the recruitment of factors required for mRNA processing, termination and mRNA export, as well as for chromatin modification and remodeling factors (Egloff & Murphy 2008).

Histone posttranslational modifications (PTM) are essential during elongation ahead and behind elongating RNA Pol II allowing efficient passage of RNA Pol II and

preventing cryptic transcription (Akey & Luger 2003; Smolle & Workman 2013).

Termination and recycling: Termination occurs when RNA Pol II reaches 1kb downstream the Poly (A) site. The newly synthesized mRNA molecule is released and further processed and RNA Pol II is removed from the DNA template and prepared to reinitiate a new round of transcription.

1.1.2 Chromatin remodeling

Chromatin is organized into various levels of condensation, allowing large amounts of genetic material fit into the nucleus. The fundamental structural and functional unit of chromatin is the nucleosome core particle. The nucleosome consists of 147 base pairs (bp) of DNA wrapped 1.7 times around an octamer of histones consisting of two H2A-H2B dimers and a H3-H4 tetramer. The organization of the genome into chromatin represents a natural barrier for transcription.

One way of changing chromatin structure is by using chromatin remodelers that use the energy of ATP hydrolysis to break existing DNA-histone contacts leading to nucleosome rearrangement. There are four major families of remodeling complexes in yeast: SWI/SNF family (SWI/SNF and RSC), ISWI family (Isw1 and Isw2), INO80 family (INO80 and SWR1) and CHD1 family (Chd1) (Gangaraju & Bartholomew 2007). Recruitment of these complexes to chromatin can be mediated by histone postranslational

modifications or by interaction with general transcription factors.

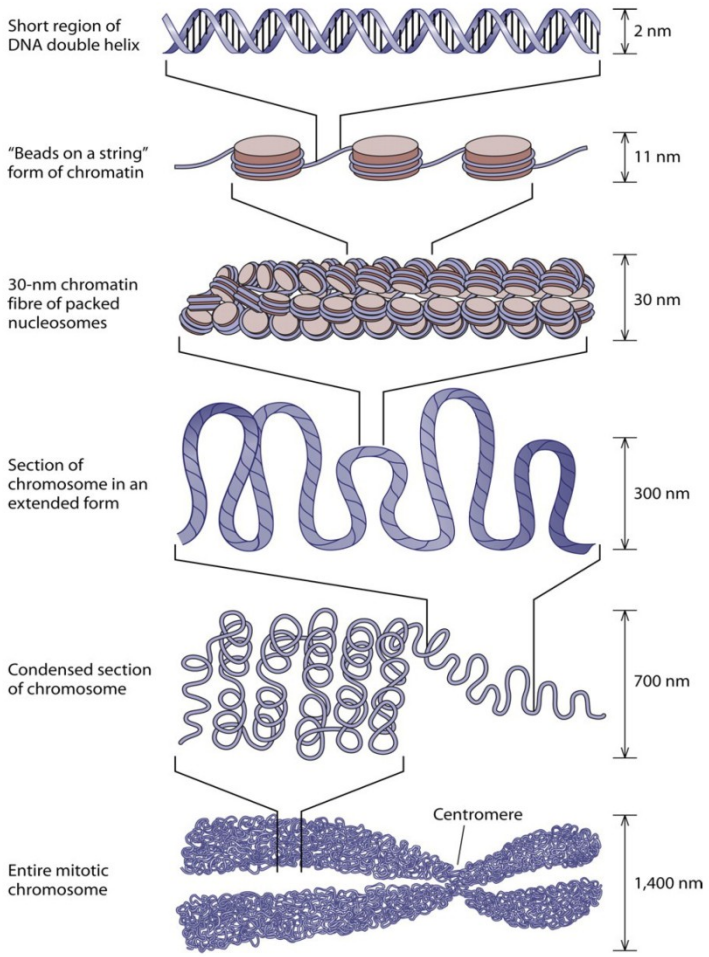


Figure 1. DNA is packaged into chromatin
In the eukaryotic nucleus, the DNA is higly compacted into chromatin. The basic subunit of chromatin is the nucleosome, which consists of an octamer composed by two copies of the four core histones (H2A, H2B, H3 and H4) that has 147 bp of DNA wrapped around it. Chromatin is organized in different compaction levels that go from the "beads on a sting", where single nucleosomes are accessible, to the entire chromosome. Figure from (Jansen & Verstrepen 2011).

Remodeling complexes can alter the chromatin structure by different mechanisms: nucleosome eviction, nucleosome sliding or histone variant incorporation. Differently to nucleosome eviction, sliding makes the nucleosome spread out over a broad region along DNA creating nucleosome fuzziness. These nucleosome rearrangements occur at both promoters and coding regions (Cairns 2009; Jiang & Pugh 2009) and the rates of remodeling depend on their genomic location and their modification status (Cairns 2009).

1.1.3 Histone modifications

Each of the four core histones, H3, H4, H2A and H2B consist of two structurally and functionally domains: a globular domain that forms the scaffold around which DNA is wrapped and flexible unstructured tails protruding from the nucleosome surface.

Core histones are subject to a vast number of postranslational modifications (PTMs), such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, succinlylation, malonylation, butyrylation, O-GlcNAcylation, hydroxylation and crotonlyation. Additionally, different flavors of some modifications appear in histones. For example lysine residues (K) can be mono, di or trimethylated and arginine (R) can be symmetrically or asymmetrically methylated. In the last years, mass spectrometry analysis have revealed a great number of new

histone PTMs (Tan et al. 2011) and also novel types of histone modifications, such as tyrosine hydroxylation, lysine crotonylation (Tan et al. 2011) and glutamine methylation (Tessarz et al. 2014).

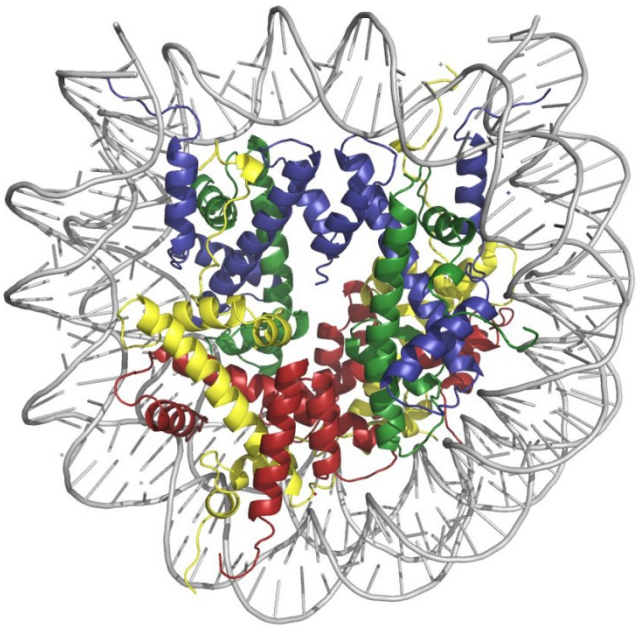


Figure 2. Crystal structure of the nucleosome core particle
The nucleosome is composed by 147 bp of DNA wrapped around an octamer of histone, which is composed by two copies of each canonical histone: H3 (blue), H4 (green), H2A (yellow) and H2B (red). This representation was made using Pymol software and the coordinates from accession number 1ID3 in the Protein Data Bank (White et al. 2001).

Modifications of histones are carried out by specialized enzymes, which can modify either a specific substrate, or several ones. For example, Set1 specifically methylates lysine 4 of histone H3 (H3K4). However, Gcn5 acetylates lysines K4, K9, K14, K18 and K23 of histone H3.

Histone PTMs can influence chromatin structure directly, by changing the charge distribution and the intra and inter-nucleosome contacts or indirectly, by recruiting downstream effectors that influence chromatin structure. For example H4K16 acetylation has been shown to directly prevent folding of chromatin into higher-order structures (Shogren-Knaak et al. 2006). Alternatively, during elongation H3K36me3 on body genes signals for the recruitment of Rpd3S histone deacetylase complex, which prevents cryptic transcription by deacetylating histones after RNA Pol II passage (Smolle & Workman 2013).

Some histone PTMs have been traditionally associated to transcriptional activation, such as H3K4me3, H3K36me3 and H2BK123ub, and some others to transcriptional repression, such as methylation of H3K9, H3K27 and H4K20. These PTMs located on the histone tails have been used in a large number of genome-wide analyses as hallmarks for actively transcribed chromatin or silenced chromatin (reviewed in (Millar & Grunstein 2006)).

However, not only the PTMs on the histone tails regulate transcription. H3K79 methylation by Dot1 is involved in transcriptional silencing and it was the first histone PTM described on the globular domain (Leeuwen et al. 2002). Since then, many PTMs involved in transcriptional regulation have been identified outside of the histone tails. For example the well studied H3K56 acetylation (Williams et al. 2008) or the recently identified H4K31 ubiquitination (Kim et al. 2013),

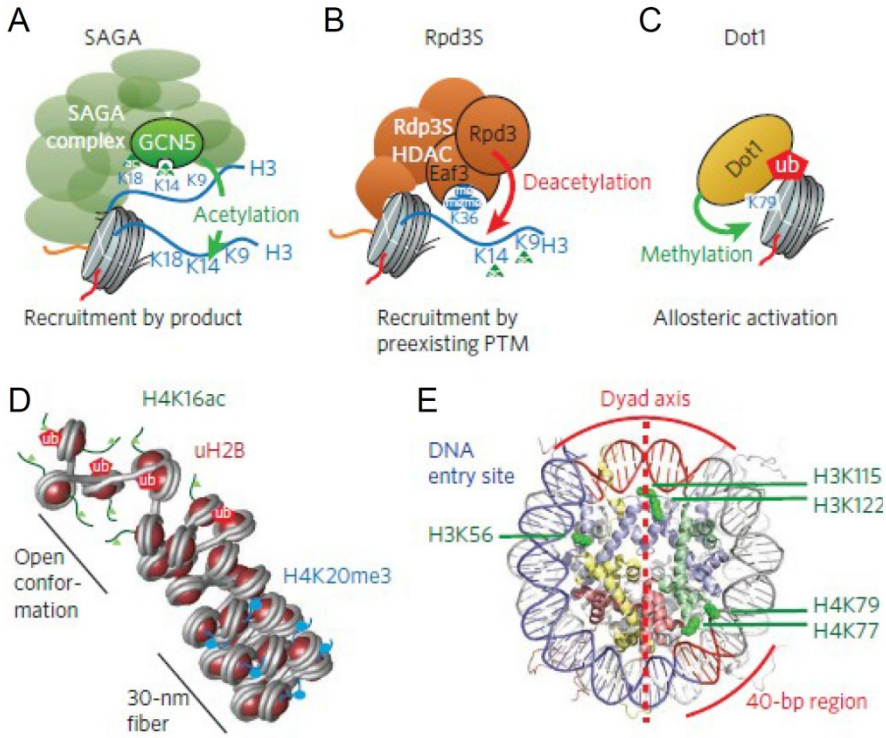


Figure 3. Control of chromatin function by histone postranlational modifications

Examples of how histone postranlational modifications can modulate chromatin structure and function. **A)** Histone modifiers can be recruited to chromatin by a histone mark and cooperate to install more copies of the same mark, as the case of the SAGA complex. **B)** Alternatively, a histone modifier can be recruited by a histone mark and act on different ones, as observed in the Rpd3S HDAC complex. **C)** There is crosstalk between histone marks; one example is the methylation of H3K79 by the mehtyltransferase Dot1, which is activated by H2B ubiquitination. **D)** Some histone marks such as H4K16ac and H4K20me3 alter the structural properties of chromatin fibers by destabilizing or stabilizing, respectively. **E)** Specific acetylation sites on the globular domain of histones H3 and H4 modulate nucleosome stability that can lead to increased DNA accessibility to downstream effectors. Figure adapted from (Fierz & Muir 2012).

H3K122 acetylation (Tropberger et al. 2013) and H3K64 acetylation (Di Cerbo et al. 2014) are all of them in the globular domain of the histones.

Recently, it has also been described that the same modification can have opposing roles in transcription depending on the chromatin environment; one interesting example of this is the well known H3K4me3. This mark, as previously mentioned, is considered a hallmark for transcription activation; however a repressive role of H3K4me3 has been described under diamide stress in *S. cerevisiae* (Weiner et al. 2012).

The understanding on the histone field has grown enormously during the last years. New ideas about PTMs requirements in different chromatin environments have emerged and numerous new PTMs have been identified. However, very little is still known about the histone residues and the histone PTMs involved in transcriptional regulation in response to stress, in this PhD thesis we will focus on their identification and characterization.

1.2 HEAT STRESS

Variation in external temperature is one of the most common environmental stresses experienced by a yeast cell. While multicellular or motile organisms can usually cope with these situations by changing the location or controlling internal homeostasis, for unicellular organisms, like yeast, an increase of extracellular temperature represents a challenging

problem for survival. In this situation, yeast cells must adapt to this new condition or perish. *S. cerevisiae* grows optimally between 25ºC and 30ºC and a sudden heat shock can cause many deleterious effects in the internal organization of the cell.

1.2.1 The damaging effects of heat

Some of the major problems provoked by heat shock are protein unfolding and unspecific aggregation, processes that lead to imbalance of protein homeostasis. Most of the morphological and phenotypic effects of heat shock can be explained by imbalance of protein homeostasis. Severe effects are also observed in the cytoskeleton. Cytoskeleton reorganization leads to the collapse of intermediary actin and tubulin networks and loss of the proper localization of organelles and intracellular transport processes. Golgi system and the endoplasmic reticulum are fragmented. Moreover, stress granules appear on the cytoplasm, containing non-translating mRNAs, translation initiation components and other proteins affecting mRNA function (Richter et al. 2010).

In addition to defects on the cytoplasm, there are also changes in membrane morphology. This changes lead to higher membrane fluidity and permeability causing a drop in cytosolic pH and imbalance in ion homeostasis (Richter et al. 2010).

Furthermore, nuclear processes are affected by heat shock. RNA splicing is strongly affected and also nucleoli

function. Nuclear granules composed of incorrectly processed ribosomal RNAs and aggregating ribosomal proteins appear in response to heat stress (Boulon et al. 2010). Together all these effects lead to cell cycle arrest and compromised cell survival. Therefore the capability to activate a rapid adaptive response is essential for cell viability.

There are two sensing mechanisms for heat stress in *S. cerevisiae*. First, the accumulation of misfolded proteins triggers the activation of Heat shock factor 1 (Hsf1), and the subsequent induction of the Heat Shock Response (see below). However, it is not clear whether is the accumulation of misfolded forms of existing proteins, misfolded forms of nascent chains or both of them the cause of the problem (Morano et al. 2012). Second, the direct sensing of temperature by primary thermosensory structures, such as DNA, RNA, proteins and lipids, which either can have a direct effect or lead to activation of signal transduction pathways (Klinkert & Narberhaus 2009; Leach & Cowen 2014).

After few minutes of exposure to heat stress, there is a rapid and massive reorganization of the transcriptional pattern of the cell. Genome-wide analysis showed that upon heat shock around 15% of the genes change their induction more than two fold (Causton et al. 2001; Gasch et al. 2000). In *S. cerevisiae* two main transcription factors control the transcriptional program in response to heat stress, the Hsf1 and the partially redundant transcription factors Msn2 and Msn4 (see below). Indeed, microarray analysis using

conditional and knockout mutants of Hsf1 and Msn2/4 respectively, suggest that these three factors are responsible for the bulk of transcriptional regulation in response to heat stress (Morano et al. 2012).

1.2.2 The Heat Shock Response

The Heat Shock Response (HSR) is an evolutionary conserved mechanism for cellular protection against high temperature. HSR is characterized by a massive, rapid and transient induction of gene expression. Most of the genes induced in the HSR encode for Heat Shock Proteins (Hsp), which maintain protein homeostasis, relieve folding defects and prevent protein aggregation and cellular damage.

In all eukaryotic cells the HSR is controlled by Hsf1. It is suggested that HSR is induced not only in response to heat stress but also in response oxidative stress, heavy metals or ethanol due to accumulation of missfolded proteins under this conditions (Richter et al. 2010).

Heat shock factor 1 (Hsf1)

Hsf1 is present in all eukaryotic cells. In mammals there are four distinct HSF isoforms: HSF1 regulates the Heat Shock Response, HSF2 is involved in developmental gene expression and the roles of HSF3 and HSF4 are less well understood, but suggested to interact with HSF1 to modulate gene expression (Akerfelt et al. 2010). In lower eukaryotes,

including Drosophila and yeast, there is only a single HSF gene that encodes for the essential gene *HSF1*.

Hsf1 is composed of four different domains: an N-terminal “winged” helix-turn-helix DNA binding domain followed by a leucine zipper motif essential for trimerization and activation, a serine-rich regulatory domain, and a carboxy-terminal transcriptional activation domain. *S. cerevisiae* Hsf1 includes an amino-terminal extension of around 150 amino acids that works as a second transcriptional activation domain (Sorger 1990). The two activation domains are required in different situations depending on the response. While the carboxy terminal activation domain is required to mediate transient and sustained responses to high temperature, the amino terminal domain is specifically required for the transient response (Sorger & Pelham 1988; Nieto-Sotelo et al. 1990)

Hsf1 binds to Heat Shock Elements (HSE) at promoter regions. A typical HSE is composed by repeating units of the sequence nGAAn. There are different types of HSE depending on the spacing and position of the repeats: perfect, gapped and stepped. The perfect HSE has three inverted repeats in a contiguous array (5’-nTTCnnGAAnnTTCn-3’) and it is present in promoter regions of genes, such as *HSP26* and *SSA1*. Gapped HSE has to consecutive inverted sequences and the third sequence separated by 5bp, and it is present in promoter regions of genes, such as *HSP82* or *CUP1*. Stepped HSE has 5bp gaps

separating all three modules. This last group of HSE is present in promoter regions of genes like *SSA3* and *HSP12*. In *S. cerevisiae* Hsf1 is constitutively bound to perfect and gapped HSE, however the binding to stepped HSE is induced by an increase in temperature (Erkine et al. 1999; Hahn et al. 2004).

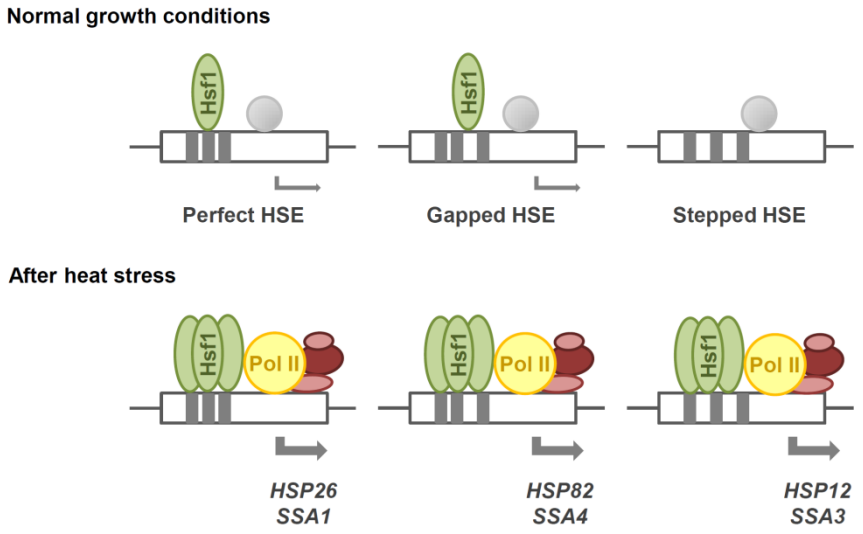


Figure 4. In *Saccharomyces cerevisiae* the Heat Shock Response is controlled by Hsf1 transcription factor
Hsf1 binds to heat shock elements (HSE) at promoter regions. There are different types of Heat Shock Elements (HSE): perfect, gapped and stepped. Hsf1 is bound at perfect and gaped HSE already in normal growth conditions allowing basal transcription of those genes. In response to heat stress, Hsf1 is rapidly recruited to all types of HSE leading to the recruitment of RNA Pol II, chromatin remodelers and histone modifiers (represented in red) that ends up in a massive and rapid transcriptional activation of the genes controlled by Hsf1. Nucleosomes are represented as grey spheres.

In mammals and *Drosophila*, trimerization of Hsf1 is a prerequisite for binding to HSEs. In these organisms, Hsf1

proteins are inactive monomers at physiological conditions, and in response to heat stress they trimerize, enter into the nucleus and bind HSEs. In *S. cerevisiae,* regulation of Hsf1 is still not well understood. Hsf1 was believed to form always trimers, although some reports have shown evidences of monomer-trimer transition regulation (Erkina & Erkine 2006). Post translational modifications on Hsf1, such as phosphorylation and sumoylation, have been linked to Hsf1 binding and transcriptional activity (Hong et al. 2001; Hilgarth et al. 2004; Guettouche et al. 2005). However binding of Hsf1 to stepped HSE does not require trimerization nor phosphorylation (Hashikawa et al. 2006). These observations suggest that there is a complex regulation of Hsf1, probably involving postranslational modifications and conformational changes depending on the architecture of the HSE. *HSP82* is a model gene used in most of the studies to understand the regulation of Hsf1 (Gross et al. 1993; Erkine et al. 1999; Zhao J et al. 2005; Zhao R et al. 2005).

1.2.3 The Environmental Stress Response

In *S. cerevisiae*, in addition to the HSR, heat stress also induces the Environmental Stress response (ESR) controlled by Msn2/4 transcription factors. The ESR is involved in the reprogramming of a significant part of the transcriptome in response to several environmental stresses, such as heat stress, osmostress, oxidative stress or nutrient starvation. Genome-wide analysis under several stress

conditions have shown that the ESR is involved in the transcriptional regulation of around 900 genes. The ESR activates around 300 genes, which are mainly involved in antioxidant and carbohydrate metabolism, protein folding and cell signaling, and also represses around 600 genes including ribosomal protein genes and protein synthesis related genes (Gasch et al. 2000; Causton et al. 2001).

Msn2/4 transcription factors

As mentioned, Msn2 and its close homolog Msn4 (referred as to Msn2/4) are required for expression of a big subset of genes in response to multiple stresses; however they are not essential for viability under normal growth conditions.

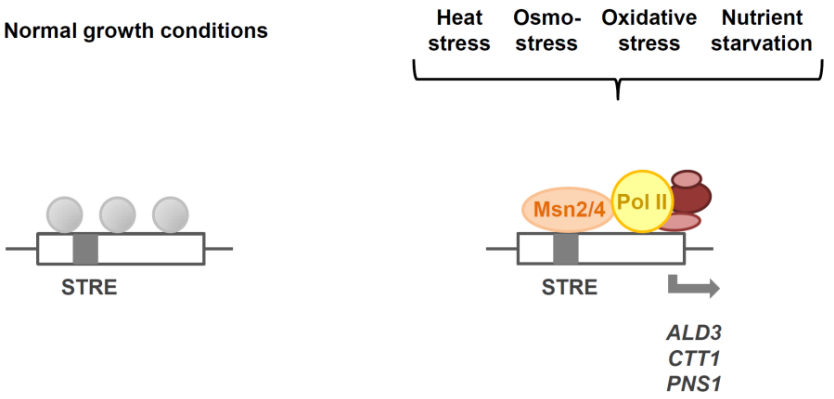


Figure 5. The Environmental Stress Response is regulated by Msn2/4 transcription factors
In response to different environmental stresses, Msn2/4 transcription factors bind to stress response elements (STRE) sequences on promoter regions. The binding of Msn2/4 recruits RNA Pol II, chromatin remodeler and histone modifiers (represented in red) and allows transcription of a subset of stress responsive genes.

Msn2/4 are Cys2His Zinc finger-type transcription factors that recognize and bind to consensus Stress Response Elements (STRE) on gene promoter regions (Martínez-Pastor et al. 1996). The STRE sequence consists of a pentameric core of CCCCT that usually appears in two or more repetitions at Msn2/4 target promoters (Moskvina et al. 1998; Treger et al. 1998). In some cases Msn2/4 binds upstream of genes to other consensus sites, in concert with other transcription factors (Capaldi et al. 2008; Ni et al. 2009).

There are several mechanisms described to regulate Msn2/4 activity. The major regulatory mechanism is the control of rapid and oscillatory shuttling of Msn2/4 between nucleus and cytoplasm (Jacquet et al. 2003). Phosphorylation by two distinct kinases, PKA and Snf1, controls Msn2 cellular localization and activity. PKA phosphorylates Msn2 on the nuclear localization signal (NLS) inhibiting nuclear import and consequently activation of gene expression (Görner et al. 1998). Snf1 kinase also phosphorylates and inhibits Msn2 function. This inhibition is counteracted by the phosphatase PP1, which directly dephosphorylates Msn2 and negatively influences Snf1 activity, setting a double mechanism to ensure Msn2 activation (Mayordomo et al. 2002; de Wever et al. 2005; Lenssen et al. 2005). Besides cellular localization, Msn2 is also controlled by degradation in the nucleus upon chronic activation (Lallet et al. 2004) and by transactivation (Boy-Marcotte et al. 1999). The TOR pathway is also involved in the regulation of Msn2/4 in response to heat stress;

however the activating upstream mechanism remains unclear.

Several studies have shown the contribution of Msn2/4 and Hsf1 for the activation of heat stress-responsive genes (Boy-Marcotte et al. 1999; Amorós & Estruch 2001; Grably et al. 2002). In one hand, there is a group of genes specifically controlled by Msn2/4, such as *ALD3* or *CTT1*. On the other hand, there is another group of genes controlled specifically by Hsf1, such as *HSP82* or *SSA3*. Additionally, there is a third group of genes that contain both STRE and HSE consensus sequences on their promoters, such as *HSP12* or *HSP26* and therefore are co-regulated by both Msn2/4 and Hsf1 transcription factors.

1.2.4 Chromatin remodeling and histone modifications upon heat stress

Chromatin remodeling is a dynamic process essential for the efficient transcriptional regulation in response to stress (Uffenbeck & Krebs 2006; Shivaswamy et al. 2008; Jiang & Pugh 2009). Few minutes after a heat shock there is a rapid nucleosome rearrangement at promoters and coding regions of stress-responsive genes in order to allow transcription (Gross et al. 1993; Erkine & Gross 2003; Zhao J et al. 2005; Erkina et al. 2008; Petesch & Lis 2012).

SWI/SNF remodeling complex is directly recruited to heat shock gene promoters during heat stress. SWI/SNF is required for nucleosome rearrangement, RNA Pol II

recruitment and Hsf1 binding on *HSP12*, which is redundantly controlled by Hsf1 and Msn2/4. However, the role of SWI/SNF is not essential at Hsf1-dependent genes, such as *HSP82* and *SSA3* (Erkina et al. 2008). Following studies showed interplay between SWI/SNF, RSC and ISWI chromatin remodeling complexes for nucleosome displacement and RNA Pol II recruitment at heat stress-induced promoters. Again different contributions of these chromatin remodeling complexes were observed in Hsf1 and Msn2/4 dependent genes (Erkina et al. 2010).

Besides chromatin remodeling, histone PTMs have also been related to heat-dependent changes in gene expression. In *HSP82* promoter there is an increase on histone acetylation preceding nucleosome displacement upon heat shock (Zhao J et al. 2005). Interestingly, the degree and the kinetics of H3 acetylation are different between different heat-responsive promoters (Erkina & Erkine 2006). Although acetylation was usually associated with transcriptional activation (Deckert & Struhl 2001; Zhao J et al. 2005; Han et al. 2008), in the past years has also been associated to transcriptional repression (de Nadal et al. 2004). Rpd3 histone deacetylase complex has a dual role in transcriptional regulation in response to heat stress. Rpd3 deacetylase complex works as a repressor at promoters of Hsf1-dependent genes (Kremer & Gross 2009), however it is essential for full activation of Msn2/4-dependent genes (see supplementary article (Ruiz-Roig et al. 2010)). These studies

indicate that a histone modifier might play different roles depending on the chromatin environment.

The role of histones in the adaptive response to heat stress has been studied before by screening histone mutant libraries. Several screens described subsets of residues within the nucleosome essential for survival upon heat stress. These screens are summarized in a database called Histonehits (http://histonehits.org).

1.3 OSMOSTRESS

A sudden change in osmotic pressure is another common stress that yeast cells can suffer. Changes in external osmolarity lead to a rapid change in water activity, which is defined as the chemical potential of free water in solution. Under normal growth conditions the water activity on the cytoplasm is lower than it surrounding environment. However, two different opposed situations can alter this balance: hypoosmotic shock that provokes a rapid water influx leading to cell swelling, and hyperosmotic shock (or osmostress) that provokes a rapid water outflow leading to cell shrinking.

In their natural environment, yeast cells can suffer sudden changes in the external osmolarity. Since water movements through the cellular membrane are very fast, the adaptive response has to be activated within seconds after the osmotic shock. The adaptive response is an active mechanism based on sensing osmotic changes and

responding to maintain optimal osmolyte concentration. Production and accumulation of glycerol restore the balance of internal osmolarity and lead to a water influx that finally restore cell volume (Hohmann 2002).

Several signaling pathways are activated in response to osmostress. The most important and best characterized of all of them is the High Osmolarity Glycerol (HOG) pathway. The HOG pathway is activated within seconds by osmotic shock and it is an essential part of the cellular adaptation to stress, since cells defective in activating the HOG pathway are not able to survive at high osmolarity (Brewster et al. 1993).

Nonetheless, changes in external osmolarity activate other signaling pathways: the cAMP-dependent Protein Kinase A (PKA) pathway (Marchler et al. 1993), the production of phosphatidylinositol-3-5-bisphosphate (Dove et al. 1997) and the Cell Wall Integrity pathway (Davenport et al. 1995). The PKA pathway mediates a general stress response observed in response to osmostress, heat stress, oxidative stress, nutrient starvation and high ethanol levels (Marchler et al. 1993). Therefore PKA probably does not respond directly to osmotic changes, actually regulation of PKA in response to stress is not well understood. Additionally it has been observed that production of phosphatidylinositol-3-5-bisphosphate is stimulated by osmostress and could act as a second messenger in osmostress signaling (Dove et al. 1997).

1.3.1 The High Osmolarity Glicerol (HOG) pathway

The HOG pathway is a stress-activated protein kinase (SAPK) cascade. This pathway is conserved through evolution. The high functional conservation between HOG pathway (in yeast) and the p38 pathway (in mammals) is illustrated by the fact that p38 MAPK can rescue osmosensitivity of *hog1* mutants (Galcheva-Gargova et al. 1994; Sheikh-hamad & Gustin 2004).

The HOG pathway is composed by membrane-associated osmosensors, and by an intracellular signaling cascade whose central core is the Hog1 MAPK.

The upstream part of the HOG pathway comprises the functionally redundant, but mechanistically distinct, Sln1 and Sho1 branches. Signal coming from either branch converges on the MAPKK Pbs2, which specifically activates Hog1 MAPK. Activated Hog1 is translocated into the nucleus where regulates transcription and cell cycle progression. Although localization of activated Hog1 is mainly nuclear, it has also targets on the cytoplasm (Saito & Posas 2012).

1.3.1.1 Signaling through the HOG pathway

As mentioned, upstream in the HOG pathway there are two independent signaling branches, the Sln1 branch and the Sho1 branch. The osmosensor of the Sln1 branch is the sensor histidine kinase Sln1, which changes its activity depending on the membrane turgor and transmits the signal through a two-component mechanism to the MAPKKKs Ssk2

and Ssk22 (Posas et al. 1996). Once activated, Ssk2/Ssk22 initiates a kinase cascade phosphorylating Pbs2 MAPKK, which specifically phosphorylates Hog1 MAPK. Differently, the Sho1 branch involves two osmosensors, Hkr1 and Msb2, which are transmembrane mucin-like proteins. Hkr1 and Mbs2 were thought to be functionally redundant; however, it was recently reported that Hrk1 and Mbs2 activated the Hog1 MAPK cascade by different mechanisms (Tanaka et al. 2014).

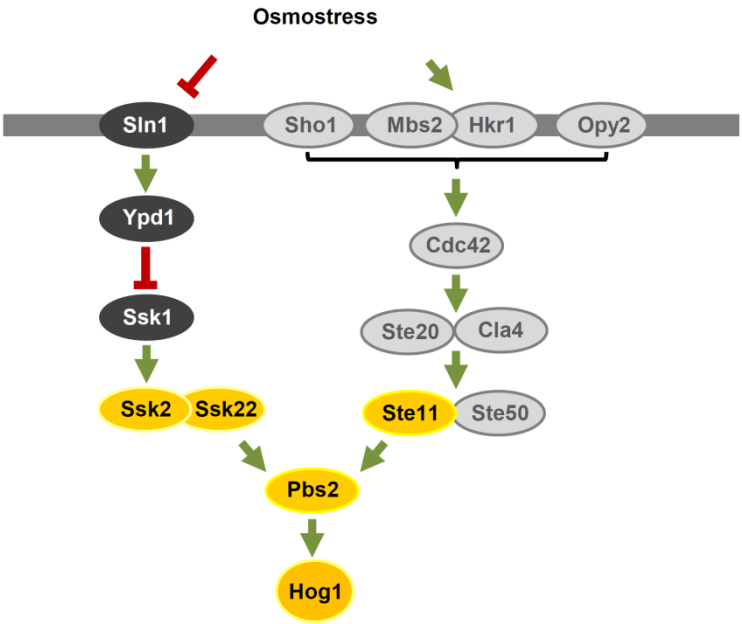


Figure 6. A schematic diagram of the HOG pathway
Two major independent upstream mechanisms lead to the activation of specific MAPKKK (Ssk2/Ssk22 and Ste11) that converge on a common MAPKK (Pbs2). Activated Pbs2 phosphorylates the MAPK Hog1 which controls the cellular adaptive response to osmostress.

Activation of the osmosensors leads to activation of the PAK-like kinases Ste20 and Cla4 by inducing their association with the membrane-bound small G-protein Cdc42 (Lamson et al. 2002). When activated, Ste20/Cla4 phosphorylate and activate the Ste11 MAPKKK (van Drogen et al. 2000), which is translocated to the membrane by interaction with Ste50 adaptor protein and by the membrane anchor protein Opy2. Activated Ste11 phosphorylates and activates Pbs2 MAPKK, which finally transmits the signal to the Hog1 MAPK (Raitt et al. 2000; van Drogen et al. 2000).

1.3.1.2 Physiological roles of Hog1

Once activated, Hog1 induces the adaptive response essential to ensure cell survival in response to osmostress. The osmoadaptive response involves metabolic adaptation, protein synthesis, cell cycle regulation and reprogramming of transcription.

Metabolic adaptation

The major strategy for survival upon osmostress is to produce and accumulate glycerol in order to maintain the water balance and restore the cell turgor (Saito & Posas 2012). Control of this glycerol accumulation is one of the most important roles of the HOG pathway in osmostress adaptation. Several mechanisms control the accumulation of glycerol: regulation of transcription, metabolic adjustments, and glycerol export and import (Hohmann 2002; Dihazi et al. 2004; Saito & Posas 2012).

Protein synthesis

Protein synthesis rapidly decreases in response to osmostress. However, expression of genes encoding functions required for stress adaptation is stimulated under these conditions and their translation has to be ensured.

It seems that Hog1 plays a role regulating protein synthesis. Microarray analysis of mRNA associated with polysomes showed a class of genes whose translation was favored in response to osmostress ("translation-activated osmostress genes"). These transcripts are recruited to polysomes in a Hog1 dependent manner, clarifying that Hog1 preferentially targets a specific subset of genes for translation (Romero-Santacreu et al. 2009; Warringer et al. 2010; Garre et al. 2013).

Cell cycle regulation

Progression through the cell cycle is regulated by the nutrient accessibility and stress stimuli. In response to osmostress, Hog1 mediates a transient cell cycle arrest in order to permit cell adaptation (Clotet & Posas 2007).

Cell cycle is divided in four phases: G1-phase, S-phase (synthesis), G2-phase and M-phase (mitosis). Hog1 regulates cell cycle progression at several phases of the cell cycle (Duch et al. 2012).

G1 phase is delayed by Hog1 activation acting through two mechanisms: directly targeting the CDK inhibitor Sic1 and

downregulating G1 cyclin expression (Escoté et al. 2004; Zapater et al. 2005; Adrover et al. 2011).

When osmostress occurs in S-phase, Hog1 phosphorylates a component of the replication machinery; Mrc1. Phosphorylation of Mrc1 by Hog1 delays early and late origin firing and slows down the progression of the replication complex preventing collision between replication and transcription machineries. S-phase delay by Mrc1 describes a novel checkpoint pathway that is independent from the DNA-damage checkpoint pathway (Duch et al. 2013).

Hog1 also controls the progression through G2 in response to osmostress downregulating Clb2 expression and Clb2/Cdc28 activity (Alexander et al. 2001; Clotet et al. 2006). Although Hog1 has been linked to exit of mitosis through the Cdc14 early anaphase release (FEAR) pathway (Reiser et al. 2006), in this case the molecular mechanism is still unclear.

Control of gene expression

Control of gene expression is tightly regulated upon stress. It is a fast process that allows the cell to change its transcriptional program within minutes in the presence of stress and to return to its basal state after the stress is removed (de Nadal et al. 2011).

Genome-wide transcriptional studies in *S. cerevisiae* have shown that 5-7% of the protein coding genes show significant changes in their expression levels after mild osmotic shock (0.4M NaCl) (Causton et al. 2001; Gasch et al.

2000). Studies using a *hog1*Δ strain showed that up to 80% of the induced transcripts are dependent on Hog1 (Rep et al. 2000; Posas et al. 2000; O´Rourke & Herskowitz 2004; Capaldi et al. 2008). The Hog1 dependent osmostress-induced genes are mainly involved in carbohydrate metabolism, protein biosynthesis, ion homeostasis, amino acid metabolism and signal transduction (Posas et al. 2000). Hog1 regulates transcription at different stages on the transcription cycle and through different mechanisms (see below).

1.3.2 Regulation of transcription by Hog1

In the presence of osmostress there is transcriptional burst of osmoresponsive genes that coincide with nuclear localization of Hog1. The MAPK controls the entire process of mRNA biogenesis: chromatin dynamics, transcription initiation, elongation, mRNA modification, stability and export (de Nadal & Posas 2010).

1.3.2.1 Chromatin remodeling by Hog1

Recruitment of chromatin remodeling complexes and histone modifiers allows accessibility of the transcription machinery to promoters and coding regions. In response to osmostress, there is a huge and rapid reorganization of nucleosomes and recruitment of RNA Pol II to osmoresponsive genes that is dependent on Hog1 (Nadal-Ribelles et al. 2012). Additionally, chromatin-remodeling

complexes such as, RSC (Mas et al. 2009), SWI/SNF (Proft & Struhl 2002) are recruited in response to osmostress in a Hog1 dependent manner. It is also described that INO80 chromatin-remodeling complex is responsible for the reposition of nucleosomes after RNA Pol II passage (Klopf et al. 2009).

Working together with chromatin remodelers are the histone modifiers, which covalently modify histones adding a control layer of regulation to the transcription process. Although histone deacetylation has been traditionally linked to transcriptional repression, in response to stress it is required for transcriptional activation. The Rpd3 histone deacetylase complex is recruited by Hog1 to osmoresponsive promoters upon an osmotic shock. Binding of Rpd3 to specific promoters leads to histone deacetylation, RNA Pol II recruitment and induction of gene expression. Interestingly Rpd3 is required for around 90% of the genes induced upon osmostress, that are Hog1 dependent (de Nadal et al. 2004). A recent report showed loss of acetylation of H3K9, H3K14 and H4K8 on the genes that are repressed upon osmostress and a small increase of acetylation on the same residues on the osmoresponsive genes, suggesting a link between these three lysines and transcriptional activation in response to osmostress (Magraner-Pardo et al. 2014).

1.3.2.2 Transcription initiation

Upon osmostress, Hog1 controls at least seven transcription factors: Msn2 and Msn4 (Schüller et al. 1994),

Hot1 (Rep et al. 2000), Sko1 (Proft et al. 2001), Smp1 (de Nadal et al. 2003), Rtg1 and Rtg3 (Ruiz-Roig et al. 2012). These transcription factors are unrelated and the activation mechanism by Hog1 is different in each case. There is specific contribution of each transcription factor to gene expression, due to different promoter specificity and different association dynamics to promoters (Capaldi et al. 2008).

Recruitment of Hog1 to osmoresponsive promoters is mediated through physical interactions with several transcription factors, which serve as anchoring platforms for the MAPK. One of the best studied mechanisms by which Hog1 regulates transcription initiation is by directly

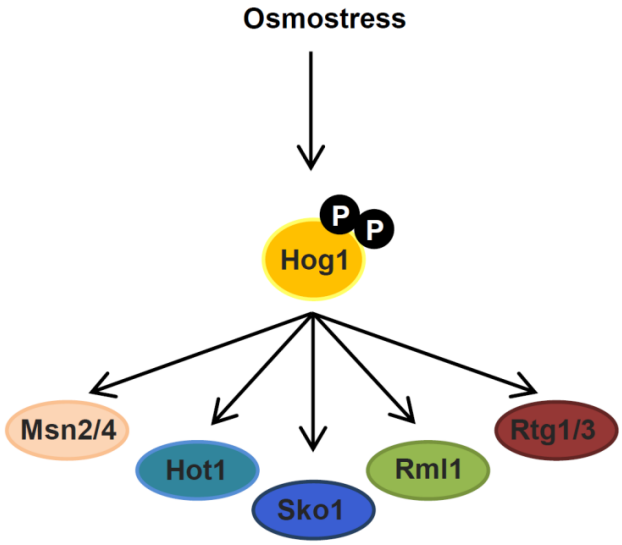


Figure 7. Transcription factors under the control of Hog1 in response to osmostress

In response to osmostress, Hog1 controls gene expression through several unrelated transcription factors: Msn2/4, Hot1, Sko1, Rml1 and Rtg1/3 that serve as anchoring platforms for binding to promoter regions. The mechanism by which Hog1 activates them is specific in each case.

phosphorylating specific transcription factors. Hog1 phosphorylates and directly interacts with Rtg1/3, Smp1, and Sko1 (Ruiz-Roig et al. 2012; de Nadal et al. 2003; Proft et al. 2001). Interestingly, Sko1 plays a dual role in transcription initiation, since in the absence of stress works as a repressor at the promoters of stress-responsive genes (together with Ssn6 and Tup1). In response to osmostress, and after Hog1 phosphorylation, Sko1 and the repressor complex are disassembled becoming a transcriptional activator (Proft & Struhl 2002).

Hot1 controls the transcription of a small group of genes, including *STL1* and *GPD1*, which encodes for genes involved in transport and biosynthesis of glycerol. Hot1 is also phosphorylated by Hog1 in response to osmostress but this phosphorylation is not crucial for gene expression, however recruitment to promoters of Hot1 and Hog1 is interdependent (Alepuz et al. 2003).

As mentioned before, Msn2/4 are the transcription factors responsible for the ESR that is induced upon several stress conditions. Upon osmostress, they induce the expression of the general stress-responsive genes, including *ALD3* and *CTT1*. Hog1 phosphorylates Msn2/4, and although this phosphorylation is not essential for transcription, the catalytic activity of Hog1 is required for Hog1 binding to *CTT1* promoter (Alepuz et al. 2001).

After binding to promoters through the different transcription factors, Hog1 recruits RNA Pol II. It has been

observed that artificial tethering of Hog1 to promoters is sufficient to initiate transcription in response to osmostress (Alepuz et al. 2003). Recent ChIP-seq and MNase-seq analysis showed that in response to osmostress there is a huge nucleosome remodeling at stress-responsive genes and RNA Pol II is redistributed through the genome and recruited to osmoresponsive genes in a Hog1 dependent manner (Nadal-Ribelles et al. 2012). Besides recruitment of RNA Pol II, Hog1 stimulates recruitment of Mediator, SAGA and SWI/SNF. Additionally, Hog1 bound to chromatin through the transcription factors can directly recruit Rpd3 histone deacetylase complex (de Nadal et al. 2004) and Ubp3 ubiquitin protease (Solé et al. 2011).

1.3.2.3 Transcription elongation

In addition to binding at promoter regions, Hog1 is also recruited at coding regions of osmoresponsive genes and travels with elongating RNA Pol II (Pokholok et al. 2006; Proft et al. 2006). Binding at coding regions is independent from binding at the promoter and only depends on the 3'UTR (Proft et al. 2006).

Hog1 also recruits Ubp3 ubiquitin protease at coding regions to modulate transcriptional elongation. Ubp3 mutants are defective in expression of osmoresponsive genes and show reduced RNA Pol II occupancy at coding regions (Solé et al. 2011). After elongation Hog1 also regulates mRNA processing, stability and export (Romero-Santacreu et al. 2009; Regot et al. 2013). Thus, Hog1 controls the

transcriptional response to osmostress by regulating every step of the transcription cycle.

1.4 TOOLS TO STUDY THE ROLE OF HISTONES IN TRANSCRIPTION

In the last few years several libraries of histone point mutations have been created and screened for many phenotypes, such as resistance to DNA damage agents, resistance to drugs, survival upon heat stress and loss of silencing. In this thesis we used two of those histone libraries, all together 569 histone point mutations from H3, H4, H2A and H2B, in order to identify the histone residues required for transcriptional regulation in response to stress. To do so, we used a single cell approach by flow citometry using Venus, a variant of the yellow fluorescence protein (YFP), as a reporter gene and we systematically scanned for defects on transcription initiation of stress-responsive genes upon heat and osmostress.

1.4.1 Histone mutant libraries

Histones are highly conserved proteins from yeast to humans, indicating the importance of each amino acid residue within the nucleosome. In the last years, large-scale studies have systematically constructed and analyzed hundreds of histone mutants (Matsubara et al. 2007; Dai et al. 2008; Nakanishi et al. 2009) providing histone libraries and phenotypic and transcriptional data sets. Two of the most

complete libraries available that have been used for several screens in *S. cerevisiae* (Dai et al. 2008; Nakanishi et al. 2008) are:

1) H3&H4 library

This library was constructed using a cassette designed to be integrated at the histone native locus with high fidelity. This cassette containing the H3/H4 genes with the specific histone mutation and the gene *URA3* was integrated at the *HHT2-HHF2*, replacing one of the two loci at which H3/H4 normally reside. The second copy of H3/H4 was deleted from the genome (Dai et al. 2008). In this collection every residue of histone H3 and H4 was mutated to alanine and the existing alanines were mutated to serines. Additionally, modifiable residues were mutated to amino acids mimicking modifiable states, for example all lysines (K) were mutated to glutamic acid (Q) to mimic acetylated state and to arginine (R) to mimic non-acetylated or methylated states and all serines (S) were mutated to aspartic acid (D) to mimic phosphorylation states.

This is one of the most complete histone libraries containing 486 mutants, 345 of them being non-essential point mutants: 150 and 195 of histone H3 and H4, respectively (Dai et al. 2008). This library also contains deletion mutants and essential histone mutations. H3 & H4 mutant library is commercially available at Open Biosystems.

2) H2A, H2B, H3 and H4 library (SHIMA)

Plasmids containing alanine point mutations within the histone genes were generated by side-directed mutagenesis.

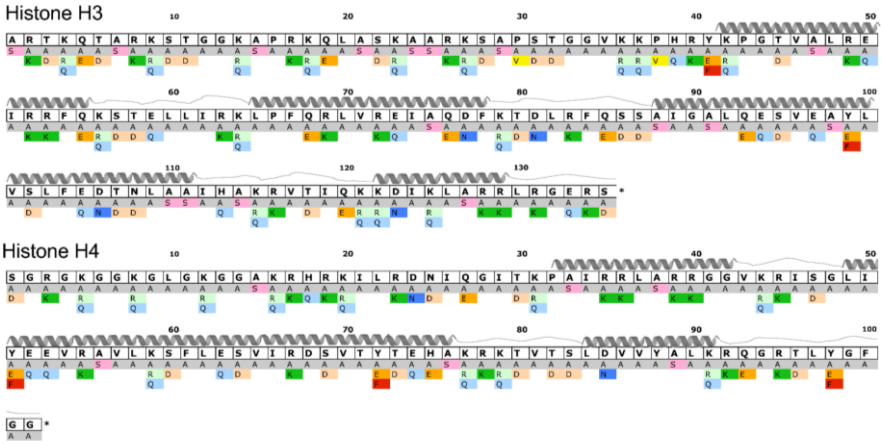


Figure 8. H3/H4 histone library
The H3/H4 library consists of mutations to alanine in all residues from histone H3 and H4 and mutations to serine in the natural alanines. Additionally, the collection contains mutations that mimic modified states of several amino acids and systematic tail deletions, totaling 486 mutants, 345 of them point mutants (Dai et al. 2008).

The library was generated by introduction of a plasmid with a wild type copy of the histones and the *URA3* gene, in order to allow deletion of the endogenous histone loci. Then, the plasmid containing the histone mutations was transformed and the original plasmid containing the wild type histones and the *URA3* gene was removed by negative selection of *URA3* on 5-fluorootic acid (5-FOA), which ensures that the entire population of histones are expressed from the new plasmid (Nakanishi et al. 2008). In this library every residue of histone H2A, H2B, H3 and H4 was mutated to alanine, except for the existing alanines. This library contains 439 point mutants: 119, 96, 112, and 112 of H2A, H2B, H3 and H4, respectively. SHIMA library is available for the scientific community upon request to Ali Shilatifard´s Laboratory.

H2A (HTA1) plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1A	S10A	G23A	V31A	N39A	P49A	Y58A	N69A	I79A	I88A	K96A	Q105A
B	G2A	K13A	L24A	H32A	Y40A	V50A	L59A	R72A	I80A	R89A	L97A	G106A
C	G3A	S15A	T25A	R33A	Q42A	Y51A	E62A	D73A	P81A	N90A	L98A	G107A
D	K4A	Q16A	F26A	L34A	R43A	L52A	I63A	N74A	R82A	D91A	G99A	V108A
E	G5A	S17A	P27A	L35A	I44A	T53A	L64A	K75A	H83A	D92A	N100A	L109A
F	G6A	R18A	V28A	R36A	G45A	V55A	E65A	K76A	L84A	E93A	V101A	P100A
G	K7A	S19A	G29A	R37A	S46A	L56A	L66A	T77A	Q85A	L94A	T102A	N111A
H	G9A	K21A	R30A	G38A	G47A	E57A	G68A	R78A	L86A	N95A	I103A	I112A

H2A (HTA1) plate 2

	L1	L2
1	H113A	S121A
2	Q114A	K123A
3	N115A	T125A
4	L116A	K126A
5	L117A	S128A
6	P118A	Q129A
7	K119A	E130A
8	K120A	L131A

H2B (HTB1) plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1A	P13A	S24A	R32A	S41A	K49A	I57A	L65A	F73A	L83A	S93A	L103A
B	K3A	E15A	T25A	S33A	S42A	Q50A	S58A	N66A	E74A	Y86A	R95A	I104A
C	E5A	K16A	S26A	K34A	Y43A	T51A	Q59A	S67A	R75A	N87A	E96A	L105A
D	K6A	K17A	T27A	R36A	I44A	H52A	K60A	F68A	I76A	K88A	I97A	P106A
E	K7A	P18A	D28A	K37A	Y45A	P53A	S61A	V69A	T78A	K89A	Q98A	G107A
F	P8A	K21A	G29A	E38A	K46A	D54A	M62A	N70A	E79A	S90A	T99A	E108A
G	S10A	K22A	K30A	T39A	V47A	T55A	S63A	D71A	S81A	T91A	V101A	L109A
H	K11A	T23A	K31A	Y40A	L48A	G56A	I64A	I72A	K82A	I92A	R102A	K111A

H2B (HTB1) plate 2

	L1	L2
1	H112A	T122A
2	V114A	K123A
3	S115A	Y124A
4	E116A	S125A
5	G117A	S126A
6	T118A	S127A
7	R119A	T128A

Figure 9. H2A/H2B histone library
Schema of the organization of the SHIMA library (H2A/H2B). This figure shows the position of each mutant on the library plates. The library contains all residues mutated to alanine, totaling 224 point mutants in H2A/H2B (Nakanishi et al. 2008).

It is worth mentioning that there is a database that summarizes the results from the all the histone screens performed in the past years (Huang et al. 2009). This database, known as Histonehits contains information from 42 assays, covering 405 of the 498 residues across yeast histones. All the data is available at http://histonehits.org Genetic analyses summarized in Histonehits show that multiple histone residues, when mutated, prevent modifications and render cells sensitive to several cellular stresses, such as heat stress or DNA damage agents like hydroxyurea, camptothecin and UV radiation. These results suggest that several histone modifications and chromatin modifying complexes might be important for transcription in response a specific stress (Huang et al. 2009).

1.4.2 Single cell analysis

Single-cell analyses have been useful to study the transcriptional response to stress and to understand the variation of stress-responsive gene expression (Pelet et al. 2011). These studies using time-lapse microscopy, flow citometry, fluorescently tagged molecules and transcription specific reporters permitted to better understand the single cell behavior in response to a specific stress, rather than the average behavior of a heterogenic population. Single cell analysis using quadruple Venus (qV) fluoresce protein under the control of stress-responsive promoters has revealed that the gene expression has a bimodal behavior rather than a uniform population response in mild stress conditions (0.1M NaCl).

In this PhD thesis we have used H3&H4 library for screening histone H3 and H4 residues and the SHIMA library for screening histones H2A and H2B residues in order to identify the histone residues required for cell survival and transcription initiation in response to heat and osmostress. To address the transcriptional approach we used flow citometry and quadruple Venus under the control of several stress-responsive promoters to analyze transcription initiation on 569 histone point mutants (195, 150, 112 and 112 from H3, H4, H2A and H2B respectively) in response to heat and osmostress.

2. OBJECTIVES

2 OBJECTIVES

Our group is interested in understanding the mechanisms by which transcription is regulated in response to environmental stresses. Because histones are known to play a key role on transcriptional regulation, the aim of this project was to give insight into the role of histone postranslational modifications in response to stress.

Specifically, the main objectives of this PhD were:

1. To identify the histone residues involved in cell survival upon heat and osmostress.

2. To identify the histone residues required for transcription initiation in response to heat and osmostress.

3. To study novel histone marks and effectors involved in transcriptional regulation.

3. MATERIAL AND METHODS

3 MATERIAL AND METHODS

3.1 YEAST STRAINS AND PLASMIDS

The H3/H4 library is commercially available at Open Biosystems. This library contains 489 mutants, 345 of them are non-essential histone point mutants. In the H3/H4 library all residues are mutated to alanine, alanines are mutated to serines and the potential modifiable residues are mutated to other amino acids that mimic modified and unmodified states. Additionally the library contains sets of 52 and 27 systematic deletion alleles of the N-terminal tail of histone H3 and H4 respectively. H2A/H2B SHIMA collection is a gift from Ali Shilatifard. It is a point mutation library of H2A, H2B, H3 and H4 where all residues are mutated to alanine, except the natural alanines. This library contains 439 (119, 96, 112 and 112 from H2A, H2B, H3 and H4, respectively) histone point mutants. From the SHIMA library we have used 112 mutants from H2A and 112 mutants from H2B.

PRS-based reporter plasmids were constructed from pSP30 (Pelet et al. 2011) by cloning the different stress induced promoters amplified by PCR from yeast genomic DNA between *SacI* and *SpeI* (see Table 1). The plasmids were integrated into wild type (BY4742α or BY5563α) and stronger expressing transformants were selected by microscopy.

Plasmid	Insert	Backbone	Source
pSP30	p*ALD3* (-664-0) qV	pRS305	*Pelet et al,2011*
pOS8	p*STL1* (-800-0) qV	pRS305	this study
pCV1	p*HSP82* (-800-0) qV	pRS305	this study

Table 1. List of plasmids

3.2 PHENOTYPIC SCREENING

345 histone mutant strains from H3/H4 library and 224 mutants from SHIMA H2A/H2B were replica pinned in quadruplicates onto YPD plates. The screen was performed using an automated system (RoTor® from Singer). Plates were incubated at 30ºC (control), 39ºC (heat stress) and on YPD plates containing 1.2M NaCl or 2M sorbitol (osmostress). The screen was performed in triplicates and plates were incubated at 30ºC or 39ºC for 3 days before scoring colony size.

3.3 GENERATION OF HISTONE LIBRARIES HARBORING REPORTER GENES

Query strains construction

BY4742 (*MATα ura3*Δ*, leu2*Δ*, his3*Δ*, lys2*Δ) and BY5563 (*MATα his3*Δ*, leu2*Δ*, ura3*Δ*, met15*Δ*, LYS2* + *can1*Δ*::MFA1pr-HIS, lyp1*Δ) strains (Tong & Boone 2005) were used to construct the query strains designed specifically for this study (see Table 2). For H3/H4 query strain construction, we used quadruple Venus (qV) fluorescence fused to the promoter of stress-responsive genes (p*ALD3*-qV,

p*HSP82*-qV and p*STL1*-qV). BY4742 was transformed with *KasI*-linearized plasmid pRS305 *pALD3-qV-LEU*, pRS305 *pHSP82-qV-LEU* and pRS305 *pSTL1-qV-LEU*, and transformants were plated on SD-*LEU* media to select for the integrated *LEU2* marker and the pRS305 backbone. For H2A/B query strain construction, BY5563 was transformed with *prMFA1-URA3-NatMX4* cassette to swap *HIS3* by *URA3-NatMX4* and obtain the BY5563 *can1*Δ*::MFA1pr-URA3-NatMX4.* Transformants were selected on YPD plates containing cloNAT. Selected clones were transformed with *KasI*-linearized plasmid pRS305 *pALD3-qV-KanMX4*, pRS305 *pHSP82-qV-KanMX4* and pRS305 *pSTL1-qV-KanMX4* and transformants were selected on YPD plates containing G418.

Mating procedure and media

Mating of the histone libraries with the query strains was performed using a Synthetic Genetic Array (SGA) Protocol (Tong & Boone 2005) with some modifications. The protocol was optimized to Singer RoTor® and for 96-well plate density. Solutions of Canavanine (L-canavanine sulfate salt), cloNAT (Nourseothricin) and G418 (Geneticine) were previously dissolved in water at 100 mg/L, filtered sterilized and stored in aliquots at 4ºC. The SGA analysis was performed as described in Figure 4. Briefly, 1) Query strains were grown overnight in 5ml of rich medium, then transferred to a 96-well plate. To generate a source of newly grown query

cells for mating to the histone libraries, the query strain culture was pinned onto rich (YPD) plates and the cells were grown for one day. 2) For the mating reaction the query strain was first pinned onto a fresh YPD plate, the histone libraries were pinned on top of the query cells, and the plates were incubated at room temperature for one day. 3) The resulting MATa/α zygotes were pinned onto medium that selects for growth of diploid cells; synthetic dextrose medium (SD) lacking uracil and leucine and SD lacking histidine and containing G418 (200mg/L) were used to select diploids from H3/H4 and H2A/B mating, respectively. **4)** Diploid cells were pinned to sporulation medium (2% agar, 1% potassium acetate, supplemented with leucine and tryptophan, lacking uracil or histidine for H3/H4 and H2A/B, respectively) and the plates were incubated for 6 days at 22ºC. **5)** To select for growth of the MATa spore progeny, spores were pinned onto SD medium (specific for each case, see below) and grown for 2 days in each selection round. Because ammonium sulfate impedes the function of G418 and cloNAT, synthetic medium containing these antibiotics was made with monosodium glutamate (MSG) as a nitrogen source: 20mg/L agar, 1.7g/L yeast nitrogen base w/o ammonium sulphate and amino acids, 1g/L monosodium glutamic acid, 2g/L amino acid drop-out (lacking specific amino acids for each case), 2% glucose, 50mg/L canavanine, and the specific antibiotics for each case. The media used for selection of H3/H4 *(MATa URA LEU NatR)* progeny was:

- First selection round: MSG lacking histidine, arginine, uracil, and containing canavanine (50mg/L) and cloNAT (200mg/L).
- Second and third selection rounds: MSG lacking histidine, arginine, uracil, leucine, and containing canavanine (50mg/L) and cloNAT (200mg/L).

The media used for selection of H2A/H2B (*MATa HIS LEU TRP kanR)* progeny was:

- First selection round: MSG lacking histidine, arginine, uracil, leucine, tryptophan and containing canavanine (50mg/L) and cloNAT (200mg/L).
- Second and third selection rounds: MSG lacking histidine, arginine, uracil, leucine, tryptophan and containing canavanine (50mg/L), cloNAT (200mg/L) and G418 (200mg/L).

After mating and selection, the final genotype of the new libraries is:

- *BY4741 MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[HHTS-HHFS]*-URA3 p-ALD3-qV-LEU2 for H3/H4.* The libraries harboring p-*STL1*-qV and p-*HSP82*-qV reporters have the same genotype as the described above.

- FY406 *MATa (hta1-htb1)::LEU2, (hta2-htb2)::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128Δ [HTA1-HTB1]*-HIS3 can1::MFA1pr-URA3-NatMX4 p-ALD3-qV-KanMX4 for H2A/H2B.* The libraries harboring p-*STL1*-qV and p-*HSP82*-

qV reporters have the same genotype as the described above.

The new histone libraries generated were stored on liquid MSG haploid selective media on 96-well plates at 4ºC and on MSG haploid selective media in 15% glycerol at -80ºC.

Strain	Genotype	Source
YCV1	BY4742 *MATα leu2::LEU2 pALD3-qV* (query strain for H3/H4)	this study
YSO63	BY4742 *MATα leu2::LEU2 pSTL1*-qV (query strain for H3/H4)	this study
YCV4	BY4742 *MATα leu2::LEU2 pHSP82*-qV (query strain for H3/H4)	this study
YCV86	BY5563 *MATα pALD3- qV –KanMX4 pcan1::pMFA1-URA3-NatMX4* (query strain for H2A/H2B)	this study
YCV88	BY5563 MATα p*STL1*- qV - *KanMX4* p*can1*::p*MFA1-URA3- NatMX4* (query strain for H2A/H2B)	this study
YCV87	BY5563 MATα p*HSP82*- qV - *KanMX4* p*can1*::p*MFA1-URA3- NatMX4* (query strain for H2A/H2B)	this study
H3/H4 library	BY4741 *MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[HHTS-HHFS]*-URA3*	*Dai et al, 2008*
H2A/H2B library	FY406 *MATa (hta1-htb1)::LEU2, (hta2-htb2)::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128Δ [HTA1-HTB1]*-HIS3*	*Nakanishi et al, 2008*

Table 2. List of yeast strains

3.4 TRANSCRIPTIONAL SCREEN BY FLOW CITOMETRY

Cell culture

Yeast strains were grown on 96 well-plates (200µl/well) and incubated at 25ºC at all steps during the screening, unless is indicated. Cells were grown in the MSG haploid selective media for 2 days. Saturated cultures were diluted 1/100, using RoTor®, in a fresh plate with filtered synthetic medium (SD-medium) and grown to log phase.

Reporter expression analysis by Flow Citometry

Cells on log phase were subjected to heat stress (39ºC) or osmostress (0.4M NaCl). Protein translation was stopped after 45 min by the addition of cycloheximide (0.1mg/ml) and then cells were incubated in dark for one hour. Fluorescence of 10.000 cells per well was measured by flow citometry (FACScanto™). Data was analyzed using Flowjo software. Results come from three independent experiments.

3.5 NORTHERN BLOT

Yeast strains were grown to log-phase on YPD medium and then subjected to heat stress (39ºC) or osmostress (0.4M NaCl) for the indicated times. Total RNA and expression of specific genes were probed using labeled PCR fragments containing the entire open reading frame of *ALD3* (1.5 kb), *HSP82* (2.1 kb), *STL1* (1.7 kb), *CTT1* (1.7kb), *SSA3* (1.9 kb) and *ENO1* (1.3 kb).

3.6 DNA MICROARRAYS

WTH3, H3T58D, WTH4 and H4S47D mutant strains were grown at 30ºC to mid-log phase and treated with 0.4M NaCl for 10 min. Total RNA was prepared using hot phenol. Hybridizations were performed comparing stressed and control conditions by triplicate. DNA microarrays were performed by Frank Holstege group at the University Medical Center Utrecht. Results were analyzed comparing gene expression upon osmostress respect to the control in each individual strain.

3.7 EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

Short histone peptides were designed to contain the wild type and the mutant of interest (see Table 3 for detailed information). These peptides were fused to GST and the recombinant protein was expressed in *E.coli* BL21 strain. *E.coli* cells were grown at 37ºC and then GST-tagged proteins were induced for 6h by adding 1mM isopropylthiogalactoside (IPTG) at 25ºC. After induction, cells were collected by centrifugation and resuspended in 1/50 volume of STET 1x buffer (10mM Tris-HCl, pH8.0, 100mM NaCl, 1mM EDTA pH8, 0.5% Triton X-100) supplemented with 2mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM benzamidine, 2ug/ml leupeptin and 2g/ml pepstatin). Cells were lysed in ice-cold by sonication and cleared by high-speed centrifugation. GST-proteins were

pulled down form supernatants with gluthatione-sepaharose beads (GE Healthcare) by mixing for 90 min at 4ºC. The gluthatione-sepharose beads were collected by brief centrifugation and washed four times in STET 1× buffer and two times in 50 mM Tris-HCl pH 8.0 buffer supplemented with 2 mM DTT. The GST-proteins were then eluted in 200 µl of 50 mM Tris-HCl pH 8.0 buffer supplemented with 2 mM DTT and 10 mM reduced gluthatione (Sigma) by rotating for 20 min at 4 °C and stored at −80 °C.

Peptide	Sequence	Lengh
WT H4S47	ARRGGVKRI**S**GLIYEEVRAV	H4 (38-57) / 20 aa
H4S47A	ARRGGVKRI**A**GLIYEEVRAV	H4 (38-57) / 20 aa
WT H3T58	LREIRRFQKS**T**ELLIRKLPFQRLV	H3 (48-71) / 24 aa
H3T58A	LREIRRFQKS**A**ELLIRKLPFQRLV	H3 (48-71) / 24 aa
H3S57A	LREIRRFQK**A**TELLIRKLPFQRLV	H3 (48-71) / 24 aa
H3T58A-S57A	LREIRRFQK**AA**ELLIRKLPFQRLV	H3 (48-71) / 24 aa

Table 3. List of short histone peptides

3.8 KINASES PURIFICATION

123 tagged kinase strains from yeast TAP collection (Ghaemmaghami et al. 2003) or GAL-ORF collection (Thermo Scientific Open Biosystems Yeast ORF Collection) were grown to mid-log phase in 50ml of YPD and collected by brief centrifugation at 4ºC. Both collections have protein A as a tag, thus purification was performed following the same protocol. Protein were extracted in buffer A (50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 15 mm EDTA, 15 mm EGTA, 2 mm DTT, 0.1% Triton X-100, 1 mm PMSF, 1 mm benzamidine, 2

mg/ml leupeptin, 2 mg/ml pepstatin) and incubated with Prot A-Sepharose beads (Sigma) for 2 h at 4ºC. Beads were washed once with lysis buffer A, 3 times with buffer A + 0.5M NaCl and two times with 50mM Tris pH8 + 2mM DTT.

3.9 *IN VITRO* KINASE ASSAYS

After purification, kinases (from yeast TAP or GAL-ORF collection) were pre-activated with kinase buffer (50mM Tris-HCl pH 7.5, 10mM MgCl2, 2mM DTT and 50µM ATP) for 5 min at 30ºC. 2µg of the substrate peptide was added to the kinase mixture together with radiolabelled γ-^{32} P ATP (0.1uCi/µl) and incubated for 30 min at 30ºC. The reaction was stopped by the addition of 5X SDS loading buffer. Labeled proteins were resolved by SDS/PAGE, transferred to a nylon membrane and detected by autoradiography. GST fused peptides and tagged kinases were identified by Western blot.

3.10 3D ANALYSIS

Tridimensional representations were done using Pymol Molecular Graphics System and the yeast nucleosome crystal structure, which accession number in the Protein Data Bank is 1ID3 (White et al. 2001).

4. RESULTS

4 RESULTS

4.1 HISTONE RESIDUES ESSENTIAL FOR CELL SURVIVAL UPON STRESS

In response to stress, cells rapidly change their physiology to adapt to the new environment and maximize cell survival. To systematically identify the histone residues that are required for cell survival in response to heat and osmostress, we performed a high-throughput screen for histone point mutations that rendered cells thermosensitive or osmosensitive. For this screen we used two histone libraries (see section 1.4.1 of the introduction). From H3/H4 library we selected the non-essential point mutants, which include 195 and 150 mutants for H3 and H4, respectively. In this library all H3 and H4 residues were mutated to alanine and additionally, the modifiable residues were mutated to amino acids that mimic modified or unmodified states. To screen H2A and H2B, we used the SHIMA library, which contains 112 point mutants for H2A and 112 for H2B. In this case all residues were mutated to alanine, but not additional mutations were performed, which explains the lower number of mutants analyzed from H2A/H2B when comparing to H3/H4.

The screen was performed by robotically pinning the 569 histone point mutants (195, 150, 112 and 112 from H3, H4, H2A and H2B, respectively) onto YPD agar plates and incubating at 30ºC (control), at 39ºC (heat stress) and onto YPD plates with or without 1.2M NaCl or 2M sorbitol (osmostress). Colony size was scored and compared to wild

type cells after 2, 3 and 4 days. Mutants with no growth or smaller colonies at 39ºC were considered thermosensitive and mutants with no growth or smaller colonies at both NaCl and sorbitol-containing plates were considered osmosensitive (Fig.1A).

A

B

	Mutants analyzed	Thermo sensitive	Osmo sensitive	Comon (TS+OS)
H3	195	88 (45%)	14(7%)	9
H4	150	49 (32%)	15(10%)	13
H2A	112	16 (14%)	12 (10%)	6
H2B	112	4 (4%)	2 (2%)	1
Total	569	157	43	30

C

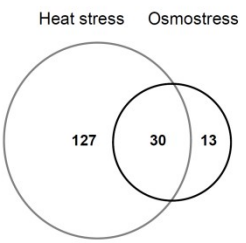


Figure 1. Identification of thermosensitive and osmosensitive histone mutants in yeast

A) From 569 histone point mutants analyzed, 157 rendered cells unable to grow at high temperature and 43 at high osmolarity. Cells were robotically pinned onto YPD plates and incubated at 30ºC (control), 39ºC (heat stress) and on plates containing 1.2M NaCl and 2M sorbitol (osmostress) for 2, 3 and 4 days before scoring colony size. A thermosensitive and an osmosensitive mutant are shown as an example (red squares). **B)** Table summarizing the result from the phenotypic screen. Abbreviations: TS, thermosensitive; OS, osmosensitive. **C)** Venn diagram showing the overlap between stress conditions. For a detailed list of the results from the phenotypic screen see **Table S1.**

4.1.1 Identification of histone residues essential for cell survival upon heat stress

The high-throughput screen upon heat stress conditions yielded 157 histone point mutations that rendered cells thermosensitive: 88, 49, 16 and 4 in histone H3, H4, H2A and H2B, respectively. These mutants represent 45%, 32%, 14% and 4% in each histone (Fig. 1B). These results not only confirmed 46 of the previously described thermosensitive histone mutants (Huang et al. 2009), but also revealed 91 novel ones (see discussion).

To characterize the spatial distribution of the mutations leading to thermosensitivity, we grouped the residues by position according to the nucleosome crystal structure (White et al. 2001; Huang et al. 2009). Histone residues can be located on the histone surface: on the lateral surface that contacts DNA or on the disk, which is the lateral surface that does not contact DNA. Histone residues can also be buried inside the histone structure or located on the unstructured tails. We found that 63% of the mutations leading to thermosensitivity were located on the histone surface (Fig. 2A). From this 63%, 24% were located on the lateral surface and 39% were located on the disk. The remaining 37% was distributed between the histone tails (12%) and buried inside the protein structure (25%) (Fig. 2A).

By sorting the histone residues that rendered cells thermosensitive by amino acid type, we observed that mutations on lysine (K), arginine (R), glutamic acid (E) and

leucine (L) generated most of the phenotypes (Fig. 2B) (see discussion).

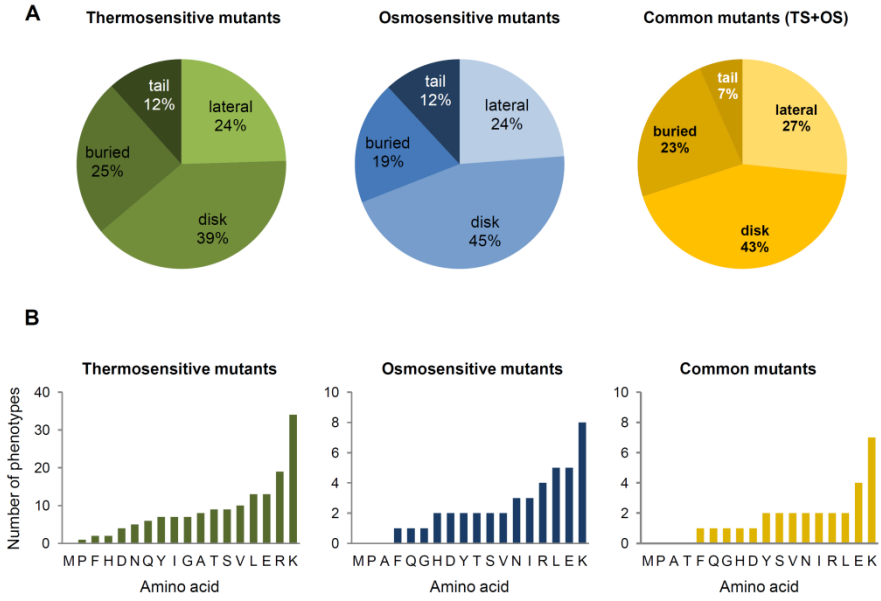


Figure 2. Spatial distribution of the histone residues necessary for cell survival upon stress

A) Distribution on the nucleosome of the thermo and osmosensitive mutants identified in the screen. Histone residues can be located on the tails, inside the protein structure (buried) or located on the histone surface that contacts DNA (lateral) or that does not contact DNA (disk). **B)** Frequency of growth defects generated by mutations on each amino acid type.

4.1.2 Identification of histone residues essential for cell survival upon osmostress

After testing cell survival at high osmolarity, we found 43 mutants that were not able to grow neither at 1.2M NaCl nor at 2M Sorbitol. We report here for the first time the subset of histone residues that are required for cell survival upon osmostress (see discussion). From the 43 osmosensitive

mutants, 14, 15, 12 and 2 mutants were identified in histone H3, H4, H2A and H2B, respectively. These affected mutants represent 7%, 10%, 11% and 2% of the total number of mutants analyzed in H3, H4, H2A and H2B, respectively (Fig. 1B).

When we analyzed the spatial distribution of these 43 osmosensitive mutants, we observed a similar pattern to the thermosensitive mutants. Most of the mutations that generated osmosensitive phenotypes were located on the histone surface, in this case representing 69%: 45% located on the disk and 24% located on the lateral surface. The remaining 31% were distributed between the histone tails (12%) and buried inside the protein structure (19%) (Fig. 2A).

When we grouped the osmosensitive mutants by amino acid type, we observed that mutations generating most of the phenotypes were on exactly the same amino acid types as for the thermosensitive mutants: lysine (K), arginine (R), glutamic acid (E) and leucine (L) (Fig. 2B) (see discussion).

4.1.3 Identification of histone residues essential for cell survival upon both stresses

From the 156 and 43 mutants that rendered cells thermosensitive and osmosensitive, respectively, there were 30 mutants that were commonly affected by both stress conditions (Fig. 1C). This overlapping result suggest that there are two groups of histone residues required for cellular survival in response to stress: the ones required in response

to a specific stress situation and the ones commonly required in response to both stresses.

To characterize the mutations leading to defects in thermo and osmosensitivity, we focused on the 30 mutants common to both stresses: 9, 13, 6 and 1 identified in H3, H4, H2A and H2B, respectively (Fig. 1B and 1C). These 30 mutants represent 24% of the thermosensitive mutants and 70% of the osmosensitive mutants. We analyzed the spatial distribution on the nucleosome and the amino acid type of these 30 common residues. The spatial distribution was similar to the one described for the individual phenotypes, 70% of the residues were located on the protein surface (lateral and disk) compared to 63% and 69% of the thermosensitive and osmosensitive mutants, respectively. However significantly less tail residues were found in this group: 7% of the common mutants compared to 12% of the single phenotypes (Fig. 2A). We observed that mutations on lysines and glutamic acid lead to most of the phenotypes, similarly that the pattern observed when looking at the thermosensitive or osmosensitive mutants individually (Fig. 2B).

We next asked where the 30 common mutations were located in the tridimensional structure of the nucleosome. To visualize this, we represented the phenotypic screening results using the crystal structure of the yeast nucleosome (White et al. 2001) downloaded from the Protein Data Bank (PDB) (Berman et al. 2000) with accession number 1ID3. We

observed that the mutations on residues that yielded both phenotypes were distributed in clusters on the nucleosome. Some of these clusters coincide with the nucleosome entry sites (Fig. 3).

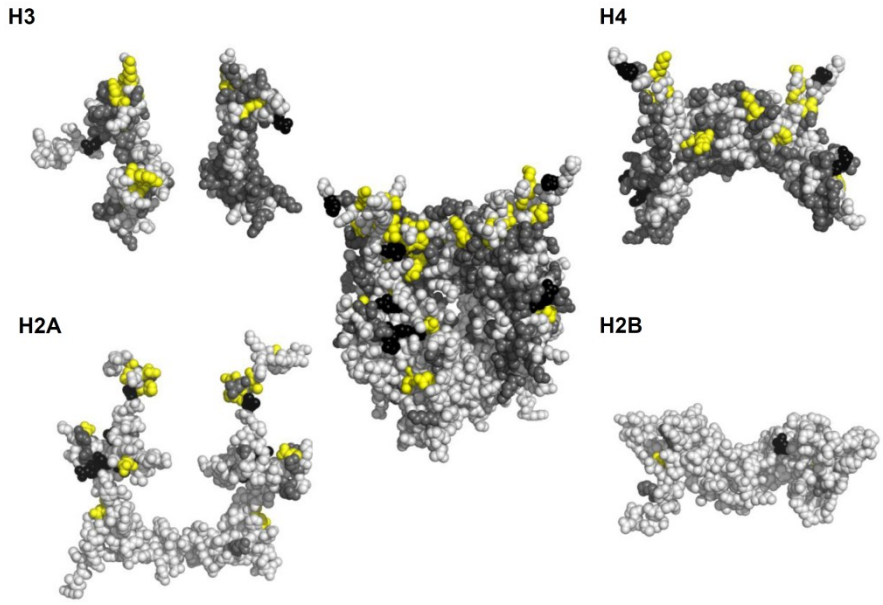


Figure 3. Position map of the mutants with thermosensitive and osmosensitive phenotype
Thermosensitive and osmosensitive phenotypes are represented in grey and black, respectively in the tridimensional structure of the four core histones. Mutations leading to both phenotypes are represented in yellow. Figures were made using Pymol software and the coordinates from the crystal structure of the yeast nucleosome (White et al. 2001) downloaded from the Protein Data Bank with accession number 1ID3.

4.2 HISTONE RESIDUES ESSENTIAL FOR TRANSCRIPTION UPON STRESS

In response to environmental fluctuations, yeast cells rapidly change their gene expression pattern by inducing a

rapid and massive transcription of stress-responsive genes (Gasch et al. 2000; Causton et al. 2001). It is known that histones play a key role in the regulation of transcription and there are a great variety of histone PTMs linked to transcription activation and repression (see section 1.1.3 of the introduction). However, very little is known about the histone PTMs that are required for the transcriptional regulation in response to stress. To identify the specific histone residues involved in transcriptional regulation upon stress, we performed a high-throughput screen for histone point mutations that affected transcription upon heat and osmostress by measuring transcription initiation (determined by the expression of a fluorescence reporter) at single cells by flow citometry.

4.2.1 Generation of histone libraries harboring reporter genes

To quantify the transcriptional output induced by heat and osmostress, we used a reporter system based on quadruple Venus (qV) fluorescent protein expressed under the control of a desired promoter (Pelet et al. 2011). For this project we used three different stress-responsive promoters regulated by the main transcription factors involved in the transcriptional regulation to stress. *ALD3* promoter is induced in response to both heat and osmostress and regulated by Msn2 and Msn4 transcription factors; *HSP82* promoter is induced in response to heat stress and is activated by the

Hsf1 transcription factor, and the *STL1* promoter, induced in response to osmostress and regulated by Hot1 and Sko1. The use of these promoters should allow the comparison of the histone requirements necessary for the transcriptional regulation upon heat stress (comparing p*ALD3*-qV and p*HSP82*-qV) and upon osmostress (comparing p*ALD3*-qV and p*STL1*-qV). Also these analyses should allow the comparison between heat and osmostress by analyzing p*ALD3*-qV in response to both stress conditions.

To generate the histone libraries harboring these reporter genes, we followed three steps: 1) Creation of the query strains by genomically integrating the reporter genes (p*ALD3*-qV, p*HSP82*-qV or p*STL1*-qV) into a wild type BY MATα background strain (BY4742 for H3/H4 and BY5563 for H2A/H2B); 2) Mating of the H3/H4 and H2A/H2B histone libraries (MAT a) with the three query strains (BY MATα p*ALD3*-qV, p*HSP82*-qV or p*STL1*-qV); and 3) Sporulation and selection of the haploid cells containing the histone mutation and the reporter gene. Basically we followed the Synthetic Genetic Array (SGA) protocol (Tong & Boone 2005) with some modifications (see Figure 4 for a schematic representation of the process and material and methods for detailed information). Following this approach, six new histone libraries were generated:

1. H3/H4 library harboring p*ALD3*-qV (345 mutants)
2. H3/H4 library harboring pHSP82-qV (345 mutants)
3. H3/H4 library harboring pSTL1-qV (345 mutants)

4. H2A/H2B library harboring pALD3-qV (224 mutants)
5. H2A/H2B library harboring pHSP82-qV(224 mutants)
6. H2A/H2B library harboring pSTL1-qV (224 mutants).

The total number of yeast strains generated for screening purposes was 1707.

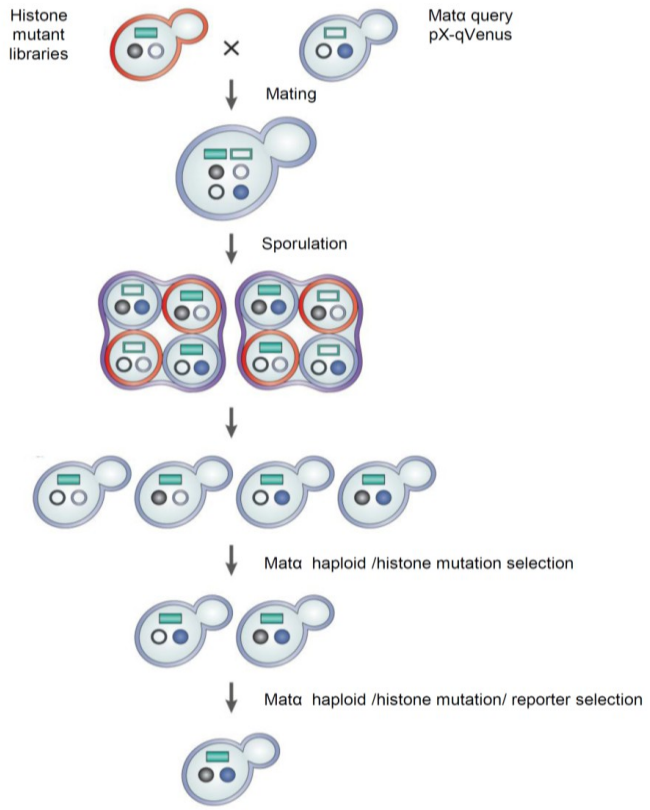


Figure 4. Experimental strategy to generate histone mutant libraries harboring reporter genes
The synthetic genetic array (SGA) methodology was used to construct histone mutant libraries harboring reporter genes. All the procedure was performed at 96-well plates density using an automated robot (RoTor® from Singer).The histone mutant libraries (MATa) were mated with query strains (MATα), diploid cells were selected and sporulated. The MATa progeny with the histone mutation and the reporter gene was selected by three consecutive rounds in specific selection media for each library. Adapted from (Tong & Boone 2005).

4.2.2 Single cell transcriptional screening

We systematically screened these six new histone libraries for mutations leading to defects on transcription initiation in response to heat and osmostress. To measure fluorescence of the reporter genes, yeast cells were grown to mid-log phase in 96 well-plates and subjected to stress for 45 minutes, at 39ºC or 0.4M of NaCl. Then, fluorescence of 10.000 cells per condition was measured by flow cytometry using a 96 well-plate reader device (FACScanto™). The results were analyzed by comparing the induction of the reporter after stress in each individual mutant with the induction in wild type cells. The differences between mutant and wild type cells were classified in three categories: 1) down-regulation, when fluorescence of the reporter upon stress was lower in mutant than in wild type cells (≥10% reduction); 2) up-regulation, when fluorescence of the reporter upon stress was higher in mutant than in wild type cells (≥10% increase); and 3) basal, when the mutant cells already had transcriptional defects at physiological conditions in the absence of stress (Fig. 5).

All together 60 mutants were identified to have p*ALD3*-qV expression defects upon heat stress and 54 mutants on the same reporter upon osmostress. 108 mutants showed p*HSP82*-qV expression defects upon heat stress and 70 showed p*STL1*-qV expression defects upon osmostress. The number of mutants in each histone and the type of transcriptional defect is showed in detail in Figure 6A.

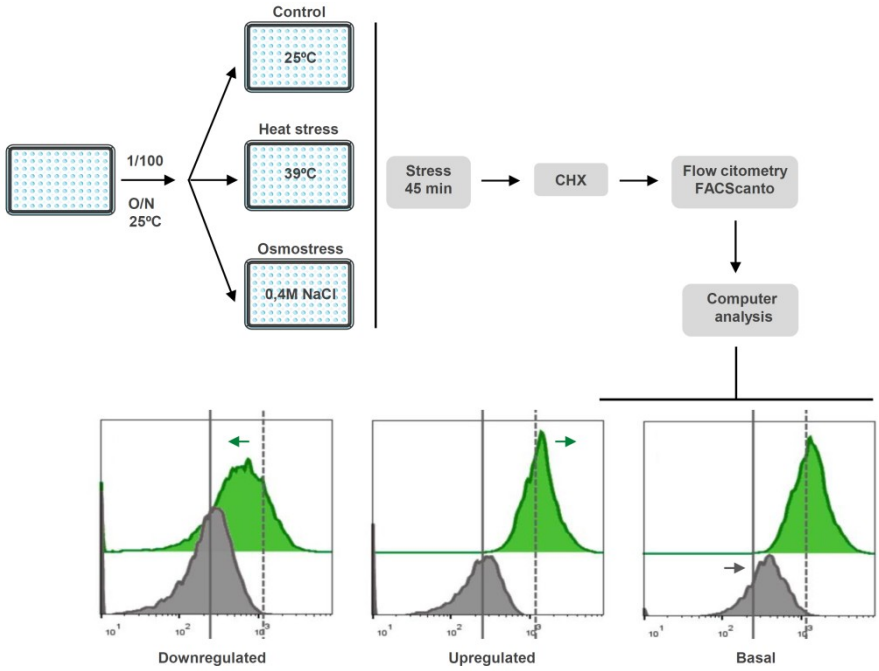


Figure 5. Experimental strategy of the single-cell transcriptional screening
Yeast cells were diluted using an automated robot (RoTor®) and grown at 25ºC to mid-log phase, then cells were subjected to heat (39ºC) or osmostress (0.4M NaCl) for 45 min. Ciclohexamide (CHX) was added and after one hour in dark, fluorescence of 10.000 cells per condition was measured by flow citometry using a 96-well plate reader (FACScanto™). Results were analyzed using Flowjo. The grey line and dashed grey line represent the level of transcription of the wild type (WT) at basal conditions and in response to stress, respectively. Mutants showing a transcriptional defect were classified into three categories: downregulated (lower transcription than the WT), upregulated (higher transcription than the WT) and basal (transcription at normal growth conditions in the mutant was higher or lower than in the WT).

In order to validate the defects observed in the single-cell screening by flow citometry, we assessed transcription of the endogenous gene by Northern blot in 18 mutant strains. In 80% of the cases we observed the same transcriptional defect in the endogenous gene and in the reporter gene,

giving a high degree of confidence to the results obtained by the screening. H4K91R is an example of this validation (Fig. 6B), H4K91R showed reduced p*ALD3*qV expression by flow citometry upon heat stress and Northern blot analysis confirmed this reduction on the endogenous *ALD3* gene.

A

HEAT STRESS

	ALD3					HSP82				
	H3	H4	H2A	H2B	Total	H3	H4	H2A	H2B	Total
DOWN	17	18	5	0	40	17	3	16	11	47
UP	2	1	1	15	19	2	1	1	1	5
BASAL	1	0	0	0	1	4	9	16	27	56
AFFECTED	**20**	**19**	**6**	**15**	**60**	**23**	**13**	**33**	**39**	**108**
NO	**130**	**103**	**101**	**93**	**427**	**120**	**114**	**32**	**45**	**311**
n/d	45	28	5	4	82	52	23	47	28	150
	195	150	112	112	569	195	150	112	112	569

OSMOSTRESS

	ALD3					STL1				
	H3	H4	H2A	H2B	Total	H3	H4	H2A	H2B	Total
DOWN	6	20	7	4	37	18	12	9	19	58
UP	3	5	4	4	16	8	1	2	1	12
BASAL	1	0	0	0	1	0	0	0	0	0
AFFECTED	**10**	**25**	**11**	**8**	**54**	**26**	**13**	**11**	**20**	**70**
NO	**135**	**93**	**96**	**100**	**424**	**156**	**129**	**95**	**88**	**468**
n/d	50	32	5	4	91	13	8	6	4	31
	195	150	112	112	569	195	150	112	112	569

B

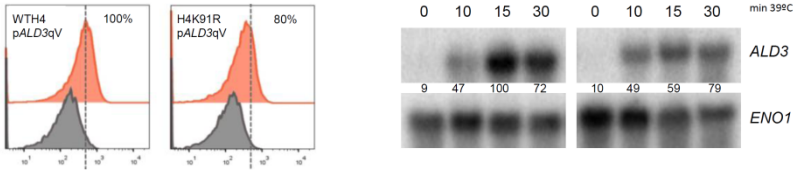
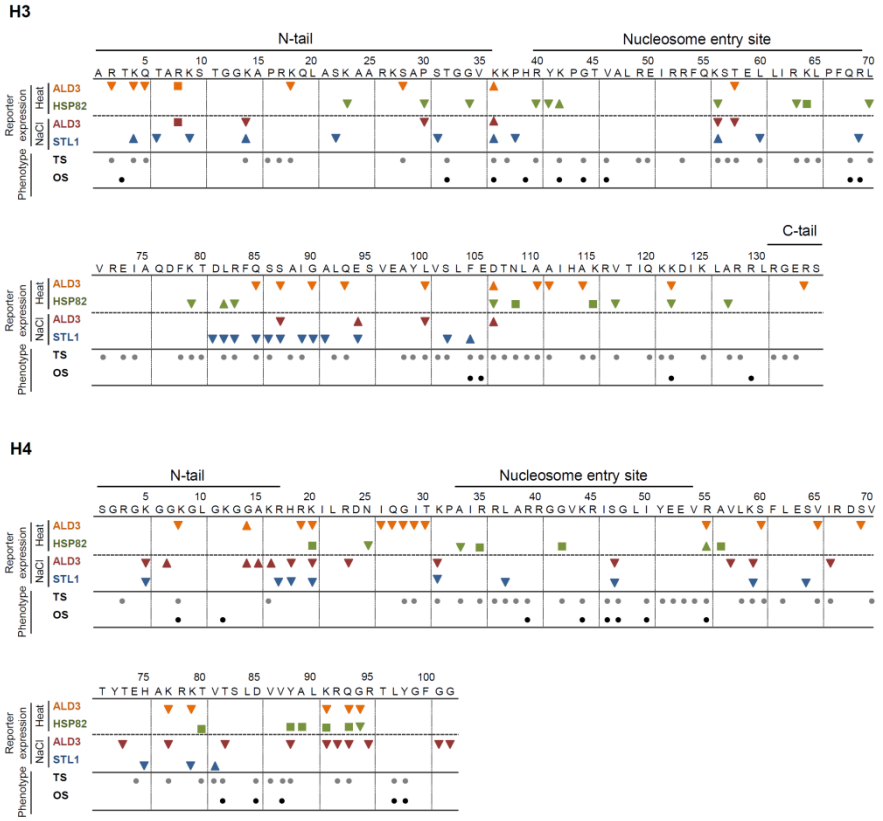


Figure 6. Overall results from the single cell transcriptional screen
A) Table summarizing the defects on transcription initiation of the histone mutants identified leading to transcriptional defects in response to heat and osmostress. Abbreviations: Down, down-regulation; up, up-regulation; No, no affected; n/d, no data. **B)** Wild type (WT) and H4K91R mutant strains harboring the quadruple-Venus (qV) fluorescence reporter driven by *ALD3* promoter (left) were measured by flow citometry before and after heat stress (39ºC for 45 min) (left). WT and H4K91R mutant strains were subjected to heat stress (39ºC) for the indicated length of time and total RNA was assayed by Northern blot for transcript levels of *ALD3* and *ENO1* (as a loading control) (right). The values of maximum reporter gene/ endogenous gene expression of the wild type strain were used as 100% reference.

To visualize the location of the histone residues involved in the transcriptional regulation of different stress-responsive genes upon different stress conditions, we represented all the data obtained from the screens in the primary (Fig. 7) and tridimensional structure (Fig. 8) of the four core histones. This way of representing the results allow us to have a general vision and to identify clusters of residues that could potentially be transcriptional regulatory regions or serve as structural platforms for the recruitment of histone modifying enzimes. We also observe that most of the mutants that show both thermo and osmosensitive phenotypes are located on the nucleosome entry region of the nucleosome.



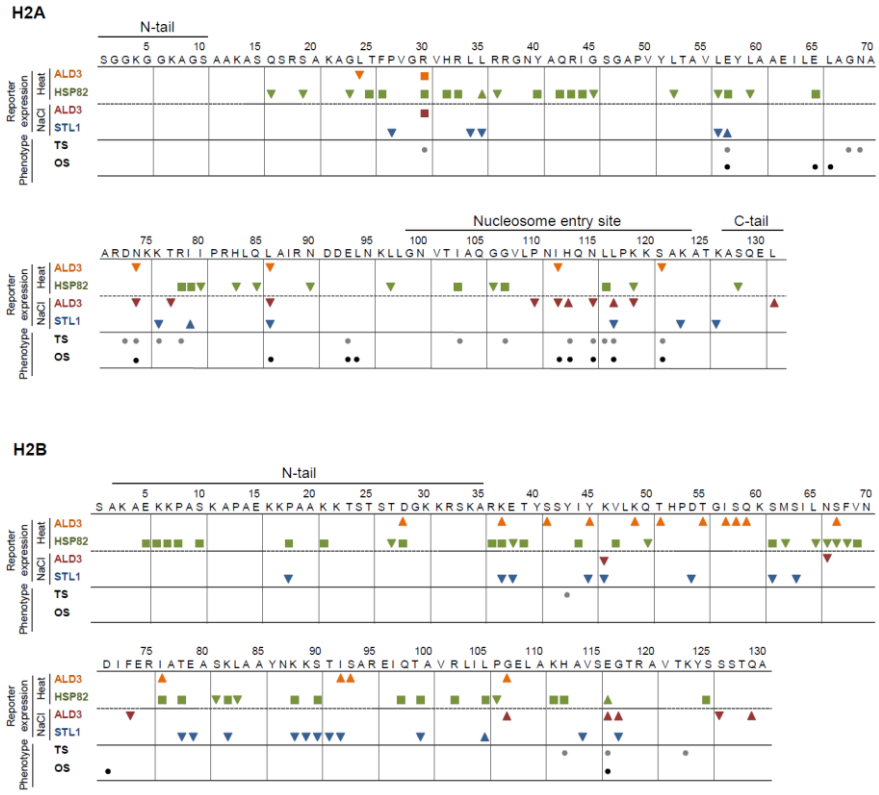


Figure 7. Summary of the results from the phenotypic and transcriptional screens

The transcriptional defects in response to stress are represented in the primary structure of each core histone for each reporter and each condition. Transcriptional defects in response to heat stress are shown in orange (p*ALD3*-qV) and in green (p*HSP82*-qV) and in response to osmostress are shown in red (p*ALD3*-qV) and in blue (p*STL1*-qV). The types of transcriptional defects are represented as follows: ▲ up-regulation, higher expression in mutant than in wild type cells in response to stress; ▼down-regulation, lower expression in mutant that in wild type cells in response to stress; ■ transcriptional defects at normal growth conditions in mutant cells. Growth defects from the phenotypic screening are also represented in this summary as ● thermosensitive and ● osmo-sensitive.

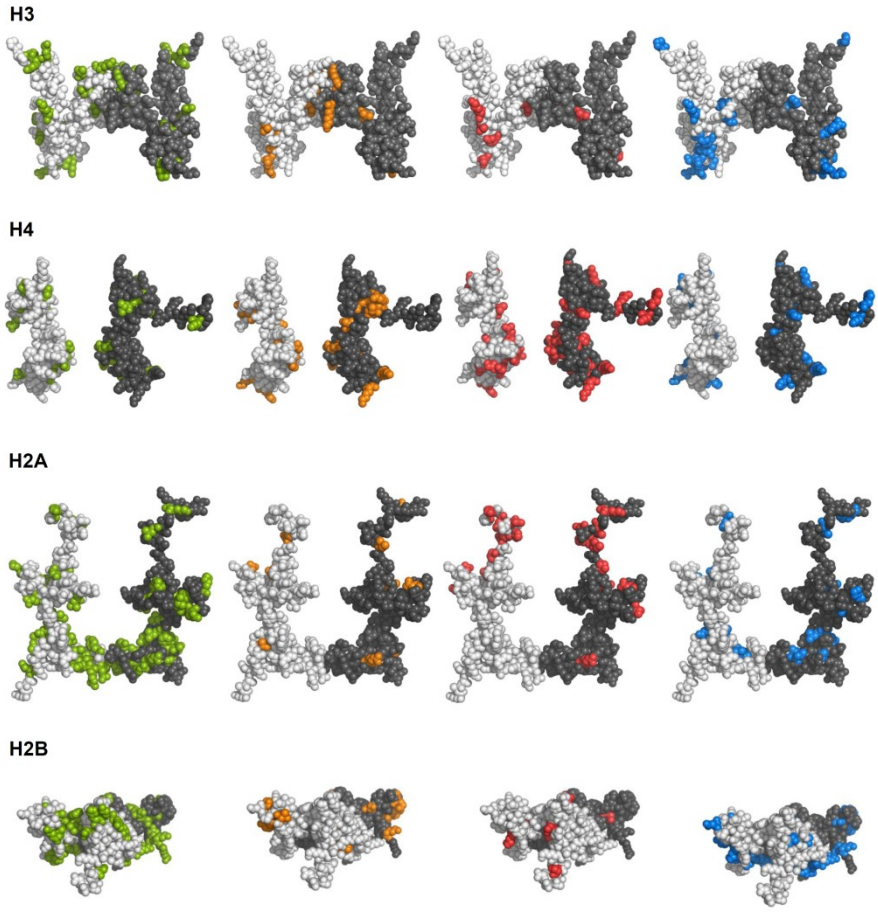


Figure 8. Tridimensional representation of the residues necessary for transcriptional regulation upon heat and osmostress
Transcriptional defects in response to heat stress are shown in orange (p*ALD3*-qV) and in green (p*HSP82*-qV) and in response to osmostress are shown in red (p*ALD3*-qV) and in blue (p*STL1*-qV). Figures were made using Pymol software and the coordinates from the crystal structure of the yeast nucleosome (White et al. 2001) downloaded from the Protein Data Bank with accession number 1ID3.

4.2.3 Identification of histone residues required for transcription upon heat stress

Single cells analysis of transcription by flow citometry upon heat stress allowed us to identify 60 and 108 mutants with defects on transcription on p*ALD3*-qV and p*HSP82*-qV, respectively (Fig. 6A). Out of the 60 mutants that showed defects on p*ALD3*-qV expression, 40 showed less expression of p*ALD3*-qV than the wild type, 19 showed higher expression and only one mutant showed transcriptional defects at normal growth conditions (basal). It is worth noting that out of the 40 mutants with lower expression of the reporter, none was a residue from H2B. Moreover, from the 19 mutants with higher expression of p*ALD3*-qV, 15 residues were from H2B.

From the 108 mutants with defects on p*HSP82*-qV transcription, 47 showed lower expression of the reporter than the wild type: 17, 3, 16 and 11 from H3, H4, H2A and H2B, respectively. H4 being the histone contributing less to p*HSP82*-qV transcriptional activation. 5 of the 108 mutants showed higher expression of the reporter than wild type cells, and 56 of the 108 mutants showed defects on transcription already at physiological conditions, in the absence of stress. Since 4, 9, 16 and 27 mutants of H3, H4, H2A and H2B, respectively, showed basal transcriptional defects, H2A and H2B seem to be the main players on the basal repression of *HSP82*.

From the 60 and 108 mutants with transcriptional defects on p*ALD3*-qV and p*HSP82*-qV, respectively, 8

mutants showed defects on both reporters in response to heat stress (Fig. 9). From these 8 common mutants, only 3 showed the same type of defect (downregulation of p*ALD3*-qV and p*HSP82*-qV). These results showed that the residues involved in *ALD3* transcription (controlled by Msn2/4 transcription factors) are different from the residues necessary for *HSP82* transcription (controlled by Hsf1 transcription factor), suggesting specific histone requirements for each subset of genes (see discussion).

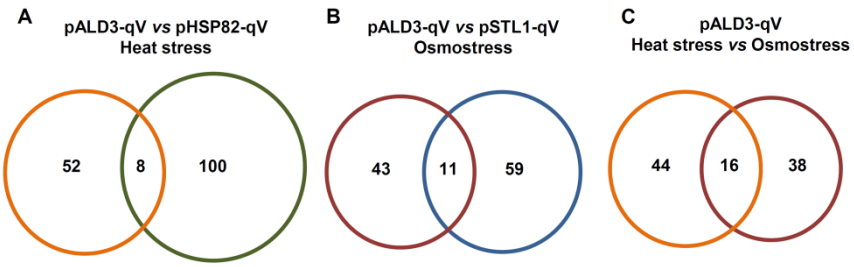


Figure 9. Venn diagrams representing the overlap of transcriptional defects
A) Overlap of p*ALD3*-qV (orange) and p*HSP82*-qV (green) in response to heat stress. **B)** Overlap of p*ALD3*-qV (red) and p*STL1*-qV (blue) in response to osmostress and **C)** Overlap between p*ALD3*-qV in response to heat (orange) and osmostress (red).

4.2.4 Identification of histone residues required for transcription upon osmostress

In response to osmostress, we identified 54 and 70 mutants showing transcriptional defects on p*ALD3*-qV and p*STL1*-qV, respectively (Fig. 6A). From the 54 mutants with defects on p*ALD3*-qV, 37 showed lower expression of the reporter than the wild type: 6, 20, 7 and 4 residues from H3,

H4, H2A and H2B, respectively, being in this case H4 the main player on p*ALD3*-qV transcriptional activation in response to osmostress. We identified 16 mutants that showed higher expression of the reporter and only one mutant showed a basal p*ALD3*-qV transcriptional defect, the same one identified in response to heat stress (H3R8A).

In response to osmostress we also identified 70 mutants with defects on p*STL1*-qV transcription, 58 of them showed lower and 12 higher expression of the reporter in the mutant compared to the wild type. No mutants showed basal transcriptional defects on p*STL1*-qV.

When comparing the mutants that showed transcriptional defects upon osmostress, we observed that from the 54 and 70 mutants with defects on p*ALD3*-qV and p*STL1*-qV, respectively, 11 mutants showed transcriptional defects on both reporters (Fig. 9). It is worth noting that 9 of the 11 common mutants showed the same type of transcriptional defect on both reporters: 8 mutants showed lower expression and 1 mutant higher expression of both reporters when compared to wild type. These results suggest that there is a subset of histone residues required for initiation of transcription in response to osmostress required for both promoters, independently on the transcription factor involved in the induction of those genes: *ALD3 (*Msn2/4) and *STL1* (Hot1/Sko1). We also observed a subset of histone residues involved specifically in the activation p*ALD3*-qV and another subset involved in the activation of p*STL1*-qV, suggesting

certain specificity depending on the promoter (see discussion).

4.2.5 Histone residues required for *ALD3* transcription upon both stresses

In order to compare the transcriptional defects upon heat and osmostress, we focused on *ALD3*, which is regulated by Msn2/4 transcription factor in response to both heat and osmostress. When we analyzed p*ALD3*-qV reporter we identified 60 and 54 mutants with transcriptional defects in response to heat and osmostress, respectively. 16 of those mutants were affected in both stress conditions (Fig. 9). The type of defect was in all the cases the same, 1 mutant showed basal transcriptional defects, 5 mutants higher expression and 9 mutants lower expression of the reporter compared to the wild type in response to both heat and osmostress, suggesting a common regulatory pattern between stress conditions.

We also observed several residues that seemed to be specific for one stress condition. There is a clear example on H4, where residues from I26 to T30 showed defects in p*ALD3*-qV only in response to heat stress, suggesting a specific region that is required depending on the stress condition. Another example on H4 is mutation of the last two amino acids G101 and G102, which impaired transcriptional activation of p*ALD3*-qV only in response to osmostress.

Taken together, our data suggests that the regulation of these promoters involves common regulatory mechanism between stresses and specific regulatory mechanisms for each specific situation.

4.2.6 Mutations on modifiable residues lead to transcriptional defects

To further characterize the residues showing transcriptional defects in response to stress, we analyzed their spatial distribution on the nucleosome and the amino acid type (Fig. 10).

When we compared the spatial distribution of the residues involved in p*ALD3*-qV transcriptional regulation in response to heat and osmostress, we observed that, although only around 30% are common between stresses, the overall spatial distribution pattern is similar in both stress conditions (Fig. 10A). However, bigger differences were observed when comparing p*HSP82*-qV and p*ALD3*-qV in response to heat stress or p*STL1*-qV and p*ALD3*-qV in response to osmostress. We found that more buried and lateral residues were involved in the transcriptional regulation of p*HSP82*-qV and p*STL1*-qV, when compared to p*ALD3*-qV (Fig. 10A). It is worth noting that upon both stresses, tail residues were generating significantly more transcriptional defects than growth defects (Fig. 2A and 10A) (see discussion).

By sorting the histone residues that led to transcriptional defects by amino acid type we observed that

mutations on leucine (L) and modifiable amino acids such as lysine (K), arginine (R) and serine (S) generated most of the transcriptional defects in response to both stresses (Fig. 10B).

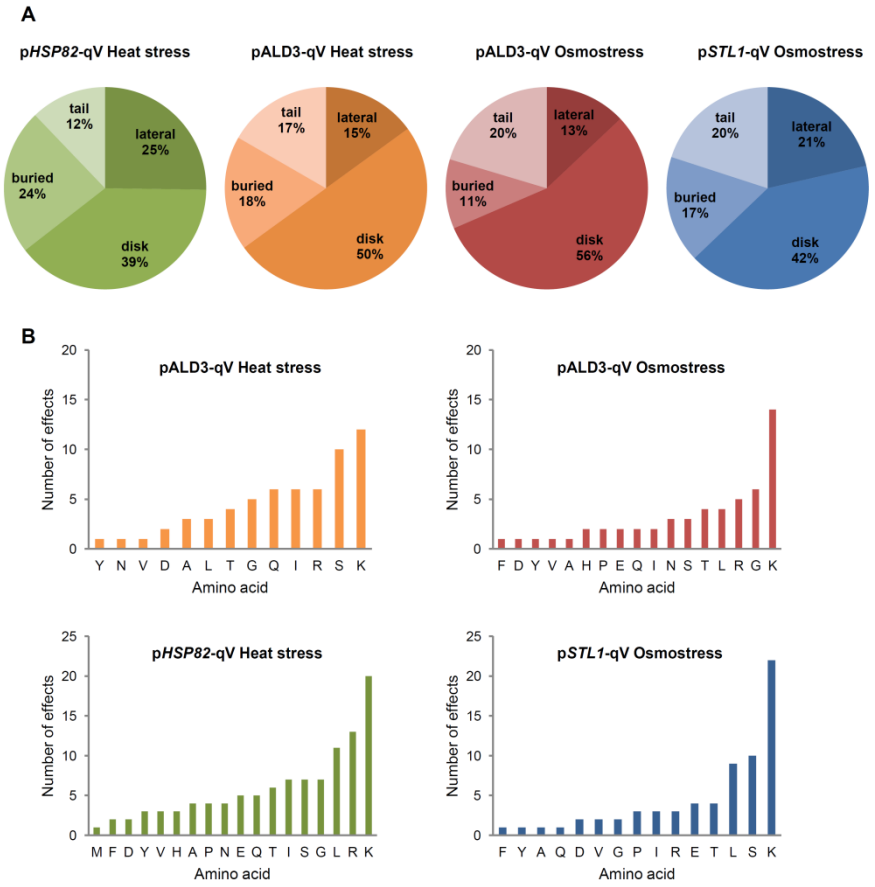


Figure 10. Spatial distribution of the histone residues necessary for transcriptional regulation upon heat and osmostress
A) Spatial distribution of the mutants identified in the transcriptional screen on the nucleosome structure. Histone residues can be located on the tails, inside the protein structure (buried) or located on the histone surface that contacts DNA (lateral) or that does not contact DNA (disk). **B)** Frequency of transcriptional defects generated by mutations on each amino acid type in each condition.

4.3 NOVEL HISTONE MARKS THAT REGULATE GENE EXPRESSION

After global analysis of the histone residues required for the proper response to stress, we then focused on some specific examples of histone H3 and H4 residues indentified in the transcriptional screening for further characterization. Results of H4T30, H4K79, H4S47, H3S57 and H3T58 are presented.

4.3.1 Region I26-T30 on H4 is required for transcriptional regulation upon heat stress

The analysis of the transcriptional screening yielded an interesting observation. Several regions in histones showed clusters of amino acids with similar transcriptional defects (Fig. 7). We focused our attention on the region located close to the N-terminal tail of H4 that comprises amino acids from I26 to T30. Mutations of all these amino acids resulted in transcriptional defects on p*ALD3*-qV in response to heat stress. Additionally to the transcriptional defects, two of the mutants included in this region, G28A and I29A showed slower growth at high temperature in the phenotypic screening.

To confirm the thermosensitivity of these two mutants, we manually spotted wild type and all the mutants from the region (H4I26A, Q27A, Q27E, G28A, I29A, T30A and T30D) onto YPD plates and incubated for 3 days at 30ºC (control) and 39ºC (heat stress). We confirmed that indeed, G28A and

I29A mutant cells were unable to grow at 39ºC and additionally we observed than T30D mutant cells were also slightly thermosensitive (Fig. 11).

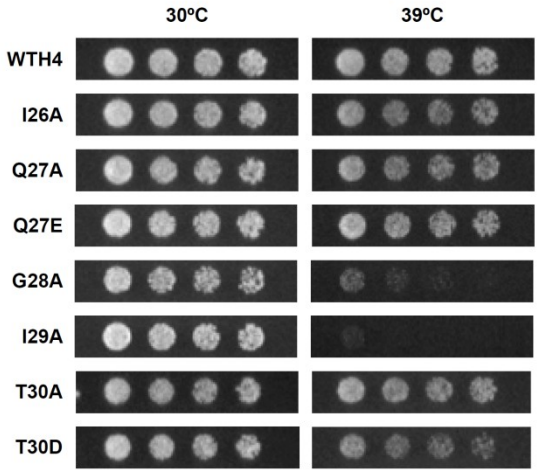


Figure 11. Impaired cell growth of H4G28A, I29A and T30D mutants upon heat stress
Wild type (WT) and all the mutant strains comprised in the H4 region (I26-T30) were grown to mid-log phase and spotted, making serial dilutions on YPD plates. Cells were incubated at 30 or 39ºC for 3 days.

To confirm the transcriptional defects observed by flow citometry, we performed Northern blot assays and we observed that indeed, all the mutants of the region had reduced *ALD3* expression and no defects were detected on *HSP82* in response to heat stress (Fig. 12). As described in the introduction, the massive transcriptional regulation in response to heat stress is governed by Msn2/4 and Hsf1 transcription factors. Msn2/4 are responsible for a subset of genes, such as *ALD3* and *CTT1*, that are induced upon several stress conditions, among them heat stress. Hsf1 is

the transcription factor responsible for the Heat Shock Response (HSR) and regulates genes such as *HSP82* and *SSA3*. To further characterize the region, we assessed mRNA levels of additional Msn2/4-dependent genes (*CTT1*) and Hsf1-dependent genes (*SSA3*). We observed that indeed, all the mutant strains showed impairment on *CTT1* while *SSA3* expression was similar in the mutants than in wild type cells (Fig. 12B). These results suggest that this region could be involved specifically in the regulation of Msn2/4 dependent genes in response to heat stress.

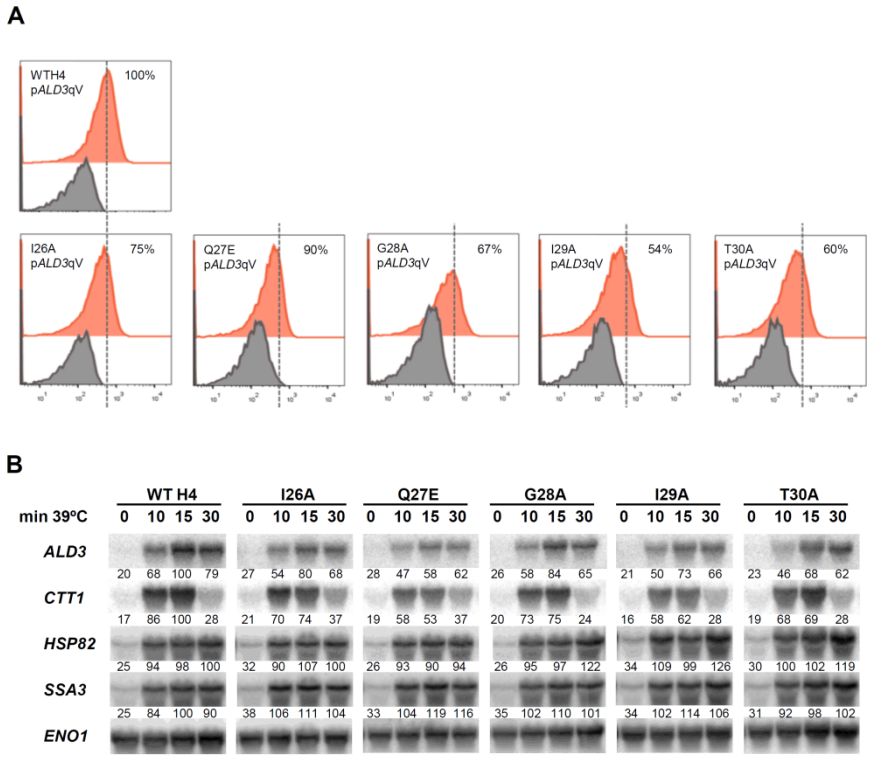


Figure 12. A novel region on histone H4 is required for transcription upon heat stress

A) Wild type (WT) and the indicated mutant strains harboring the quadruple-Venus (qV) fluorescence reporter driven by *ALD3* promoter

were measured by flow citometry before and after heat stress (39ºC for 45 min). **B)** WT and the indicated mutant strains were grown to mid-log phase and then subjected to heat stress (39ºC) for the indicated length of time. Total RNA was assayed by Northern blot for transcript levels of the indicated genes and *ENO1* (as a loading control). For panels **A** and **B,** the values of maximum gene expression of the wild type strain were used as 100% reference.

Within this region, Q27 and T30 are two modifiable residues. To test whether phosphorylation on T30 could be important to regulate transcription, we assessed the effect of changing it to alanine (T30A) and the corresponding phosphomimetic mutant (T30D) by Northern in response to heat stress. We found that the T30D phosphomimetic mutant could partially rescue the transcriptional defect of the alanine mutant (Figure 13), suggesting that phosphorylation on T30 could be involved in the regulatory mechanism of the region.

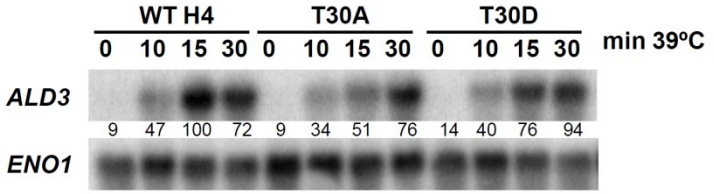


Figure 13. H4T30D phospho-mimetic mutant partially rescues the transcriptional defects upon heat stress
Wild type (WT), H4T30A and T30D mutant strains were grown to mid-log phase and then subjected to heat stress (39ºC) for the indicated length of time. Total RNA was assayed by Northern blot for transcript levels of *ALD3* and *ENO1* (as a loading control). The values of maximum gene expression of the wild type strain were used as 100% reference.

It is worth mention that the representation on the tridimensional structure of the nucleosome shows that all residues with the exception of I29 are located on the nucleosome surface, being easily accessible for histone modifiers or chromatin remodeling complexes (Fig. 14).

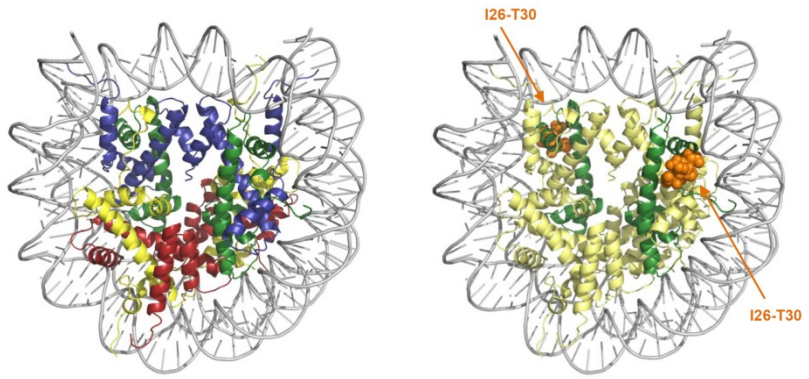


Figure 14 Localization of the H4 region on the nucleosome surface
A representation of the tridimensional structure of the nucleosome is shown (left). Histone H3 is represented in green, H4 in blue, H2A in yellow and H2B in red. The same representation highlighting only histone H4 in green is presented (right) to show the location of H4 region (orange) on the nucleosome surface. Figures were made using Pymol software and the coordinates from the crystal structure of the yeast nucleosome (White et al. 2001) downloaded from the Protein Data Bank with accession number 1ID3.

Taken together, our data indicates that we have identified a novel region on histone H4 required for activation of transcription in response to heat stress. Our data also suggest that this H4 region is required for regulation of Msn2/4 dependent genes in response to heat stress and that this regulation might be through modification of T30, a phosphorylable threonine located on the nucleosome surface.

Futher experiments need to be done to clarify the regulatory mechanism of H4 region (see discussion).

4.3.2 H4K79 is required for transcriptional regulation upon heat stress

p*ALD3*-qV transcription was strongly reduced in H4K79A and H4K79Q mutants upon heat stress (Fig. 15A). H4K79 is located in the L2 loop of H4, which interacts with H3L1 loop, generating a DNA binding surface (Luger et al. 1997). The fact that lysine (K) is a modifiable residue makes H4K79 an interesting candidate for further studies.

To validate these results, we performed Northern blot assays in response to heat stress and we observed a significantly reduced transcription of Msn2/4 dependent genes (*ALD3* and *CTT1*) on H4K79A and H4K79Q mutants, however expression of Hsf1 dependent genes (*HSP82* and *SSA3*), was similar to wild type (Fig. 15B). Furthermore, no defects on transcription were observed on H4K79R mutant cells when analyzed upon heat stress by Northern.

Often mutations of lysine (K) to glutamine (Q) are used to mimic lysine acetylated states, while mutations to arginine (R) are used to mimic non-acetylated or methylated states. Conversely, lysine mutated to alanine (A) prevents any modification event. Since mutations to K79A and K79Q led to transcriptional defects and K79R showed no defects on transcription we can hypothesize that methylation of H4K79 could be important to regulate transcription activation in

response to heat stress. Furthermore the position of H4K79 on the nucleosome surface makes this residue a potential modifiable lysine and accessible for histone modifiers and chromatin remodeling complexes (Fig. 16).

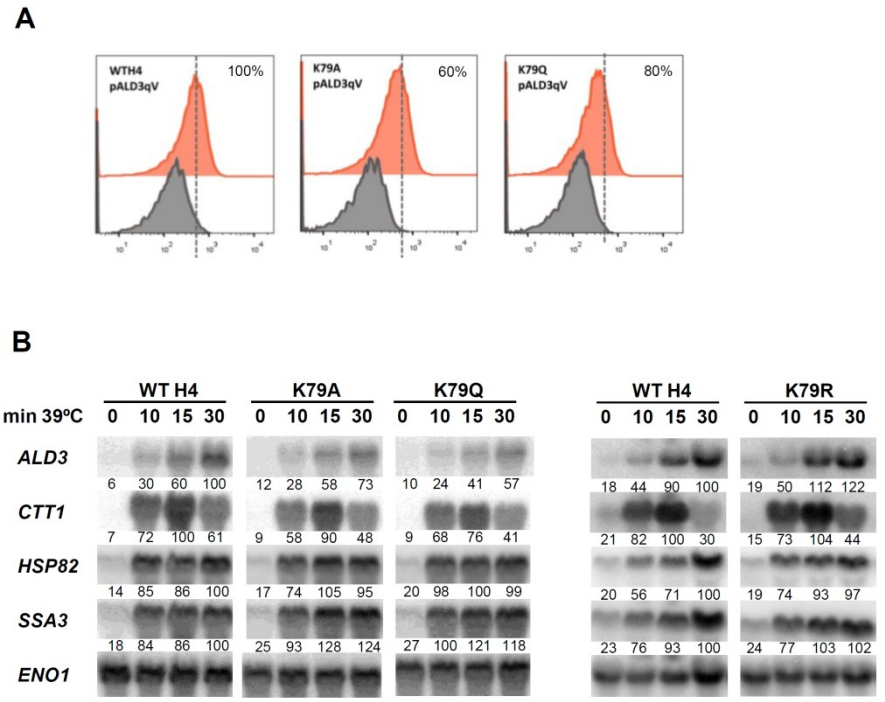


Figure 15. H4K79 is required for transcription upon heat stress
A) Wild type (WT), H4K79A and K79Q mutant strains harboring the quadruple-Venus (qV) fluorescence reporter driven by *ALD3* promoter were measured by flow citometry before and after heat stress (39ºC for 45 min). The values of maximum reporter gene expression of the wild type strain were used as 100% reference. **B)** Wild type (WT) and H4K79A, K79R and K79Q mutant strains were grown to mid-log phase and then subjected to heat stress (39ºC) for the indicated length of time. Total RNA was assayed by Northern blot for transcript levels of the indicated genes and *ENO1* (as a loading control). For panels **A** and **B**, the values of maximum gene expression of the wild type strain were used as 100% reference.

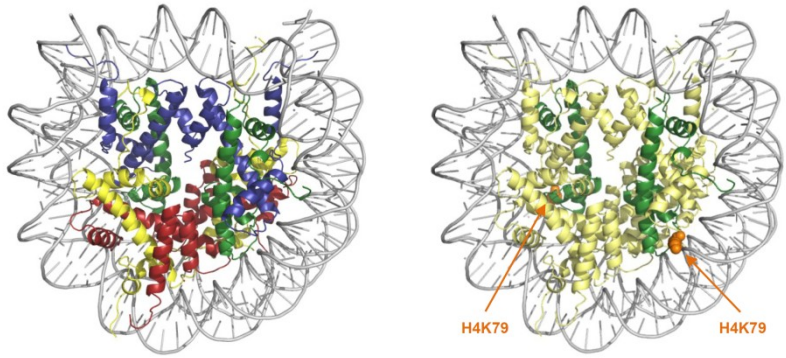


Figure 16. Localization of HK79 on the nucleosome surface
A representation of the tridimensional structure of the nucleosome is shown (left). Histone H3 is represented in green, H4 in blue, H2A in yellow and H2B in red. The same representation highlighting only histone H4 in green is presented (right) to show the location of H4K79 (orange) on the nucleosome surface. Figures were made using Pymol software and the coordinates from the crystal structure of the yeast nucleosome (White et al. 2001) downloaded from the Protein Data Bank with accession number 1ID3.

Taken together our data suggest that H4K79 is involved in transcriptional regulation of Msn2/4 dependent genes specifically in response to heat stress and that this regulation is likely through the methylation of H4K79. Further experiments have to be done in order to confirm our hypothesis (see discussion).

4.3.3 H4S47 regulates transcription in response to osmostress

Among the mutants with defective transcription upon osmostress, H4S47D showed a strong defect on both p*ALD3*-qV and p*STL1*-qV reporter genes. Additionally, H4S47D

mutation rendered cells osmosensitive, suggesting that this mutation may play an important role in the general adaptive response to osmostress.

To confirm the slower growth at high osmolarity that we observed in the phenotypic screening, we manually spotted wild type, H4S47A and S47D mutant cells onto YPD plates with or without 1.2M NaCl or 2M Sorbitol. We confirmed that indeed H4S47D mutant cells were osmosensitive, while H4S47A mutant cells growth was similar to wild type cells (Fig. 17).

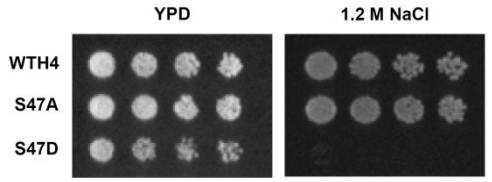


Figure 17. Impaired cell growth of H4S47D mutant upon osmostress
Wild type (WT), H4S47A and S47D mutant strains were grown to mid-log phase and spotted, making serial dilutions on YPD plates with or without 1.2M NaCl. Growth at 30ºC was scored after 3 days.

To further confirm the transcriptional defects identified in the screening by flow citometry, we assessed mRNA levels of the endogenous *ALD3* and *STL1* genes before and after addition of NaCl. Northern blot analysis showed that expression of *ALD3* was lower in H4S47D mutant than in wild type cells, confirming the result from the screening. However, expression of *STL1* was higher in H4S47D mutant than in wild type cells, contrary to the defect observed by flow

citometry in the screening (Fig. 18); this discrepancy could be due to differences in regulation of transcription initiation (analyzed using the reporter p*STL1*-qV) and transcription elongation (see discussion).

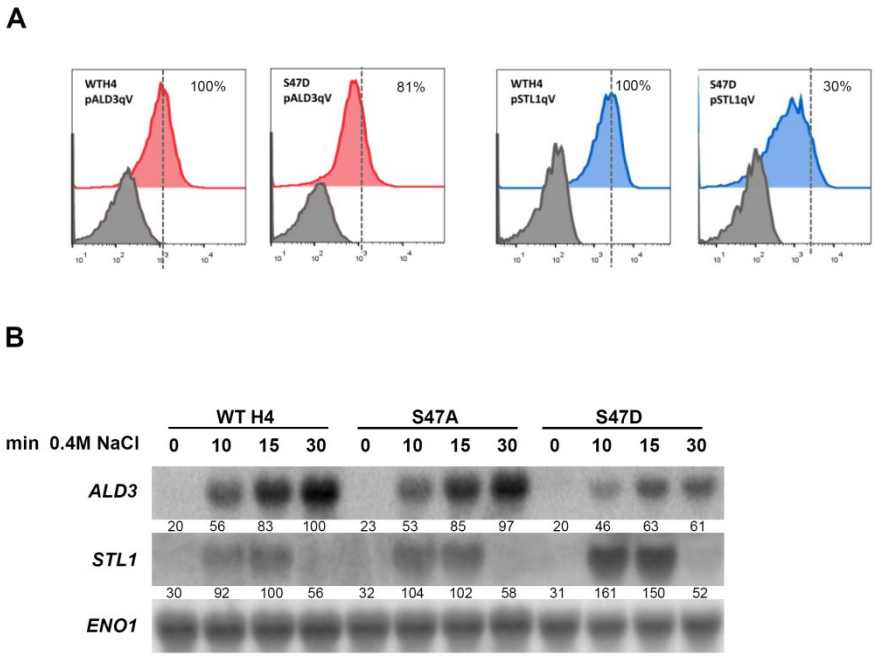


Figure 18. H4S47 required for transcription upon osmostress
A) Wild type (WT) and H4S47D mutant strains harboring the quadruple-Venus (qV) fluorescence reporter driven by *ALD3* or *STL1* promoters were measured by flow citometry before and after osmostress (0.4M NaCl for 45 min). **B)** Wild type (WT), H4S47A and S47D mutant strains were grown to mid-log phase and then subjected to osmostress (0.4M NaCl) for the indicated length of time. Total RNA was assayed by Northern blot for transcript levels of the indicated genes and *ENO1* (as a loading control). Quantification data normalized to the loading control. For panels **A** and **B**, the values of maximum gene expression of the wild type strain were used as 100% reference.

To have an overall picture of the transcriptional response of H4S47D mutant in response to osmostress, we

performed genome-wide analysis by using DNA microarrays. We compared gene induction upon osmostress (0.4M NaCl, 10 min) in wild type and H4S47D mutant strains. The results showed that out of the 622 genes induced more than two fold in response to osmostress, 261 (including *STL1*) were upregulated in the H4S47D mutant and 24 (including Msn2/4-dependent genes) were downregulated. These results confirmed the Northern blot results and provided an overall vision of the transcription of osmoresponsive genes in the H4S47D mutant strain, showing a clear tendency for upregulation.

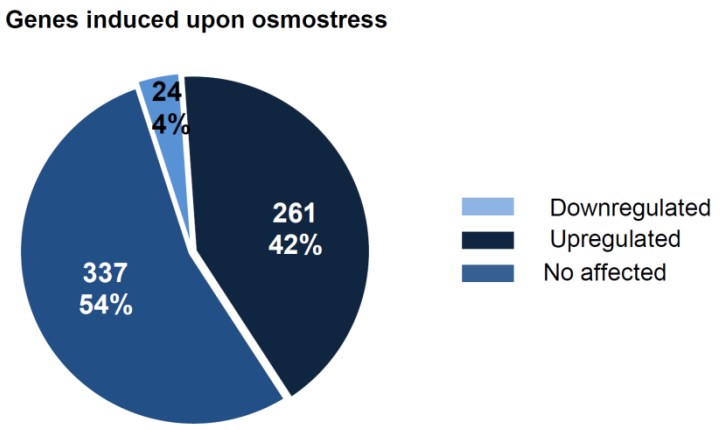


Figure 19. H4S47 is required for transcriptional regulation of osmoresponsive genes

Genes induced at least twofold under osmostress (0.4M NaCl for 10 min) were grouped into three categories according to the gene expression levels in the H4S47D mutant compared to the wild type (WT): downregulated (gene expression was at least 25% reduced in the mutant), upregulated (gene expression was at least 25% increased in the mutant) and no affected. Microarrays were performed by Frank Holstege Laboratory in Ultrech University Medical Center.

4.3.4 H4S47 is phosphorylated *in vitro* by several kinases

Since the H4S47D phosphomimetic mutant showed clear defects on transcriptional regulation in response to osmostress, we designed a biochemical screen to identify putative kinases able to phosphorylate this particular residue in histone H4 (Fig. 20). First, we individually purified all the existing kinases in yeast (123) using the TAP and Gal-ORF collections. Then, we performed *in vitro* kinase assay for individually testing these kinases and short histone peptides carrying the wild type S47 or the non-phosphorylatable version (S47A) as substrates. The histone peptides were fused to GST expressed and purified from *E.coli* (Fig.20).

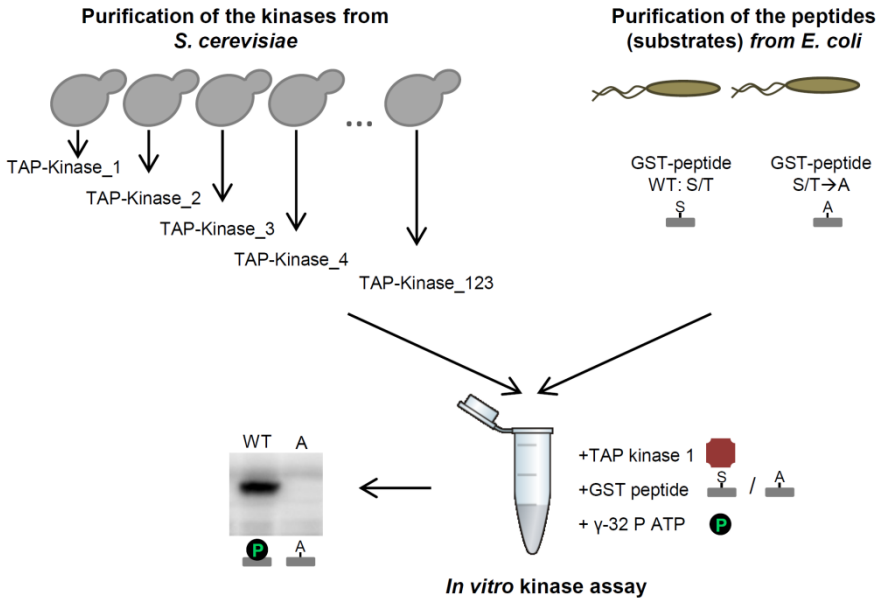


Figure 20. Experimental strategy to asses phosphorylation events by *in vitro* kinase assay

123 tagged kinases were purified from yeast cells and GST-peptides (substrates) were purified from *E.coli*. Each kinase was individually tested by *in vitro* kinase assay with the WT and mutated version of the peptide. The mixture of pre-activated kinase, GST-peptide and radiolabelled γ-32 P ATP was incubated at 30ºC for 30 min. Labeled peptides were resolved by SDS/PAGE, transferred to a nylon membrane and detected by autoradiography.

We found that 5 out of 123 kinases tested yielded a positive result in the *in vitro* kinase assay. These kinases were able to phosphorylate the wild type but not the mutated version of the peptide (Fig. 21). The five kinases were: Ste20, Cla4, Skm1, Cdc28 and Kin3. It is worth noting that Ste20, Cla4 are two kinases involved in the activation of the signaling cascade in the Sho1 branch of the HOG pathway. Of note, PAK2 kinase, orthologue of Ste20, Cla4 and Skm1 in human cells (Hofmann et al. 2004), has been found to phosphorylate H4S47 in human cells (see discussion).

H4S47D mutant was a good example to validate the results from the phenotypic and transcriptional screens performed in this study. Furthermore, it supports the potential of our screens to detect residues and to find novel histone marks and modifying activities required for the transcriptional regulation in response to stress not only in yeast but in evolutionarily conserved organisms (see discussion). Further experiments in order to assess the biological relevance of this mark in response to stress will be described in the discussion.

A

Peptide	Sequence	Lengh
WT H4S47	ARRGGVKRI**S**GLIYEEVRAV	H4 (38-57) / 20 aa
H4S47A	ARRGGVKRI**A**GLIYEEVRAV	H4 (38-57) / 20 aa

B

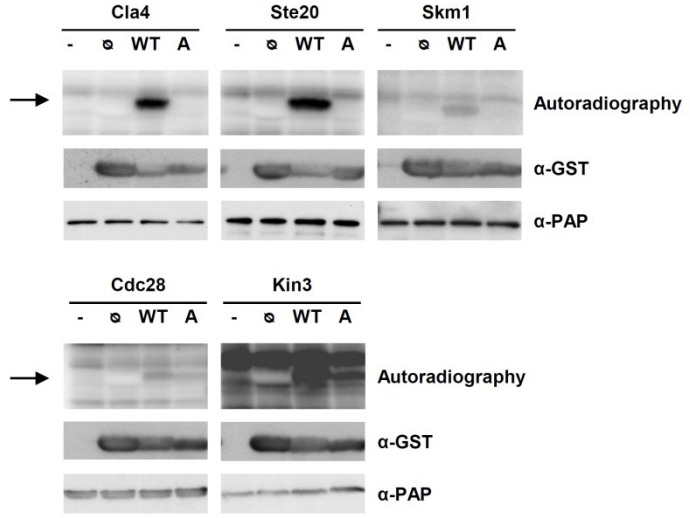


Figure 21. H4S47 is phosphorylated *in vitro* by several kinases
A) Table of the peptides used as substrates for the *in vitro* kinase assays. Peptides were fused to GST, cloned, expressed and purified from *E. coli.* **B)** Results of the *in vitro* kinase assay. 123 tagged kinases were purified from yeast and individually tested using GST-peptides as substrates. After pre-activation in kinase buffer containing ATP, the substrates were added in the presence of γ32P radiolabelled ATP. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. Tagged kinases and GST-tagged histone peptides were detected by Western blot.

4.3.5 H3T58 is involved in transcriptional regulation upon heat and osmostress

One of the mutants showing a transcriptional defect in p*ALD3*-qV expression in response to both heat and osmostress conditions was H3T58D (Fig. 22A). We assessed endogenous mRNA levels upon heat (39ºC) and osmostress

(0.4M NaCl) in order to confirm the defects observed at the transcriptional level by flow citometry. Northern blot analysis showed that H3T58D mutant cells have reduced expression of *ALD3* in response to heat and osmosstress, confirming the results observed by flow citometry in the screening (Fig. 22B).

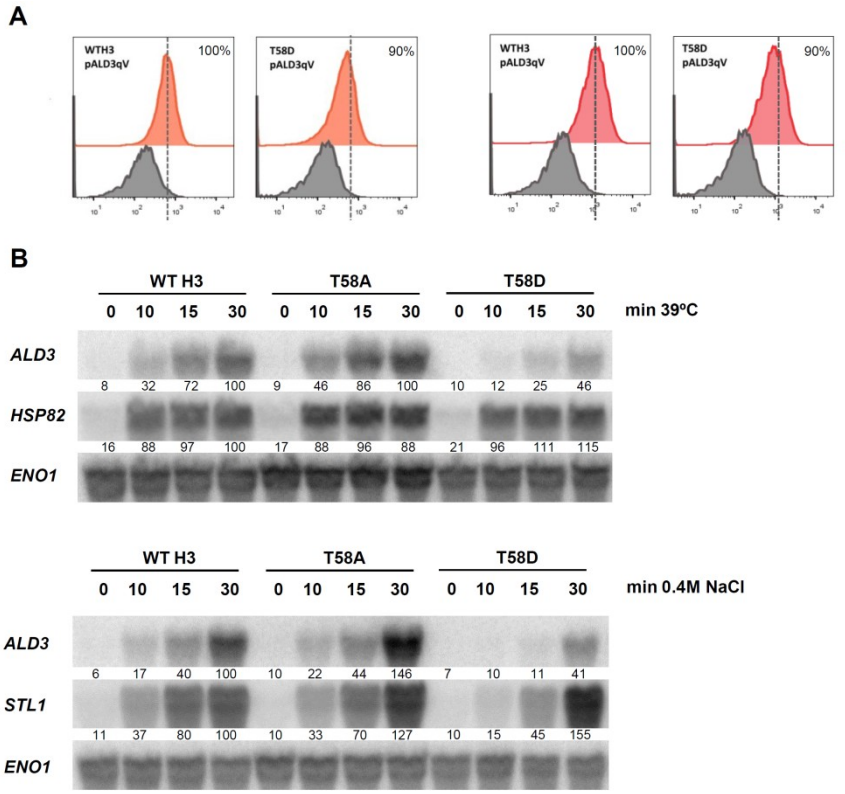


Figure 22. H3T58D is required for transcription upon stress
A) Wild type (WT), H3T58 and T58D mutant strains harboring the quadruple Venus (qV) fluorescence reporter driven by *ALD3* promoter were measured by flow citometry before and after heat (39ºC) and osmostress (0.4M NaCl) for 45 min. **B)** WT, H3T58A and T58D mutant strains were grown to mid-log phase and then subjected to heat (39ºC) and osmostress (0.4M NaCl) for the indicated length of time. Total RNA was assayed by Northern blot for transcript levels of the indicated genes and *ENO1* (as a loading control). Quantification data normalized to the loading control. In panels **A** and **B**, the values of maximum gene expression of the wild type strain were used as 100% reference.

To further characterize the role of this threonine in transcriptional regulation upon stress, we performed a global gene expression analysis comparing wild type and H3T58D mutant strains in response to osmostress. DNA microarray analysis showed that around half of the genes induced upon osmostress were affected by the histone mutation H3T58D. Out of the 622 genes induced at least twofold in response to osmostress (0.4M NaCl, for 10 min), 324 genes (including Msn2/4 dependent genes) showed a significant reduction in gene expression in the histone mutant. Thus, microarray analysis indicates that H3T58 is involved in a global manner in the transcriptional activation of osmoresponsive genes (Fig. 23). This analysis confirmed the results from the Northern blot and provided an overall vision of the transcription of osmoresponsive genes in the H3T58D mutant strain. Interestingly, in this case DNA microarray analysis showed a clear tendency to downregulation, contrary to the results observed in the H4T47D mutant strain (Fig.19).

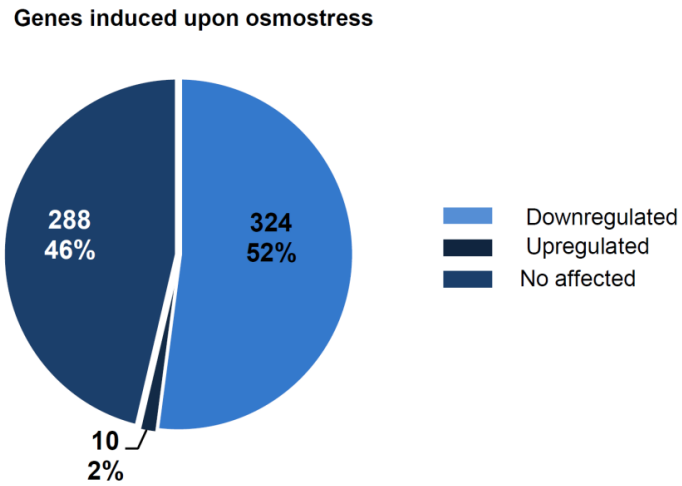


Figure 23. H3T58 is required for transcriptional regulation of osmoresponsive genes
Genes induced at least twofold under osmostress (0.4M NaCl for 10 min) were grouped into three categories according to the gene expression levels in the H4S47D mutant compared to the wild type (WT): downregulated (gene expression was at least 25% reduced in the mutant), upregulated (gene expression was at least 25% increased in the mutant) and no affected. Microarrays were performed by Frank Holstege Laboratory in Ultrech University Medical Center.

4.3.6 T58 and S57 are phosphorylated *in vitro* by several kinases

The clear defects on transcription in response to heat and osmostress of the mutant H3T58D, the absence of PTMs previously identified for this residue and the lack of any information related to transcriptional regulation made this residue an interesting target for further studies. Thus, we next performed *in vitro* phosphorylation assays to shed some light on to this unknown molecular mechanism. As described before, we attempted to identify putative kinases that could target this residue. To find the kinase responsible for T58 phosphorylation, we performed *in vitro* kinase assays for 123 tagged purified kinases in yeast using short peptides fused to GST as substrates (as described in section 4.3.1.2 and Figure 23).

From the 123 kinases tested, we found 10 kinases able to phosphorylate the wild type peptide. Of those, three kinases, Pkp2, Mek1 and Vhs1, showed a reduction of phosphorylation signal in T58A when compared to wild type peptide (Fig. 24). Remarkably, we also found some kinases

showing a greater phosphorylation signal in the T58A peptide than in the wild type. These results led us to re-design the substrate by mutating also H3S57A, a serine next to T58. Then, we generated four different peptides: wild type, T58A, S57A and the double mutant T58A-S57A, all of them fused to GST as previously described (Figure 20). Using the new substrates, we repeated the *in vitro* kinase assay with the 123 kinases and we identified 10 kinases able to phosphorylate S57 *in vitro*, 7 of them specifically phosphorylated this residue (Rad53, Snf1, Psk1, Elm1, Kin1 Kin3 and Mrk1) and 3 kinases phosphorylated both T58 and S57 (Pkp2, Mek1 and Vhs1) (Fig. 24).

After finding that H3S57 and H3T58 were phosphorylated *in vitro* by common kinases, we asked whether H3S57 was also involved in transcriptional regulation of stress-responsive genes. Northern blot analysis showed a clear reduction of *ALD3* transcription in response to heat and osmostress when H3S57D mutant was compared to the wild type strain (Fig. 25). Therefore, the H3S57D mutant strain displayed a similar gene expression pattern as the H3T58D strain upon both stress conditions.

A

Peptide	Sequence	Lengh
WT H3T58	LREIRRFQKSTELLIRKLPFQRLV	H3 (48-71) / 24 aa
H3T58A	LREIRRFQKSAELLIRKLPFQRLV	H3 (48-71) / 24 aa
H3S57A	LREIRRFQKATELLIRKLPFQRLV	H3 (48-71) / 24 aa
H3T58A-S57A	LREIRRFQKAAELLIRKLPFQRLV	H3 (48-71) / 24 aa

B

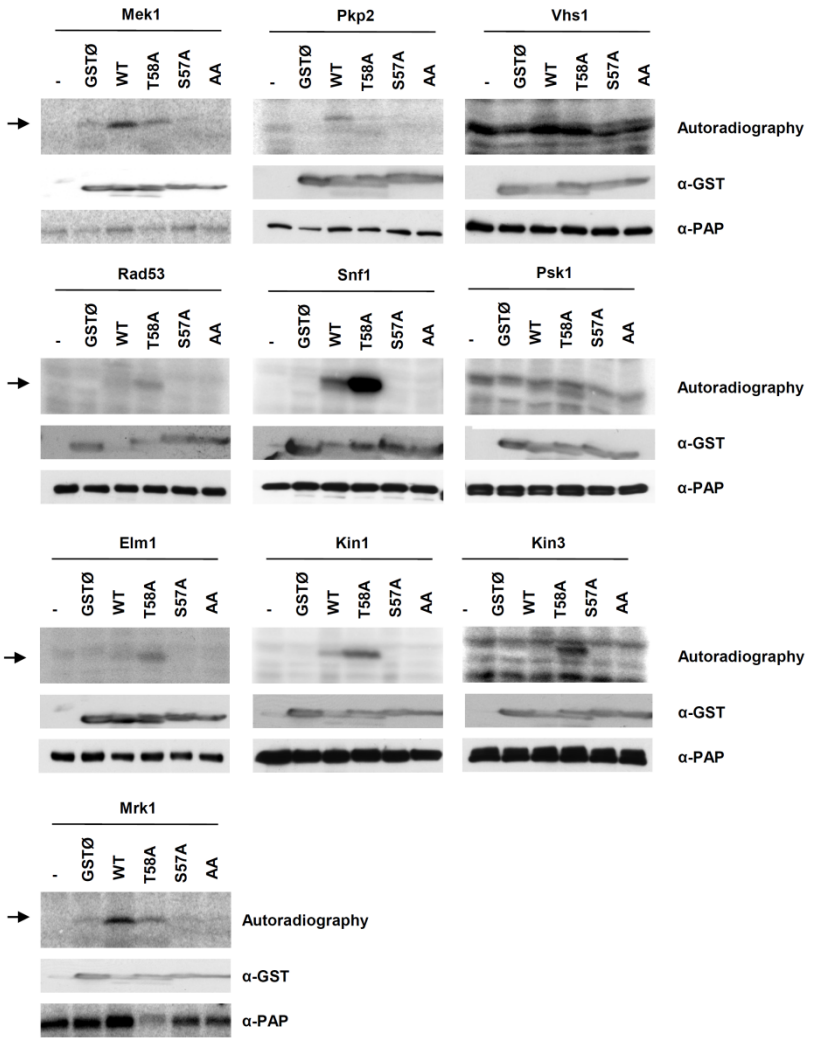


Figure 24. Results of the *in vitro* kinase assays for H3T58 and H3S57
A) Table of the peptides used as substrates for the *in vitro* kinase assays. All peptides were fused to GST, cloned, expressed and purified from *E. coli*. **B)** Results of the *in vitro* kinase assays. Performed as in Figure 21.

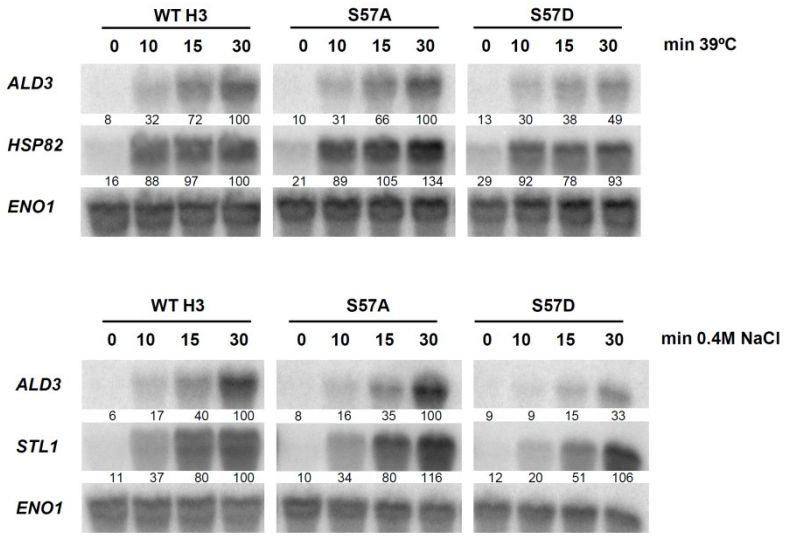


Figure 25. H3S57 is required for transcription upon stress
Wild type (WT), H3S57A and S57D mutant strains were grown to mid-log phase and then subjected to heat stress (39ºC) and osmostress (0.4M NaCl) for the indicated length of time. Total RNA was assayed by Northern blot for transcript levels of the indicated genes and *ENO1* (as a loading control). Quantification data normalized to the loading control. The values of maximun gene expression of the wild type strain were used as 100% reference.

Further experiments need to be done to elucidate which is the kinase responsible for the phosphorylation of H3S57 and T58 and which is the role of these phosphorylations on the regulation of transcription upon stress (see discussion).

Supplementary tables

Table S1. List of the histone mutants that rendered cells thermosensitive and/or osmosensitive upon heat and osmostress, respectively. Mutants showing both phenotypes are highlighted in grey.

Histone	Mutant	Position	TS	OS
H3	A1S	tail	NO	NO
H3	R2A	tail	YES	NO
H3	T3D	tail	NO	YES
H3	K4A	tail	YES	NO
H3	K4R	tail	YES	NO
H3	K4Q	tail	YES	NO
H3	Q5A	tail	YES	NO
H3	K14A	tail	YES	NO
H3	P16A	tail	YES	NO
H3	R17A	tail	YES	NO
H3	K18Q	tail	YES	NO
H3	S28A	tail	YES	NO
H3	T32D	tail	n/d	YES
H3	K36A	tail	YES	YES
H3	K36K	tail	YES	NO
H3	K36Q	tail	YES	NO
H3	K37A	tail	YES	NO
H3	H39A	lateral	NO	YES
H3	R40A	lateral	YES	NO
H3	K42A	lateral	YES	YES
H3	G44A	lateral	YES	YES
H3	V46A	lateral	YES	YES
H3	R49A	lateral	YES	NO
H3	E50A	disk	YES	NO
H3	E50Q	disk	YES	NO
H3	R53A	disk	YES	NO
H3	K56A	lateral	YES	NO
H3	K56Q	lateral	YES	NO
H3	S57D	disk	YES	NO
H3	T58A	disk	YES	NO
H3	T58D	disk	YES	NO
H3	Q68A	disk	YES	YES
H3	R69A	lateral	NO	YES
H3	L70A	buried	YES	NO
H3	V71A	buried	YES	NO
H3	E73A	disk	YES	NO
H3	I74A	buried	YES	NO
H3	F78A	disk	YES	NO
H3	K79R	disk	YES	NO
H3	T80A	disk	YES	NO
H3	T80D	disk	YES	NO
H3	L82A	disk	YES	NO
H3	R83A	lateral	YES	NO

Histone	Mutant	Position	TS	OS
H3	Q85A	lateral	YES	NO
H3	S86A	lateral	YES	NO
H3	S86D	lateral	YES	NO
H3	A88S	buried	YES	NO
H3	I89A	disk	YES	NO
H3	L92A	buried	YES	NO
H3	Q93E	buried	YES	NO
H3	A98S	disk	YES	NO
H3	Y99A	buried	YES	NO
H3	Y99E	buried	YES	NO
H3	L100A	buried	YES	NO
H3	V101A	buried	YES	NO
H3	S102A	disk	NO	NO
H3	S102D	disk	YES	NO
H3	F104A	buried	YES	YES
H3	E105Q	disk	YES	YES
H3	D106A	disk	YES	NO
H3	D106N	disk	YES	NO
H3	T107A	buried	YES	NO
H3	N108A	buried	YES	NO
H3	L109A	disk	YES	NO
H3	A110S	buried	YES	NO
H3	A111S	buried	YES	NO
H3	A114S	disk	YES	NO
H3	K115Q	lateral	YES	NO
H3	V117A	lateral	YES	NO
H3	Q120A	lateral	YES	NO
H3	K121A	disk	YES	NO
H3	K122R	disk	YES	YES
H3	K122Q	disk	YES	YES
H3	K125A	disk	YES	NO
H3	K125R	disk	YES	NO
H3	K125Q	disk	YES	NO
H3	A127S	buried	YES	NO
H3	R128A	disk	YES	NO
H3	R128K	disk	YES	NO
H3	R129K	disk	NO	YES
H3	R131A	disk	YES	NO
H3	R131K	disk	YES	NO
H3	G132A	disk	YES	NO
H3	E133A	disk	YES	NO
H3	E133Q	disk	YES	NO
H4	R3K	tail	YES	NO
H4	K8Q	tail	YES	YES
H4	G9A	tail	YES	NO
H4	K12Q	tail	NO	YES
H4	K16A	tail	YES	NO
H4	N25A	disk	YES	NO
H4	N25D	disk	NO	YES
H4	G28A	disk	YES	NO

Histone	Mutant	Position	TS	OS
H4	I29A	buried	YES	NO
H4	T30D	lateral	NO	YES
H4	K31A	lateral	YES	NO
H4	K31Q	lateral	YES	NO
H4	A33S	buried	YES	NO
H4	R35A	lateral	YES	NO
H4	R36K	lateral	YES	NO
H4	L37A	buried	YES	NO
H4	A38S	buried	YES	NO
H4	R39K	lateral	YES	YES
H4	G42A	buried	YES	NO
H4	K44A	lateral	YES	YE
H4	K44R	lateral	YES	YES
H4	I46A	lateral	YES	YES
H4	S47A	lateral	YES	NO
H4	S47D	lateral	YES	YES
H4	I50A	buried	YES	YES
H4	Y51A	disk	YES	NO
H4	E52Q	disk	YES	NO
H4	E53A	disk	YES	NO
H4	E53Q	disk	YES	NO
H4	V54A	buried	YES	NO
H4	R55A	disk	YES	YES
H4	L58A	buried	YES	NO
H4	K59Q	disk	YES	NO
H4	S60D	disk	YES	NO
H4	L62A	buried	YES	NO
H4	V65A	buried	YES	NO
H4	I66A	buried	YES	NO
H4	V70A	buried	YES	NO
H4	E74A	disk	YES	NO
H4	K77R	lateral	YES	NO
H4	K79Q	lateral	YES	NO
H4	T80A	lateral	YES	NO
H4	V81A	buried	YES	NO
H4	T82A	lateral	YES	NO
H4	T82D	lateral	YES	NO
H4	D85A	buried	YES	YES
H4	V86A	buried	YES	NO
H4	V87A	buried	YES	YES
H4	Y88A	disk	YES	NO
H4	R92K	disk	YES	NO
H4	Q93A	disk	YES	NO
H4	Q93E	disk	YES	NO
H4	L97A	buried	YES	YES
H4	Y98A	buried	YES	YES
H4	Y98E	buried	YES	YES
H2A	R30A	lateral	YES	NO
H2A	E57A	disk	YES	YES

Histone	Mutant	Position	TS	OS
H2A	E65A	disk	NO	YES
H2A	L66A	disk	NO	YES
H2A	G68A	buried	YES	NO
H2A	N69A	disk	YES	NO
H2A	R72A	disk	NO	YES
H2A	D73A	disk	YES	NO
H2A	N74A	disk	YES	YES
H2A	K76A	lateral	YES	NO
H2A	R78A	lateral	YES	NO
H2A	L86A	disk	NO	YES
H2A	E93A	disk	YES	YES
H2A	L94A	buried	NO	YES
H2A	I103A	buried	YES	NO
H2A	G107A	disk	YES	NO
H2A	I112A	disk	NO	YES
H2A	H113A	disk	YES	YES
H2A	N115A	disk	YES	NO
H2A	L116A	buried	YES	NO
H2A	L117A	disk	YES	NO
H2A	S121A	disk	YES	YES
H2B	Y43A	lateral	YES	NO
H2B	D71A		NO	YES
H2B	H112A	disk	YES	NO
H2B	E116A	disk	YES	YES
H2B	K123A	disk	YES	NO

Table S2. List of histone mutants that lead to transcriptional defects upon heat and/or osmostress. Mutants showing a defect on p*ALD3*qV expression in response to both stresses are highlighted in grey.

			Heat stress		Osmostress	
Histone	Mutant	Position	*ALD3*	*HSP82*	*ALD3*	*STL1*
H3	R2A	tail	YES	NO	NO	NO
H3	K4A	tail	YES	NO	NO	YES UP
H3	K4R	tail	NO	n/d	NO	YES UP
H3	K4Q	tail	NO	n/d	NO	YES UP
H3	Q5A	tail	YES	n/d	n/d	NO
H3	T6D	tail	NO	NO	NO	YES
H3	R8A	tail	B&UP	n/d	B&UP	NO
H3	K9A	tail	NO	NO	n/d	YES
H3	K14A	tail	NO	NO	YES	NO
H3	K14R	tail	NO	NO	NO	YES UP
H3	K14Q	tail	NO	NO	NO	YES UP
H3	K18R	tail	YES	NO	NO	NO
H3	S22A	tail	NO	NO	NO	YES
H3	K23Q	tail	NO	YES	NO	NO
H3	S28A	tail	YES	NO	NO	NO
H3	P30V	tail	NO	YES	YES	NO
H3	S31D	tail	NO	NO	NO	YES
H3	G34A	tail	NO	YES	NO	NO
H3	K36R	tail	YES UP	n/d	YESUP	YES UP
H3	P38A	disk	NO	NO	NO	YES
H3	R40A	lateral	n/d	YES	n/d	NO
H3	Y41F	lateral	NO	YES	NO	NO
H3	K42A	lateral	NO	YES UP	NO	NO
H3	K42R	lateral	n/d	YES	n/d	NO
H3	K56A	lateral	n/d	NO	n/d	YES UP
H3	K56R	lateral	NO	YES	YES	NO
H3	T58D	disk	YES	NO	YES	NO
H3	L60A	disk	n/d	NO	n/d	YES
H3	R63A	lateral	NO	YES	NO	NO
H3	K64R	lateral	NO	B&UP	NO	NO
H3	R69A	lateral	n/d	NO	n/d	YES
H3	L70A	buried	NO	YES	n/d	n/d
H3	K79R	disk	NO	YES	NO	NO
H3	D81A	disk	NO	NO	n/d	YES
H3	L82A	disk	NO	YES UP	NO	YES
H3	R83A	lateral	NO	NO	NO	YES
H3	R83K	lateral	NO	YES	NO	NO
H3	Q93A	buried	YES	n/d	NO	n/d
H3	Q85A	lateral	n/d	NO	n/d	YES
H3	Q85E	lateral	YES	NO	NO	NO
H3	S86D	lateral	NO	NO	NO	YES
H3	S87A	disk	YES	NO	NO	n/d
H3	S87D	disk	YES	NO	YES	YES
H3	I89A	disk	n/d	n/d	n/d	YES
H3	G90A	disk	YES	NO	NO	YES

			Heat stress		Osmostress	
Histone	Mutant	Position	*ALD3*	*HSP82*	*ALD3*	*STL1*
H3	A91S	buried	NO	NO	NO	YES
H3	E94A	disk	NO	NO	n/d	YES
H3	E94Q	disk	NO	NO	YES UP	NO
H3	L100A	buried	YES	n/d	YES	NO
H3	S102D	disk	NO	NO	NO	YES
H3	F104A	buried	n/d	n/d	n/d	YES UP
H3	D106A	disk	YES UP	NO	YESUP	NO
H3	D106N	disk	n/d	YES	n/d	NO
H3	N108A	buried	NO	BASAL	NO	NO
H3	A110S	buried	YES	NO	NO	NO
H3	A111S	buried	YES	n/d	NO	NO
H3	A114S	disk	YES	NO	NO	NO
H3	K115A	lateral	NO	B&UP	NO	NO
H3	K115R	lateral	NO	YES	NO	NO
H3	V117A	lateral	n/d	YES	n/d	NO
H3	K122A	disk	YES	NO	NO	NO
H3	K122R	disk	NO	B&UP	NO	NO
H3	K122Q	disk	n/d	B&UP	NO	NO
H3	A127S	buried	NO	YES	NO	NO
H3	R134A	disk	YES	NO	NO	NO
H4	K5A	tail	NO	NO	YES UP	NO
H4	K5R	tail	NO	n/d	YES	NO
H4	K5Q	tail	NO	NO	YES	NO
H4	G7A	tail	NO	n/d	YES UP	NO
H4	K8Q	tail	YES	NO	NO	NO
H4	G14A	tail	YES UP	NO	YES UP	NO
H4	A15S	tail	NO	NO	YES UP	NO
H4	K16R	tail	NO	NO	YES UP	NO
H4	R17K	tail	NO	NO	NO	YES
H4	H18Q	disk	NO	NO	YES	YES
H4	R19A	disk	YES	NO	NO	NO
H4	K20A	disk	YES	BASAL	NO	YES
H4	K20Q	disk	NO	NO	YES	YES
H4	R23K	lateral	NO	NO	YES	NO
H4	N25A	disk	NO	YES	NO	NO
H4	I26A	disk	YES	NO	NO	NO
H4	Q27E	disk	YES	n/d	NO	NO
H4	G28A	disk	YES	n/d	NO	NO
H4	I29A	buried	YES	NO	NO	NO
H4	T30A	lateral	YES	NO	NO	NO
H4	K31R	lateral	NO	NO	YES	YES
H4	A33S	buried	NO	YES	NO	NO
H4	R35A	lateral	n/d	BASAL	n/d	NO
H4	L37A	buried	NO	NO	NO	YES
H4	G42A	buried	n/d	BASAL	n/d	NO
H4	S47D	lateral	NO	NO	YES	YES
H4	R55A	disk	YES	YESUP	NO	NO
H4	A56S	disk	NO	BASAL	n/d	NO
H4	V57A	buried	NO	NO	YES	NO
H4	K59R	disk	NO	NO	YES	YES

			Heat stress		Osmostress	
Histone	Mutant	Position	*ALD3*	*HSP82*	*ALD3*	*STL1*
H4	K59Q	disk	NO	NO	YES	NO
H4	S60A	disk	YES	NO	NO	NO
H4	S64D	disk	NO	NO	NO	YES
H4	V65A	buried	YES	NO	NO	NO
H4	I66A	buried	NO	NO	YES	NO
H4	S69A	buried	YES	NO	NO	NO
H4	T73A	buried	NO	NO	YES	NO
H4	H75Q	buried	n/d	NO	NO	YES
H4	K77Q	lateral	YES	NO	YES	NO
H4	K79A	lateral	YES	NO	NO	YES
H4	K79Q	lateral	YES	NO	NO	NO
H4	T80A	lateral	n/d	B&UP	n/d	n/d
H4	V81A	buried	n/d	NO	n/d	YES UP
H4	T82D	disk	NO	NO	YES	NO
H4	Y88A	disk	n/d	B&UP	n/d	n/d
H4	Y88F	disk	NO	NO	YES	NO
H4	A89S	buried	NO	B&UP	n/d	NO
H4	K91A	disk	n/d	B&UP	n/d	NO
H4	K91R	disk	YES	NO	YES	NO
H4	R92A	disk	NO	NO	YES	NO
H4	Q93A	disk	NO	BASAL	NO	NO
H4	Q93E	disk	YES	NO	YES	NO
H4	G94A	disk	YES	YES	n/d	NO
H4	R95K	disk	NO	NO	YES	NO
H4	G101A	disk	NO	n/d	YES	NO
H4	G102A	disk	NO	n/d	YES	NO
H2A	Q16A	lateral	NO	YES	NO	NO
H2A	S19A	disk	NO	YES	NO	NO
H2A	G23A	disk	NO	YES	NO	NO
H2A	L24A	disk	YES	n/d	NO	NO
H2A	T25A	disk	NO	B&UP	NO	NO
H2A	F26A	buried	NO	BASAL	NO	NO
H2A	P27A	disk	NO	n/d	NO	YES
H2A	R30A	lateral	YES UP	B&UP	YES UP	NO
H2A	H32A	disk	NO	B&UP	NO	NO
H2A	R33A	lateral	NO	B&UP	NO	NO
H2A	L34A	disk	NO	n/d	NO	YES
H2A	L35A	buried	NO	YES UP	NO	YES
H2A	R36A	lateral	NO	YES	NO	NO
H2A	Y40A	disk	NO	B&UP	NO	NO
H2A	Q42A	lateral	NO	BASAL	NO	NO
H2A	R43A	lateral	NO	B&UP	NO	NO
H2A	I44A	lateral	NO	B&UP	NO	NO
H2A	G45A	lateral	NO	YES	NO	NO
H2A	L52A	buried	NO	YES	NO	NO
H2A	L56A	buried	NO	YES	NO	YES
H2A	E57A	disk	NO	B&UP	NO	YES UP
H2A	L59A	buried	NO	YES	NO	NO
H2A	E65A	disk	NO	BASAL	NO	NO
H2A	N74A	disk	YES	n/d	YES	NO

			Heat stress		Osmostress	
Histone	Mutant	Position	*ALD3*	*HSP82*	*ALD3*	*STL1*
H2A	K76A	lateral	NO	n/d	NO	YES
H2A	T77A	lateral	NO	n/d	YES	NO
H2A	R78A	lateral	NO	B&UP	NO	NO
H2A	I79A	buried	NO	B&UP	NO	YES UP
H2A	I80A	disk	NO	YES	NO	NO
H2A	H83A	buried	NO	YES	NO	NO
H2A	Q85A	disk	NO	YES	NO	NO
H2A	L86A	disk	YES	n/d	YES	YES
H2A	N90A	disk	NO	YES	NO	NO
H2A	L97A	disk	NO	YES	NO	NO
H2A	I103A	buried	NO	B&UP	NO	NO
H2A	G106A	buried	NO	YES	NO	NO
H2A	G107A	disk	NO	B&UP	NO	NO
H2A	P110A	disk	NO	NO	YES	NO
H2A	I112A	disk	YES	n/d	YES	NO
H2A	H133A	disk	NO	n/d	YES UP	NO
H2A	N115A	disk	NO	n/d	YES	NO
H2A	L116A	buried	NO	B&UP	NO	NO
H2A	L117A	disk	NO	n/d	YES UP	YES
H2A	K119A	disk	n/d	YES	YES	NO
H2A	S121A	disk	YES	n/d	NO	NO
H2A	K123A	disk	NO	n/d	NO	YES
H2A	K126A	tail	NO	NO	NO	YES
H2A	S128A	tail	NO	YES	NO	NO
H2A	L131A	tail	NO	n/d	NO	YES
H2B	E5A	tail	NO	BASAL	NO	NO
H2B	K6A	tail	NO	BASAL	NO	NO
H2B	K7A	tail	NO	BASAL	NO	NO
H2B	P8A	tail	NO	B&UP	NO	NO
H2B	K21A	tail	NO	BASAL	NO	NO
H2B	T27A	tail	NO	YES	NO	NO
H2B	D28A	tail	YES UP	B&UP	NO	NO
H2B	R36A	lateral	NO	BASAL	NO	NO
H2B	K37A	lateral	YES UP	BASAL	NO	YES
H2B	E38A	lateral	NO	YES	NO	YES
H2B	T39A	disk	NO	B&UP	NO	NO
H2B	S41A	disk	YES UP	n/d	NO	NO
H2B	I44A	buried	NO	BASAL	NO	NO
H2B	Y45A	lateral	YES UP	NO	NO	YES
H2B	K46A	lateral	NO	NO	YES	YES
H2B	V47A	buried	NO	BASAL	NO	NO
H2B	K49A	disk	YES UP	n/d	NO	NO
H2B	Q50A	disk	NO	YES	NO	NO
H2B	T51A	disk	YES UP	NO	NO	NO
H2B	D54A	disk	NO	NO	NO	YES
H2B	T55A	buried	YES UP	NO	NO	NO
H2B	I57A	disk	YES UP	NO	NO	n/d
H2B	S58A	disk	YES UP	NO	NO	NO
H2B	Q59A	lateral	YES UP	NO	NO	NO
H2B	S61A	buried	NO	BASAL	NO	YES

			Heat stress		Osmostress	
Histone	Mutant	Position	*ALD3*	*HSP82*	*ALD3*	*STL1*
H2B	M62A	disk	NO	YES	NO	NO
H2B	S63A	disk	NO	NO	NO	YES
H2B	L65A		NO	YES	NO	NO
H2B	N66A	disk	NO	YES	YES	NO
H2B	S67A	buried	YES UP	YES	NO	NO
H2B	F68A	buried	NO	YES	NO	NO
H2B	V69A	buried	NO	BASAL	NO	NO
H2B	F73A	buried	NO	n/d	YES	NO
H2B	I76A	buried	YES UP	BASAL	NO	NO
H2B	T78A	disk	NO	B&UP	NO	YES
H2B	E79A	buried	NO	NO	NO	YES
H2B	S81A	disk	NO	YES	NO	NO
H2B	K82A	disk	NO	BASAL	NO	YES
H2B	L83A	buried	NO	YES	NO	NO
H2B	K88A	disk	NO	B&UP	NO	YES
H2B	K89A	lateral	NO	n/d	NO	YES
H2B	S90A	lateral	NO	BASAL	NO	YES
H2B	T91A	lateral	NO	NO	NO	YES
H2B	I92A	buried	YES UP	NO	NO	YES
H2B	S93A	disk	YES UP	n/d	NO	NO
H2B	I97A	buried	NO	BASAL	NO	NO
H2B	T99A	disk	NO	B&UP	NO	NO
H2B	R102A	disk	NO	B&UP	NO	NO
H2B	L105A	disk	NO	B&UP	NO	YES UP
H2B	P106A	disk	NO	YES	NO	NO
H2B	G107A	disk	YES UP	n/d	YES UP	NO
H2B	K111A	disk	NO	B&UP	NO	NO
H2B	H112A	disk	NO	B&UP	NO	NO
H2B	V114A	disk	NO	n/d	NO	YES
H2B	E116A	disk	NO	YES UP	YES UP	NO
H2B	G117A	buried	NO	NO	YES UP	YES
H2B	S125A	disk	NO	BASAL	NO	NO
H2B	S126A	disk	NO	n/d	YES	NO
H2B	Q129A	disk	NO	n/d	YES UP	NO

5. DISCUSSION

5 DISCUSSION

Identification of histone residues required for cell survival upon stress

In this study, we described a global map of histone residues required for cell survival in response to heat and osmostress. We performed a high-throughput screen for histone residues essential for cell survival at high temperature and high osmolarity. The screening identified 157 histone mutations (88, 49, 16 and 4 in histone H3, H4, H2A and H2B, respectively) that rendered cells thermosensitive and 43 (14, 15, 12 and 2 in histone H3, H4, H2A and H2B, respectively) that rendered cells osmosensitive.

Similar studies in histones H3 and H4 were previously performed in response to heat stress (Huang et al. 2009). According to Histonehits (http://www.histonehits.org), a database that summarizes all the previously reported histone screens, there are 65 H3/H4 mutants reported to be thermosensitive, our screen confirmed 46 of them. This observation supports the reliability of the data provided by our screen. Additionally, 91 new histone residues essential for cell survival in response to heat stress were identified, indicating a higher sensitivity than the reported screens. In addition, we also provide data for H2A/H2B that was not done before. In contrast to high temperature, a screen on the relevance of histone residues upon high osmolarity has not been reported before. Here, we show that 43 histone

residues are required for maximal cell survival upon osmostress.

A higher number of H3/H4 mutants were found to show defects on growth in response to stress in contrast to H2A/H2B. This difference could be for several reasons: 1) The fact of having several mutations of the same residue could explain the higher number of mutants found in H3/H4 library in contrast to H2A/H2B. H3/H4 library besides the alanine mutations, it also contains mutations mimicking modified and unmodified states, for example mutations of lysine H3K4 to K4A, K4R and K4Q rendered cells thermosensitive. 2) These differences could also be explained by the strain background, since 45% of the H3/H4 mutants previously described as thermosensitive in Histonehits and confirmed as thermosensitive in our screen, are not thermosensitive when we analyzed the histone mutant strains from the SHIMA library (data not shown). Thus lower sensitivity of the SHIMA background might cause an underestimation of the results from H2A/B screen. 3) Another possible explanation could be the lower degree of evolutionary conservation. The sequence identity between yeast (*S. cerevisiae*) and human histones is lower in histones H2A (71%) and H2B (63%) than in histones H3 (92%) and H4 (90%).

Furthermore, we identified a higher number of thermosensitive *versus* osmosensitive histone mutants, this result could be due to the astringency of the osmosensitivity

analysis, since only the mutants with slower growth at both NaCl and Sorbitol conditions were considered osmosensitive.

Our screening reveals that most of the histone residues involved in cellular survival upon heat and osmostress are lysine (K), arginine (R), glutamic acid (E) and leucine (L). It is worth noting that lysine and arginine are positively charged amino acids whereas glutamic acid is negatively charged. Furthermore, these three amino acids are putative sites of a great variety of posttranslational modifications (PTMs), indicating that modifiable charged residues are most likely to generate altered growth phenotypes. Our conclusion is in agreement with previously described data in Histonehits, where after summarizing 42 histone phenotypic screens from different labs, they concluded that highly conserved and modifiable residues lead to stronger growth defects (Huang et al. 2009).

From 156 thermosensitive and 43 osmosensitive mutants, 30 showed sensitivity to both stresses. This overlapping suggest a double regulatory mechanism for cell survival in response to stress: a subset of residues commonly required in response to both stress conditions and also a specific subset of histone residues specifically required for each stress situation. A similar effect was previously described by our group when comparing genome-wide genetic screens searching for gene mutations that rendered cells thermosensitive (see supplementary article (Ruiz-Roig et al. 2010)) and osmosensitive (Zapater et al. 2007). Only 18%

of the genes required for survival at high temperature were also crucial for survival at high osmolarity, suggesting that the response to each stress is limited to a specific subset of cellular activities (Ruiz-Roig et al. 2010).

A global map of histone residues essential for transcription upon stress

Nucleosomes are natural barriers for transcription. In response to stress there is massive nucleosome reorganization in order to activate transcription of stress-responsive genes. This nucleosome reorganization has been characterized genome-wide upon heat stress (Zanton & Pugh 2004) and upon osmostress (Nadal-Ribelles et al. 2012). In this thesis we have identified a set of histone residues essential for transcription regulation in response to heat and osmostress. We identified 60 and 108 mutants involved in *ALD3* and *HSP82* transcription initiation upon heat stress, respectively. In response to osmostress, we identified 54 and 70 histone residues involved in *ALD3* and *STL1* transcription initiation, respectively. Thus, this study provides a global map of the nucleosomal requirements for transcriptional regulation in response to stress conditions.

Identification of histone residues required for transcription initiation upon heat stress

In response to a sudden increase of temperature or osmolarity in the media, cells rapidly activate a transcriptional

program changing the gene expression pattern by activating stress-responsive genes and downregulating housekeeping genes in few minutes (Gasch et al. 2000; Causton et al. 2001). Hsf1 and Msn2/4 are the main transcription factors responsible for the transcriptional response upon heat stress.

We observed a poor overlapping of histone residues required for transcriptional regulation between *ALD3* (Msn2/4-dependent gene) and *HSP82* (Hsf1-dependent gene) in response to heat stress. Indeed, only three histone residues (from 60 and 108 residues required for *ALD3* and *HSP82*, respectively) are commonly required for transcription activation of both genes in response to heat stress. These results suggest that the subset of histone residues required in transcription initiation in response to heat stress is specific for each subset of genes, controlled by a specific transcription factor. Our results agree with previous studies from our group and others (Ruiz-Roig et al. 2010; Sadeh et al. 2011) about different requirements of histone modifiers and chromatin remodelers in response to heat stress depending on the stress-responsive gene. The Rpd3L Histone deacetylase complex (HDAC) is essential for transcription activation of Msn2/4 dependent genes in response to heat stress, acting as an activator of transcription. However Hsf1-dependent genes are activated independently of Rpd3L, which instead seems to contribute to basal repression (Kremer & Gross 2009; Ruiz-Roig et al. 2010). Another example is Snf2, the catalytic subunit of SWI/SNF chromatin remodeling complex.

Snf2 is essential for transcriptional activation of *HSP12* (Mns2/4 and Hsf1 dependent gene), however it is not necessary for the induction of *HSP82* (Hsf1 dependent gene) in response to heat stress (Erkina et al. 2008). Besides SWI/SNF, also the role of RSC and ISWI remodeling complexes in response to heat stress is different depending on the transcription factor involved and promoter context (Erkina et al. 2010). These previously reported examples agree with our findings and confirm that transcription in response to heat stress is specifically regulated depending on the chromatin context and the transcription factors involved in each case.

Identification of histone residues required for transcription initiation upon osmostress

An overlap of the histone residues required for transcriptional regulation was observed between *ALD3* and *STL1* in response to osmostress. *ALD3* is controlled by Msn2/4 in response to several stress conditions, among them osmostress and *STL1* is controlled by Hot1 and Sko1 transcription factors in response to osmostress (Proft et al. 2005). Out of 54 and 70 mutants identified on *ALD3* and *STL1*, respectively 9 mutants showed the same transcriptional defect in response to osmostress in both genes. In this case, the histone residues required for transcriptional regulation seem to be divided in two classes: those that are specifically required for the transcriptional

regulation of a subset of genes governed by a specific transcription factor and those that are necessary for the regulation of transcription of genes independently on the transcription factor that controls their expression. One example that supports common requirements for the regulation of osmosresponsive genes has been previously reported from our group, Rpd3 HDAC is recruited to *ALD3* and *STL1* promoters by Hog1 in response to osmostress and this recruitment is essential for transcription activation of both genes (de Nadal et al. 2004).

In our screen we also identified histone residues that are required specifically for *ALD3* or *STL1* and interestingly some of them are clustered in regions along the nucleosome. For example in histone H3, nine residues comprised between D81 and A91 and in H2B, residues from N87 to I92 are required for p*STL1*-qV expression in response to osmostress. Another region was identified in histone H4 to be required for p*ALD3*-qV expression in response to osmostress, comprising five residues between G14 and K20. These data suggest that specific histone residues are required for transcriptional regulation of specific subsets of genes.

A common subset of histone residues involved in *ALD3* transcription upon heat and osmostress

Around 25% of the histone mutants with transcriptional defects on *ALD3* in response to heat stress were also required to respond to osmostress. Since there are common

histone residues required for the transcription of the same genes upon different stress conditions, these results suggest that there would be common regulators important for the transcriptional response to several stress conditions. It would be interesting to identify the histone modifying activities or remodeling complexes responsible for the modification of the newly identified histone residues and shed some light into the transcriptional regulatory mechanism commonly induced upon several stress conditions. An example of common histone modifier between stress conditions was previously reported by our group, Rpd3 histone deacetylase complex is necessary for transcription activation of *ALD3* in response to heat stress (Ruiz-Roig et al. 2010) and osmostress (de Nadal et al. 2004). Other studies confirmed Rpd3 as an activator of Msn2/4 dependent genes and showed that single deletions of several Msn2/4-interacting proteins significantly affected Msn2/4 transcriptional activity under heat stress, osmostress and oxidative stress to a similar extent (Sadeh et al. 2011). Our screening agrees with these reports and indicates that some of the transcriptional defects are independent of the type of stress condition when analyzing Msn2/4 dependent genes.

Our single cell screen is a powerful tool for identifying histone marks essential for transcription upon stress

Among the residues identified in our screening, there are several examples of residues that are known to be

modified during transcription. For instance tri-methylation of H3K4 by Set1 (H3K4me3) is a mark that correlates with active transcription (Santos-rosa et al. 2002; Bernstein et al. 2002; Guenther et al. 2007). However, a repressive role for H3K4me3 in response to diamide stress has recently been shown in *S. cerevisiae* (Weiner et al. 2012). Interestingly, results from our screening also pointed out to the dual role for H3K4 methylation. Whereas in response to heat stress H3K4A mutant showed lower levels of p*ALD3*-qV expression, in response to osmostress H3K4 mutants showed higher levels of p*ALD3*-qV transcription. This suggests an activating role for H3K4 in response to heat stress and a repressive role in response to osmostress.

In addition to identify specific residues required for transcription, in some cases we also characterized the potential postranslational modification required for it. One example is H3R2. Methylation of this arginine in the tail of histone H3 has been reported to be essential for H3K4me3 (Kirmizis et al. 2007; Nakanishi et al. 2008) and the double mark H3R2me2s-H3K4me3 has been analyzed genome-wide and linked to active promoters (Yuan et al. 2012). In response to heat stress H3R2A mutant showed lower levels of p*ALD3*-qV transcription than wild type cells, however H3R2K (the “methyl-mimetic” mutant) showed normal p*ALD3*-qV levels, suggesting that methylation of H3R2 is required for p*ALD3*-qV transcription in response to heat stress.

Another example is H3R8. In human cells, methylation of H3R8 by PRMT5 negatively regulates expression of tumor suppressor genes, acting as a repressive mark (Pal et al. 2004). Accordingly, in our screening we observed that expression of p*ALD3*-qV was already induced in the absence of stress in the H3R8A mutant. However, H3R8K mutant, that could be mimicking a methylated state, showed similar transcriptional levels to the wild type, suggesting that a potential methylation on H3R8 could be repressing *ALD3* under normal growth conditions. Other examples of histone residues whose PTMs have previously described to regulate transcription have been identified in our screen to have transcriptional defects in response to stress such as, H3K36me3 (Strahl et al. 2002), H4K20me (Nishioka et al. 2002), H3K56ac (Williams et al. 2008), H3K122ac (Tropberger et al. 2013), and H4K31ub (Kim et al. 2013). This point out that the data collected in this thesis will be important to define new residues and histone postranslational modifications required for gene expression.

H2A and H2B seem to be involved in the promoter repression of *HSP82* under normal growth conditions

We identified a high number of H2A and H2B mutants showing basal transcriptional defects on *HSP82* in response to heat stress. *HSP82* promoter is supposed to have an open conformation even on the absence of heat shock as a result of Hsf1 binding (Gross et al. 1993; Erkine et al. 1999) and,

nucleosome displacement at *HSP82* promoter occurs within seconds of temperature shift (Zhao J et al. 2005). The fact of having open promoter architecture, the transcription factor already bound to the promoter at basal conditions, and a rapid nucleosome eviction upon heat shock indicates that this promoter is not repressed under normal growth conditions. The absence of promoter repression could lead to transcription induction under small changes in nucleosome stability. H2A-H2B dimers are easily interchanged and displaced from the H3-H4 tetramer. Actually some studies reveal that RNA Pol II can transcribe through hexamers after one of the two H2A-H2B dimers is displaced (Kulaeva et al. 2010; Bintu et al. 2011). These observations could explain the high number of H2A/H2B mutants that show basal transcription of *HSP82* in the absence of stress. However further confirmation of these basal defects should be done.

Mutations on potentially modifiable residues lead to transcriptional phenotypes

Similarly to the phenotypic screening, mutations on modifiable residues such as lysine, arginine and serine led to most of the transcriptional defects in response to heat and osmostress. This indicates that changes on modifiable amino acids are more likely to affect cell survival and transcription in response to stress. Mutations on leucines were also highly represented in the screen. Leucine is a hydrophobic amino acid that usually is buried in folded proteins; actually 71% of

the leucines in the nucleosome are buried or located right next to a buried residue. Thus, mutations in leucines might slightly affect histone structure and subsequentially nucleosome conformation.

A high number of mutations on tail residues were identified to have transcriptional defects. It is know that deletions on histone tails lead to higher chromatin structure packaging (Shogren-Knaak et al. 2006) and affect nucleosome stability (Iwasaki et al. 2013). And also that numerous histone PTMs on the histone tails are involved in histone crosstalk and transcriptional regulation (Lee et al. 2010). Our screening confirmed the role in transcription of several histone residues located on the tails, becoming a powerful tool to identify novel histone residues essential for transcriptional regulation upon stress.

Region I26-T30 on histone H4 is required for transcriptional regulation upon heat stress

Our transcriptional screen identified a region close to the N-terminal tail of histone H4 that comprises residues I26, Q27, G28, I29 and T30. Mutations on all these residues showed reduced p*ALD3*-qV expression by flow citometry upon heat stress, results that were confirmed by Northern. The H4 region is specifically required for transcription activation of Msn2/4 dependent genes in response to heat stress, however is not required for transcription activation of Hsf1 dependent genes. This transcription factor dependency

in response to heat stress has been previously observed by our group (Ruiz-Roig et al. 2010). Rpd3L HDAC is necessary for Msn2/4 dependent genes in response to heat stress, however is not required for Hsf1 dependent genes (Ruiz-Roig et al. 2010).

Additionally to the role in transcription, two mutants of the region were identified in the phenotypic screening as thermosensitive. By manually spotting all mutants from the region we confirmed that indeed, G28A and I29A mutant cells were unable to grow at high temperature and additionally we observed that T30D mutant cells were slightly thermosensitive when compared to wild type cells. This weak phenotype could be the reason why this mutant was not identified as thermosensitive during the phenotypic screen.

Among this region there are two modifiable residues, Q27 and T30. Gene expression analysis of the alanine and the phosphomimetic mutants identified a partial rescue of the transcriptional defect on the T30D mutant in contrast to the T30A. This suggest that phosphorylation on this threonine would be required for transcription. The tridimensional representation of H4 region shows that this region is located on the surface on the nucleosome being accessible for histone modifiers and chromatin remodeling complexes and could be working as a platform for the recruitment of the kinase responsible for T30 phosphorylation. It is also interesting to mention, that in the absence of phosphorylation (T30A) transcription of Msn2/4 genes is impaired and in the

static phosphorylation state (T30D) transcription is partially restored, however cells grow slower at high temperature, suggesting that phosphorylation seems to be dynamic and that its regulation is necessary for the proper cellular adaptation to heat stress.

Taken together, our results suggest that H4 region is required for transcriptional activation of Msn2/4 dependent genes in response to heat stress and that phosphorylation on T30 might contribute to its regulatory mechanism. Further experiments need to be done in order to identify the kinase responsible for T30 phosphorylation and the role of this region genome-wide upon heat stress. Moreover, production of an antibody will be useful to identify the kinase and to monitor the dynamics of this mark genome-wide in response to heat stress.

H4K79 is required for transcriptional regulation upon heat stress

Data from the single cell transcriptional screen and Northern blots indicates that H4K79 is required for transcription of Msn2/4 dependent genes in response to heat stress. Moreover, our data suggest that methylation of H4K79 could be regulating transcription activation in response to heat stress.

In *S. cerevisiae* mutations on H4K79 affect both telomeric and rDNA silencing (Hyland et al. 2005). Several studies have identified by mass spectrometry or mass

fingerprinting PTMs in H4K79 such as acetylation, formylation, propionylation, butyrylation, sumoylation and ubiquitination (Zhang et al. 2003; Hyland et al. 2005; Chen et al. 2007; Tan et al. 2011; Kim et al. 2013; Arnaudo & Garcia 2013), however no role in transcritpion has been described to date for the known PTMs on this site. H4K79 monomethylation was reported in HeLa cells to be associated with Suv39h1 methyltransferase (Robin et al. 2007). H4K79me1, K79me2 and K79me3 were identified in *Toxoplasma gondii* (Nardelli et al. 2013), however till date, methylation on H4K79 has not been reported in *S. cerevisiae*.

Our results suggest that methylation of H4K79 could be involved in the transcriptional activation of genes induced upon heat stress. Since in HeLa cells H4K79 methylation has been identified when co-immunoprecitating Suv39h1, it is worth to study whether Set2 (Suv39h1 orthologue in yeast) is the responsible for H4K79 methylation in response to heat stress. Of note, *set2Δ* mutant shows impairment of *ALD3* transcription in response to heat stress (data not shown). Thus, it is worth to further characterize the molecular mechanisms involved in transcriptional regulation and find out which is the methyltransferase responsible for this modification. The generation of an antibody specific to H4K79 methylation would provide a great tool for studying the role of this mark genome-wide in response to heat stress.

H4S47 is required for transcriptional regulation upon osmostress

H4S47D mutant was identified in the transcriptional screening for displaying defects on p*ALD3*-qV and p*STL1*-qV in response to osmostress. In both cases, expression of the reporters was lower in the mutant than in wild type cells. *ALD3* transcriptional defect was validated by Northern, however, *STL1* expression was higher in the mutant than in wild type cells, contrary to the defect shown by flow citometry in the transcriptional screening. This discordant result between the reporter p*STL1*qV and the endogenous *STL1* may be caused by effects of this residue on initiation and elongation since we measured initiation by assessing the reporter gene whereas mRNA biogenesis includes the entire transcription cycle on the endogenous gene. In response to osmostress, in addition to recruitment to promoters, Hog1 is also recruited to coding regions of osmoresponsive genes (Pokholok et al. 2006; Proft et al. 2006) acting as an elongation factor. Studies from our group have shown that Hog1 interacts with the RSC complex to mediate its recruitment to osmoresponsive genes, where RSC is required for the massive nucleosome rearrangement in response to osmostress (Mas et al. 2009). The possibility that H4S47 is involved in transcription elongation of *STL1* could be a reason why the defects observed on the promoter do not correspond to the defects observed on the whole gene.

To better characterize the role of H4S47D regulating osmoresponsive genes, microarray analysis were performed in wild type and H4S47D mutant strains before and after addition of NaCl. Microarrays analysis showed that 46% of the genes induced upon osmostress were affected in the S47D mutant. The overall effect was upregulation of gene expression in the H4S47D mutant. It is worth noting that one of the genes induced upon osmostress with higher expression in H4S47D mutant was *STL1,* confirming the result from the Northern blot and suggesting a role for H4S47 during transcription elongation in response to osmostress.

Often serine (S) and threonine (T) are mutated to glutamic acid (D) to mimic phosphorylation; conversely the mutation to alanine (A) prevents potential phosphorylation on these residues. H4S47D phospho-mimetic mutant showed defects on both growth and transcriptional regulation upon osmostress, while H4S47A mutant does not show any of these defects. It is important to notice that this is a static situation, rather than the naturally dynamic phosphorylation-dephosphorylation cycle occurring in nature. However, this artificial situation provides useful information and all together our data suggest that the dynamics of H4S47 phosphorylation may be a key point on transcriptional regulation upon osmostress.

H4S47 phosphorylation was previously detected *in vitro*, however the role of this phosphorylation was unknown (Benner et al. 1995). Although H4S47 phosphorylation has

not been directly associated with transcription, recently was reported to be preferentially enriched in H3.3 containing nucleosomes and phosphorylated by PAK2 kinase and dephosphorylated by PP1α, PP1β and Wip-1 phosphatases in human cells (Kang et al. 2011; Zhang et al. 2013). Thus, we asked whether this mechanism would be conserved from yeast to human and which yeast kinases would play this role. In agreement with the data reported in human cells, among the five kinases identified by *in vitro* kinase assays to phosphorylate H4S47 were Ste20, Cla4 and Skm1 that together constitute the PAK family in *S. cerevisiae,* confirming the evolutionary conservation of this mechanism from yeast to human. It is an interesting observation, though, that Ste20 and Cla4 are kinases of the HOG pathway. They are associated with the cellular membrane in the Sho1 branch, leading to activation of the MAPK signaling cascade in response to osmostress (Raitt et al. 2000; van Drogen et al. 2000). These data suggest that Ste20 and Cla4 besides having the well described role in activating the HOG pathway, they also might be required in the nucleus for the proper transcriptional regulation of stress-responsive genes. In fact, Ste20, Cla4 and Skm1 have been shown to be in the nucleus downregulating genes involved in sterol uptake in *S. cerevisiae* (Lin et al. 2009). These observations support the results from our kinases assays and suggest that novel pair kinase-histone substrate can be identified using this approach.

Further experiments need to be done in order to better characterize the role of H4S47 phosphorylation in transcriptional regulation in response to osmostress. Next steps will be guided by the production of an antibody specific for H4S47 phosphorylation, since the commercial one does not react with *S. cerevisiae* (data not shown). This will allow us visualize the presence of this mark genome-wide, the dependency of this mark on transcriptional regulators, such as Hog1 or chromatin remodelers, and the dynamics of H4S47 phosphorylation on promoters and coding regions in response to osmostress.

H3T58 and S57 are required for transcriptional regulation upon heat and osmostress

H3T58D was one of the 16 mutations that led to transcriptional defects on *ALD3* in response to both stress conditions. Moreover, we observed the same transcriptional defects by Northern blot upon heat and osmostress on the neighbor residue H3S57 when mutated to aspartic acid (D). The fact that S57D was not identified in the transcriptional screen (that assessed transcription initiation), but it was shown to have transcriptional defects by Northern, suggest that S57 could be required during transcription elongation rather than during transcription initiation. Another explanation for the same defect on both mutants could be that one residue is modified in response to stress and the residue next

to it could be important for this modification, acting as a platform for the modifying factor.

Genome-wide analysis using DNA microarrays showed that H3T58 was required for transcription of 54% of the osmoresponsive genes. On the contrary that happened in H4S47D mutant, in this case most of the genes affected in the H3T58D mutant showed reduced expression. The fact that the T58D phospho-mimetic mutant had problems to activate around half of the osmoresponsive genes suggests that the dynamics of phosphorylation and dephosphorylation of this threonine might be part of the regulatory mechanism responsible for transcriptional induction in response to stress.

Assuming that the phosphorylation on H3T58 and H3S57 residues regulates transcription in response to stress, we performed *in vitro* kinase assays in order to identify the responsible enzymes. *In vitro* kinase assays identified three kinases that phosphorylated T58 (Pkp2, Mek1 and Vhs1) and 10 kinases (the previous three plus new ones) that phosphorylated S57 (Vhs1, Pkp2, Mrk1, Mek1, Rad53, Snf1, Psk1, Elm1, Kin1 and Kin3). In this context it is interesting to mention that in human studies MSK1 has been linked to H3S57 phosphorylation *in vitro* (Winter et al. 2008). One of the 10 kinases identified to phosphorylate H3S57 in our kinase assays was Snf1, the orthologue of MSK1 in *S. cerevisiae*. Snf1 has been reported to phosphorylate H3S10 and together with Gcn5 regulate transcription in *S. cerevisiae* (Lo et al. 2001). Snf1 has also been reported to

phosphorylate and downregulate Msn2/4 in basal conditions (de Wever et al. 2005) and interestingly to act as an activator of Msn2/4 dependent genes in response to osmostress, heat stress and oxidative stress (Sadeh et al. 2011). These observations suggest that Snf1 could be repressing Msn2/4 dependent genes under normal growth conditions in addition to directly inhibiting Msn2 (Mayordomo et al. 2002; de Wever et al. 2005; Lenssen et al. 2005).

The role of H3S57 in transcription was completely unknown, however during the course of this work it was reported in human cells that phosphorylation of H3S57 by DYRK1a activates transcription of cytokine genes in megakaryoblastic leukemia associated to Down's syndrome (Jang et al. 2014). While this report linked H3S57 phosphorylation to transcription activation of a subset of genes, our results suggest that H3S57 phosphorylation impedes the proper transcription activation of the genes induced in response to heat and osmostress. This controversy suggests specific roles of S57 phosphorylation dependent on the cellular and chromatin context.

Taken together our results indicate that H3T58 and S57 are essential for induction of stress-responsive genes upon heat and osmostress. While H3T58 seems to be required during transcription initiation, H3S57 could be required during transcription elongation. It is worth to put efforts on discovering the specific kinase for H3T58 and S57 in response to heat and osmostress. The generation of

antibodies specific for T58 and S57 would be a very powerful tool to study the dynamics of these marks in promoters and coding regions in order to elucidate their role in transcription initiation and elongation in response to stress.

In conclusion the results obtained in this PhD thesis revealed a comprehensive map of histone residues essential for cellular adaptation in response to stress. Interestingly, our transcriptional screen is a powerful tool to identify novel histone residues and in some cases also de histone modifications required for proper gene expression upon heat and osmostress.

6. CONCLUSIONS

6 CONCLUSIONS

1. A high throughput screen reveals histone residues required for cell survival upon heat and osmostress.

2. Single cell transcriptional screens reveal histone residues required for transcription initiation of stress-responsive genes upon heat and osmostress.

3. In general, the histone residues required for gene expression seem to be specific for each subset of genes and/or each specific stress condition.

4. A subset of histone residues required for *ALD3* transcription in response to both stresses represents a common regulatory pattern between stress conditions.

5. Mutations on modifiable residues located on the histone surface lead to most of the growth and transcriptional phenotypes in response to heat and osmostress.

6. H4S47 regulates transcription of osmoresponsive genes and the dynamics of its phosphorylation seem to be a key regulatory mechanism.

7. H3T58 and S57 are involved in transcriptional regulation of stress-responsive genes upon heat and osmostress.

8. The region on H4 that comprises from I26 to T30 is required for transcription of Msn2/4-dependent genes in response to heat stress.

9. H4K79 is required for transcription of Msn2/4-dependent genes in response to heat stress.

10. This genetic approach has provided a global map of histone residues essential for cellular adaptation to stress and it has proved to yield relevant information on specific histone marks not characterized to date.

7. SUPPLEMENTARY ARTICLE

The Rpd3L HDAC complex is essential for the heat stress response in yeast

Clàudia Ruiz-Roig, Cristina Viéitez, Francesc Posas
and Eulàlia de Nadal

Molecular Microbiology (2010)
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The Rpd3L HDAC complex is essential for the heat stress response in yeast

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Molecular Microbiology (2010). Volume 76, issue 4, pages: 1049-1062

Yeast cells exposed to a sudden increase of temperature suffer deleterious effects on the cellular organization that lead to an imbalance of protein homeostasis and cell cycle arrest. In order to adapt and survive to heat stress yeast cells induce a rapid and massive reorganization of their transcriptional program.

In this study we performed a high throughput screen for genes required for growth at high temperature in order to identify the activities necessary for cellular survival upon heat stress. The screen identified 277 yeast genes essential for cell survival upon heat stress, among them Rpd3 histone deacetylase complex was enriched. We performed genome-wide analysis and showed that Rpd3 was involved in the transcriptional regulation upon heat stress.

Hsf1 and Msn2/4 transcription factors are the main regulators of transcription in response to heat stress in *S. cerevisiae*. Interestingly, we observed that Rpd3 was recruited to Msn2/4 dependent genes upon heat stress and

its catalytic activity was required for the induction of transcription on Msn2/4 dependent genes upon heat stress, however the deacetylase was not required for induction of Hsf1 dependent genes. We further analyzed the mechanism underling the Msn2/4-dependent genes regulation and showed that is the large, but not the small Rpd3 complex the responsible for cellular adaptation in response to heat stress. Recruitment of Rpd3L complex to Msn2/4 promoters is dependent on the transcription factors and leads to recruitment of RNA Pol II

In this study we described a new role for Rpd3 as an activator of transcription upon heat stress.

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

8. REFERENCES

8 REFERENCES

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