Role of Histone Modifications in Transcription Regulation upon Environmental Stress in Eukaryotes

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知를 탐구하는 모든 분들에게 존경을

All my respects to whom seek for wisdom and knowledge

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Barcelona wouldn’t be as special without Tutti flat and my friends. All those times, I get to become who I am now thanks to all of you. Difficult moments and happy moments all turned into happy times because I was with you. I learned how to be happy and what a happy life can be by being with you.

지구 반대편에서도 응원을 아끼지 않고 사랑을 보내주신 부모님이 계셨기에 오늘의 배한솔이 있다고 생각합니다. 배움과 열정을 삶의 자산으로 가르쳐 주신 부모님께 감사드리고, 사랑합니다. 현재의 배한솔이 있는 순간까지 옆에서 응원과 도움을 주신 동국대학교 교수님, 그리고 어려움때 행복할 때나 함께해 준 친구와 동료들에게 정말 감사드립니다. `

ABSTRACT

Epigenetic modification serves as a crucial mechanism in regulating gene expression and facilitating cellular adaptation to environmental stress. This study aimed to identify histone residues and their associated post-translational modifications (PTMs) that contribute to stress response and adaptation in the eukaryotic model organism *Saccharomyces cerevisiae*. A comprehensive set of histone mutants was analyzed to understand the transcriptional and phenotypic effects of potential histone PTMs. Through rigorous selection criteria and extensive experimental validation, we refined our list of candidate histone residues for further study. Histone H3 lysine 64 (H3-K64) emerged as a key player, exhibiting significant transcriptional effects in a PTM-dependent manner. We postulated the PTM involved in this process and investigated the potential regulatory mechanisms of those PTMs involved in this process. Our findings suggest a potential association of the stress response modulating transcription factor, Msn2, and the Set domain-containing methyltransferase, Set1, in the regulation of H3-K64 methylation.

ABSTRACT

La modificación epigenética desempeña un papel crucial en la regulación de la expresión génica y en la adaptación celular al estrés ambiental. Este estudio tuvo como objetivo identificar los residuos de histonas y sus modificaciones postraduccionales (PTMs) asociadas que contribuyen a la adaptación al estrés en el modelo eucariota *S. cerevisiae*. Se analizó un conjunto de mutantes de histonas para entender los efectos transcripcionales y fenotípicos de las PTMs de histonas. Aplicando criterios de selección rigurosos y una extensa validación experimental, refinamos nuestra lista de residuos candidatos para profundizar en su estudio. La histona H3 lisina 64 (H3-K64) exhibió defectos transcripcionales significativos de manera dependiente de sus PTMs. Postulamos posibles mecanismos regulatorios y propusimos las PTMs involucradas en este proceso. Sugerimos una asociación del factor de transcripción que modula la respuesta al estrés, Msn2, y la metiltransferasa con dominio Set, Set1, en la regulación de la metilación de H3-K64.

PREFACE

Environmental stress occurs in many different forms, temperature, pH, metal and osmolarity. Study on transcriptional outcome of these stresses had been highlighted for decades in various organisms including plants, mammals, and fungus as they enhance our understanding on the adaptation and adjustment of the biological signaling against the stress. This thesis presents a comprehensive study on the role of epigenetic modifications, particularly histone residues, in gene expression regulation and cellular adaptation to environmental stress on the eukaryotic model *S. cerevisiae*. This thesis highlights two substantial parts. An extensive experimental validation of a subset of histone residue mutants to accumulate the behavioral information on candidate histone residues with a key role in transcriptional regulation in response to stress. The second part is based on the outcome of our selection based on the validation results. As a proof of concept, we characterized the function of the histone residues H3-K64 in response to stress. Our results suggested that the methyltransferase Set1 methylated H3-K64. This study provides new insights into the role of histone residues in transcriptional regulation under stress conditions.

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INTRODUCTION

1. STRESS RESPONSES IN YEAST

S. cerevisiae, commonly known as budding yeast, is a unicellular eukaryote and a type of saprotrophic fungi. It thrives in environments rich in simple organic carbon, such as plant or animal tissues, particularly those that are liquid or extremely high in moisture (Spencer et al., 1997). The highly variable nature of these habitats has led yeast cells to evolve specific mechanisms to adapt to different types of extracellular changes. These changes are categorized as environmental stresses, which include fluctuations in temperature, osmolarity, exposure to external toxic chemicals, radiation, and more. The adaptive capacity of yeasts has garnered significant attention over the past two decades. This interest extends beyond basic sciences to include medical studies, due to the high conservation of stress response signaling between yeasts and other eukaryotes, including humans (Chasman et al., 2014; X. Liu, 1997; Pincus, 2020).

Heat stress and osmolarity stress have been particularly highlighted due to the involvement of highly conserved response modulators such as heat shock transcription factor 1 (Hsf1) and multiple mitogen-activated protein kinases (MAPKs), known as stress-activated protein kinases (SAPKs). These factors have been reported to significantly impact disease models in various organisms (Liao et al., 2023; Martínez-Limón et al., 2020; Son et al., 2023). In addition, Msn2 and Msn4 (Msn2/4) transcription factors are responsible for modulating responses to more than one type of stressor and are defined as a general stress response regulator (Jacquet et al., 2003). Including these three representatives, more

molecular components are involved in the stress response cascade to maximize the cell survival and adaptation against environmental stresses. This study is particularly focused on heat and osmotic stress.

1.1 Environmental stress response

The Environmental Stress Response (ESR) is a fundamental cellular protection mechanism activated in the presence of environmental stresses, including thermal stress, osmolarity changes, and oxidative stress. Environmental stress damages macromolecules inside of the yeast cell, which includes protein abnormalities and their accumulation. Heat stress can lead to protein aggregation (Mühlhofer et al., 2019), while changes in osmolarity can cause cell wall shrinkage and improper cell volume, potentially resulting in cell cycle abnormalities (Clotet et al., 2006; González-Novo et al., 2015; Tognetti et al., 2020). Increased acidity can elevate anion levels within cells and lead to intracellular vacuole degradation (Ribeiro et al., 2021). Oxidative stress, characterized by an excessive amount of reactive oxygen species (ROS), can induce oxidation of cellular components such as proteins, lipids, and nucleic acids (Lushchak, 2010). Environmental stress, therefore, has a multifaceted impact on cells, causing damage at both the molecular and cellular levels (**Figure 1**).

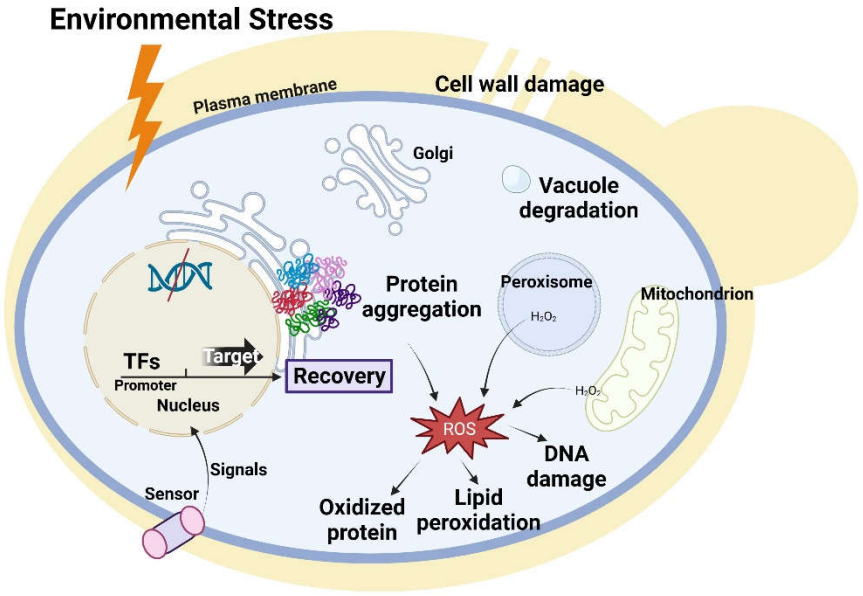


Figure 1. Schematic representation of cellular responses to environmental stress.
Environmental stress triggers a variety of cellular responses, including cell wall damage, protein aggregation, and vacuole degradation. In response to this cellular damage, the cell initiates signal transduction to activate the stress signaling pathway. This activation involves transcription factors (TFs) targeting a subset of genes, which assists the cell in adapting and surviving under stress conditions. Figure modified and referred from Lin et al., 2022.

Cells employ sensor proteins to detect stress and transmit signals to the nucleus to activate stress responses based on the type of stress sensed. This process involves triggering sensor molecules and activation of the transcription factors responsible for the response and promoting the transcription of specific genes enabling cells to recover from stress-induced damage (**Figure 1**). Notably, the transcription factors Msn2/4 play a significant role in various environmental stresses, such as heat and osmotic stress (Saini et al., 2018).

Msn2/4 are two homologous zinc-finger transcription factors

involved in inducing ESR. They target ESR genes that contain stress-response elements (STREs) and mediate the induction of these genes by binding to a specific sequence (CCCCT) within those elements. Representative genes in this STRE-containing subset include *HSP12*, *CTT1*, and *DDR2* (Martínez-Pastor et al., 1996). *HSP12*, a small heat shock gene, is characterized as one of the most abundantly induced gene by multiple stressors including heat and osmotic stress and it is associated with stress resistance (Motshwene et al., 2004; Schmitt & McEntee, 1996). The gene *CTT1*, which encodes the cytosolic catalase T, is rapidly induced upon heat stress to maintain redox balance (Krantz et al., 2004). Under heat and osmotic stress conditions, these genes fail to express when *MSN2/4* are deleted, and with Msn2 probably plays a more pronounced role than Msn4 (Martínez-Pastor et al., 1996; Schmitt & McEntee, 1996).

Another key player in the ESR signaling is the c-AMP dependent Protein Kinase A (PKA). PKA has been implicated in various cellular activities and most distinctively in growth process upon stress. Cells with PKA-deletion accumulated in G1 phase (Amigoni et al., 2015), and increased concentration of carbohydrate, glucose, and trehalose (Smith A., 1998; Zaman et al., 2009). In contrast, cells with elevated PKA activity failed to store glucose and lost their ability to adapt and grow under the environmental stress (Amigoni et al., 2015; Zaman et al., 2009). This implies that PKA plays a crucial role on cell growth under stress conditions. Its physiological effect is modulated by transcriptional changes affected through Msn2/4 where PKA is recognized as a negative

regulator of Msn2/4 (Smith A., 1998).

1.2 Heat stress response

The budding yeast's optimal growth temperature ranges between 25°C and 30°C. Upon exposure to temperatures slightly above this range, cells activate a protective transcriptional program known as the heat shock response (Lutz Nover, 1991). This response triggers physiological changes such as alterations in membrane composition and carbohydrate flux, which protect cellular components and repair damage (Verghese et al., 2012). Heat shock proteins (HSPs), modulate these responses by interacting with other proteins to activate a protective and adaptive mechanism against thermal stress (Krivoruchko & Storey, 2010). Upon sensing heat stress stimuli, cellular regulators activate multiple pathways to maintain homeostasis. Two major pathways modulating the heat stress response are the heat shock response (HSR) controlled by Hsf1 and the Msn2/4-mediated ESR (**Figure 2**).

The HSR is triggered by the activation of the transcription factor Hsf1 (**Figure 2**, pathway in the left). Hsf1, a highly conserved DNA-binding protein in eukaryotes, induces the expression of heat-responsive genes. Under basal conditions, Hsf1 is already bound to promoters, allowing a certain level of transcription. Upon heat shock, 90kDa and 70kDa heat shock proteins (Hsp90 and Hsp70) are released from Hsf1, promoting its activation (Kravats et al., 2018). The released Hsf1 is activated by trimerization, recognizes a pentameric sequence nGAAn, and binds

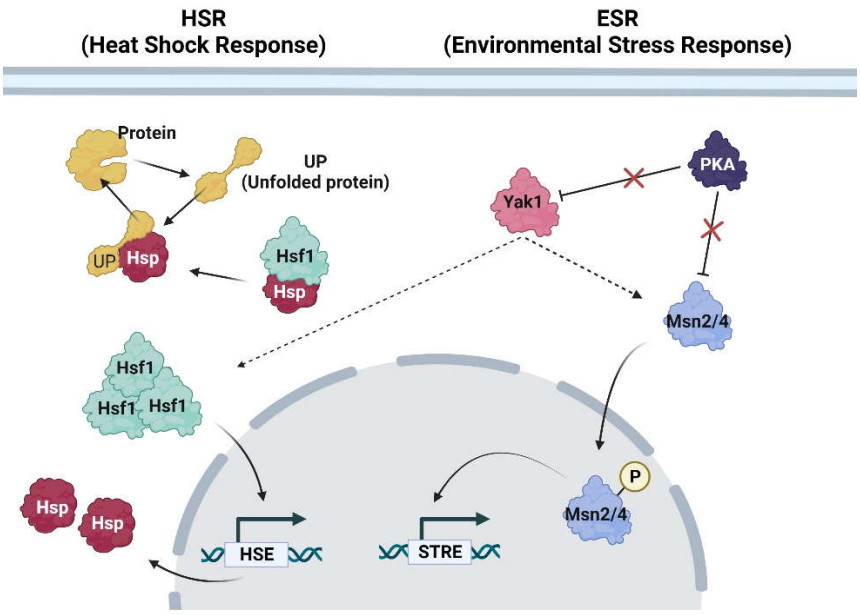


Figure 2. Heat stress responses.
Heat shock response (Left). Various stresses can lead to unfolding of proteins, calling heat shock proteins (HSPs) into action to aid refolding. Consequently, HSPs dissociate from the heat shock transcription factor 1 (Hsf1). The freed Hsf1 then becomes activated through trimerization and hyperphosphorylation, binds to the heat shock element (HSE) and mediates the upregulation of HSP genes. Environmental stress response (Right). Upon heat stress, PKA inhibition of Msn2/4 is released to activate the general environmental stress response (ESR). Moreover, the kinase Yak1 repressed by PKA also gets activated upon heat stress and it promotes Hsf1 phosphorylation to induce heat stress-responsive genes.

DNA as a trimer (Pincus et al., 2018; Sorger & Pelham, 1988).

Post-translational modification regulates Hsf1's transcriptional activity. The phosphorylation-regulated tyrosine kinase Yak1, which is inhibited by PKA, has been observed to be involved in the PKA-dependent regulation of Hsf1 (Ferguson et al., 2005; Lee et al., 2011). This multidirectional regulation of Hsf1 implies the existence of various pathways mediating the heat shock response.

In addition to the Hsf1-dependent pathway, the Msn2/4-mediated general stress response pathway is also activated upon heat stress. Upon heat exposure, PKA inhibition of Msn2/4 is released, leading to the activation of the general stress response pathway. This pathway is characterized by the induction of genes with STRE by the activated Msn2/4 (**Figure 2**, pathway on the right).

1.3 Osmostress response

Increased osmolarity can cause cells to loose internal water, leading to cell shrinkage due to the collapse of ion gradients on the plasma membrane. This immediate cellular response is a direct consequence of physio-mechanical forces under hyperosmolarity (Hohmann & Mager, 2003). Several molecular-level events occur in response to these cellular changes to enhance cell survival and adaptation to the stimulus (Alexander et al., 2001; Bellõ et al., 2001). These include cell growth arrest, glycerol accumulation, among others. Ultimately, these sequential responses represent a comprehensive cellular metabolism management strategy to maximize cell survival and adaptation under hyperosmotic stress. Upon osmostress, the high osmolarity glycerol (HOG) pathway and the stress-activated protein kinase Hog1 homolog of mammalian p38, senses and coordinates the response (de Nadal & Posas, 2022).

The primary role of Hog1 in response to osmostress is to facilitate stress adaptation by rapidly modulating metabolism and

transcription of stress-responsive genes (**Figure 3**). Intracellular glycerol accumulation is achieved by inducing Stl1, a sugar transporter-like protein that imports glycerol into the cell, and by inhibiting its export through Fps1 (Hohmann, 2002; J. Lee et al., 2013; Posas & Saito, 1997).

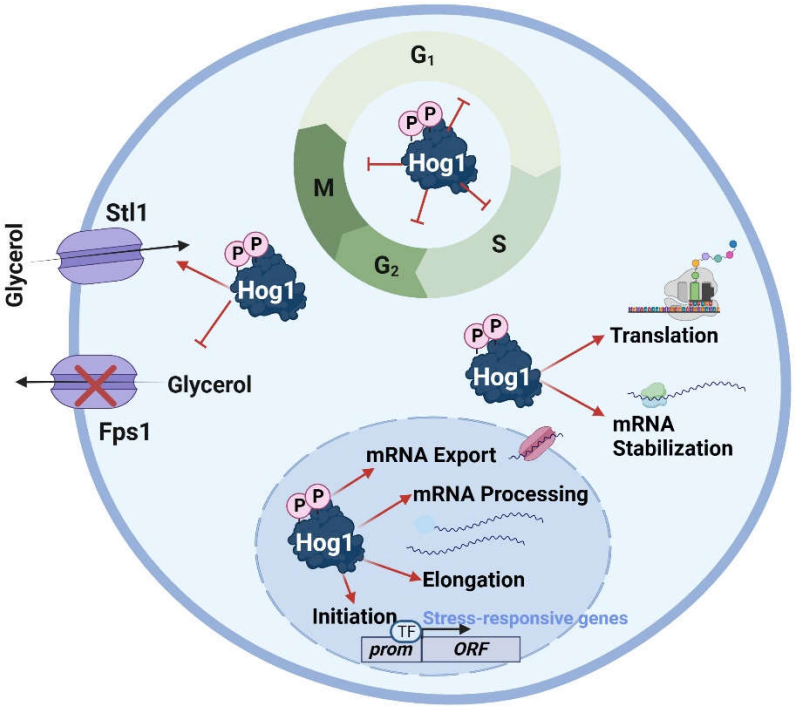


Figure 3. Stress adaptation mediated by the Hog1 MAPK upon osmostress.
Upon osmostress, activated Hog1 orchestrates several cellular functions to coordinate the adaptive response and maximize cell survival. Once activated, Hog1 controls mRNA biogenesis in both the nucleus and the cytoplasm. In the nucleus, Hog1 associates with stress-responsive loci to modulate transcription initiation and elongation. Upon stress, Hog1 modulates progression at all phases of the cell cycle by acting on the core elements of the cell cycle machinery. Hog1 also induces a cytoplasmic response that acts on glycerol and ion transporters, metabolism, and translation (Figure modified from de Nadal & Posas, 2022).

Hog1 also plays a crucial role in cell cycle regulation during osmostress. Cells exhibit a transient cell cycle delay under stress, which is essential for survival in stressed environments (Adler et al., 2022). Activated Hog1 intervenes in multiple cell cycle stages, including the G1/S transition (Escoté et al., 2004), S phase(Duch et al., 2013, 2018), G2/M transition (Clotet et al., 2006), and M phase (Jiménez et al., 2020; Tognetti et al., 2020). This stringent cell cycle regulation enables cells to adapt efficiently to stress by optimizing cell growth.

A key aspect of Hog1's function is its involvement in transcriptional regulation. Osmostress affects a large number of genes, approximately 1,000 genes from the total yeast genome (Gasch et al., 2000; Latorre et al., 2022), with 30% being Hog1-dependent (Latorre et al., 2022). Upon Hog1 activation, it facilitates transcription initiation by activating various transcription factors, such as Sko1 and Hot1, and recruiting chromatin remodelers (Nadal et al., 2003; Nadal-Ribelles et al., 2012; Proft, 2001; Proft & Struhl, 2002; Wosika & Pelet, 2020). Additionally, Hog1 interacts with mechanistic factors of transcription elongation, mRNA processing, and export within the nucleus (Proft et al., 2006; Regot et al., 2013; Silva et al., 2017).

In conclusion, the Hog1 kinase emerges as a pivotal orchestrator of the yeast's osmostress response, coordinating cellular processes to ensure survival and adaptation.

2. HISTONE POST-TRANSLATIONAL MODIFICATIONS

Yeast core histones, small basic proteins comprising the histone octamer, have been increasingly utilized as tools in recent decades. Yeasts, with only two or three redundant copies of histone genes compared to the 50 or more in mammalian and insect cells, offer a simplified model for genomic engineering (Lifton et al., 1978; Marzluff et al., 2002). This simplicity has facilitated the study of histones and enhanced our understanding of epigenetic phenomena, particularly transcriptional regulation via histone post-translational modifications (PTMs) (Bennett et al., 2021; O'Kane & Hyland, 2019).

Histone PTMs, often termed chromatin or epigenetic marks, regulate chromatin structure through interactions with associated proteins, thereby involving histone PTMs in various signaling pathways. These marks are managed by specific PTM modulators known as writers, readers, and erasers. The significance of histone PTMs has been underscored in various studies linking mutations in key regulators to disease development or progression (Gordon et al., 2015; Nacev et al., 2019; B. J. Smith & Carregari, 2022).

While general concepts exist for certain modifications, many aspects of histone biochemistry remain to be explored. Lysine, a key substrate residue, can undergo diverse modifications, including acetylation, methylation, ubiquitylation, and SUMOylation (**Figure 4**). Acetylation and methylation are the most prevalent PTMs on lysine residues (Berger, 2007; Millán-Zambrano et al., 2022)).

Current understanding of histones predominantly centers on the N-terminal tail of core histones, attributed to their structural accessibility. Given the dynamic and multifunctional nature of histone PTMs, there is a significant scope for in-depth exploration of not only the histone tails but also other potential accessible residues that remain unidentified. As indicated in **Figure 4**, both known and unknown PTMs of the histone residues are subjects of ongoing research to determine their possible modifications and functions.

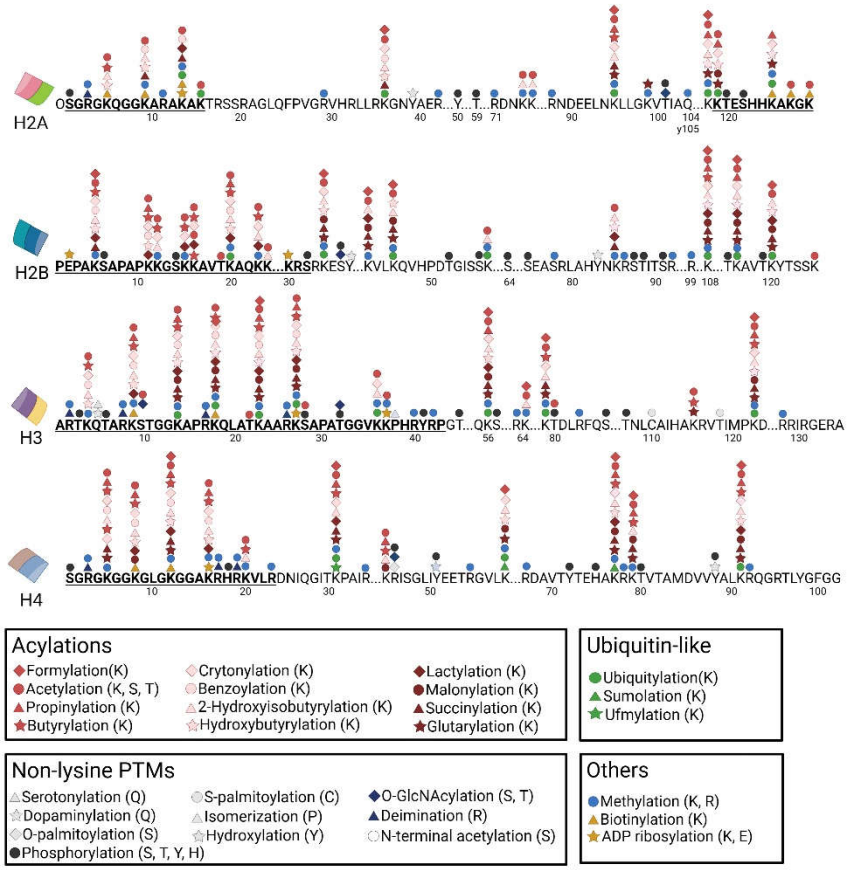


Figure 4. Sites of histone post-translational modifications

The amino acid (aa) sequence (with position numbers beneath) for histones H2A, H2B, H3 and H4. Gaps in the sequence are indicated by "…". Amino acids within the histone tails are underlined. The most common post-translational modifications (PTMs) are methylation (blue circle), phosphorylation (black circle), acetylation (red circle) and ubiquitin-like (green circle). Black and grey symbols represent modifications of non-lysine amino acids. Single-letter amino acid abbreviations are shown in brackets. Figure adapted from Millán-Zambrano et al., 2022.

2.1. Histone methylation

Histone methylation involves the covalent addition of up to three methyl (CH_3) groups to lysine and arginine residues. Lysine can undergo mono-, di-, or trimethylation, while arginine can be mono- or demethylated. Unlike acetylation, methylation does not change the residue's charge. Several hypotheses suggest that histone methylation plays a significant role in chromatin structure, particularly in higher-order folding, as it is predominantly found in the N-terminal tail of histones (Carruthers & Hansen, 2000; Martin & Zhang, 2005; Separovich & Wilkins, 2021). Another hypothesis posits that PTM-specific binding sites serve as functional domains for other proteins that mediate downstream effects (Jenuwein & Allis, 2001; Strahl & Allis, 2000).

The diverse functions and mechanisms of histone methylation are complex and not fully understood. However, it is known that certain protein domains, such as the chromodomain, Tudor domain, and PHD domain, interact with lysine methylation sites as PTM readers, influencing transcriptional outcomes (Huyen et al., 2004; Min et al., 2003; Shi et al., 2006). Six mechanistically defined methylation sites in human cells with 30 methyltransferases

(MTases) and 22 demethylases (DMases) identified. The complexity of histone methylation regulation is evident by the interrelationship of different effectors (Højfeldt et al., 2013; Husmann & Gozani, 2019; Separovich et al., 2020). Notably, the key methylation sites and their associated methyltransferases are highly conserved in yeast.

SET domain containing histone methyltransferase (MTase)

SET domain is a 130 amino acid-long domain which is named after the drosophila proteins, Suppressor of variegation 3-9 [Su(var)3-9], Enhancer of zeste [E(z)], and Trithorax (Trx). SET domain-containing proteins are able to mono-, di- or trimethylated lysine substrate by utilizing the cofactor S-adenosyl-L-methionine (SAM), but not limited to lysine of their substrates (Herz et al., 2013; Sedkov et al., 1999).

Methylation of histone H3 at lysine 4 (H3-K4) is a quintessential outcome of SET domain-containing MTase activity, observed across various organisms (Byrd & Shearn, 2003; Sebastian et al., 2009; Takahashi et al., 2011). Genome-wide studies have identified Set1 as the MTase responsible for H3-K4 methylation, particularly in euchromatin regions, with this modification being prevalent at activated promoters (Deshpande & Bryk, 2023; Ramakrishnan et al., 2016). However, the methylation activity can exert bidirectional transcriptional effects. While H3-K4 methylation is generally associated with transcriptional activation, it has also been identified as a repressive mark for the *PHO5* in yeast (Carvin & Kladde, 2004; Deshpande & Bryk, 2023; Ramakrishnan et al.,

2016). In this context, H3-K4 methylation promotes the recruitment of the Rpd3L histone deacetylase complex, including Pho23 and Cti6, leading to PHO5 repression (Wang et al., 2011). This dual functionality underscores the complex regulatory potential of histone modifications mediated by SET domain containing MTases.

2.2. Histone acetylation

Histone lysine acetylation is commonly linked with active transcription and an open chromatin state (Eberharter & Becker, 2002). The modification neutralizes the positive charge on lysine residues, leading to alterations in nucleosome-DNA interactions, which ultimately result in changes to nucleosome folding and positioning (Eberharter & Becker, 2002). The enzymes responsible for adding acetyl groups to lysine side chains are known as histone acetyltransferases (HATs), while histone deacetylases (HDACs) function as erasers by removing acetyl groups.

HATs and HDACs are essential enzymes in the dynamic regulation of histone lysine acetylation, which significantly impacts gene expression and chromatin organization. HATs, exemplified by human p300/CBP and yeast Rtt109, catalyze the transfer of acetyl groups from acetyl-CoA to lysine residues (Bazan, 2008; Liu et al., 2008), often targeting gene promoters in conjunction with DNA-binding proteins to regulate transcription. Rtt109 is notable for acetylating H3 lysine 56 (H3-K56), a modification situated within the nucleosome's globular domain. This modification is implicated in maintaining genome stability, cell cycle control, and the DNA

damage response in mammals. (Collins et al., 2007; Lin & Yuan, 2008; Yuan et al., 2009). HDACs counteract the activity of HATs by removing acetyl groups, a process requiring a zinc ion for catalytic activity (Ficner, 2009; Seto & Yoshida, 2014). Sir2 specifically deacetylates H3-K9, H3-K14, and H4-K16, which are critical for telomeric silencing (Imai et al., 2000).

Regulatory dynamics of histone acetylation

The addition of an acetyl group to histone residues results in an increased positive charge, predominantly marking the site as active for transcription, whereas the removal of the acetyl group reverts transcription to a recessive state. A pertinent instance is the acetylation of H4-K16, which has been documented to play a role in transcriptional activation and the maintenance of an open chromatin configuration (Hilfiker, 1997; Smith et al., 2003; Suka et al., 2002). In yeast, H4-K16 acetylation, mediated by the telomeric acetyltransferase Sas2, is instrumental in regulating gene silencing, impacting both telomeric gene expression and rDNA expression (Shogren-Knaak et al., 2006; Zou & Bi, 2008). Furthermore, H4-K16 interacts with Sir2, which, antagonistically, leads to the establishment of heterochromatin and subsequent gene silencing (Kimura et al., 2002; Oppikofer et al., 2011; Suka et al., 2002). H4-K16ac exemplifies that histone modifications alone may not dictate transcriptional outcomes; rather, a holistic view encompassing the gene's context and the surrounding genomic localization is essential.

2.3. Mode of action of histone modifications

The *S. cerevisiae* genome, encapsulating approximately 12 Mb of genetic information across 16 chromosomes, is compacted within the nucleus into polymeric complex chromatin (Eldarov et al., 2016), which alternates between active and repressed transcriptional states. This chromatin is composed of nucleosomes and their structural dynamics are crucial for the regulation of gene expression (Roberts & Orkin, 2004). The assembly and dynamics of nucleosomes are influenced by ionic concentration, dictating the transition from a 10-nm to a 30-nm fiber structure or a supramolecular globule, depending on the Mg^{2+} concentration (Tolsma & Hansen, 2019). These structural variations are governed by histone post-translational modifications, which are central to the regulation of chromatin structure and composition, setting the stage for the action of histone modifications.

Structural perturbations

Histone modifications such as acetylation and phosphorylation alter the charge of histones, reducing their interaction with DNA and leading to a more relaxed chromatin structure that facilitates access for transcriptional machinery. These modifications are not only abundant but also exhibit intriguing patterns and interactions. For example, trimethylation of H3-K9 keeps HP1 (heterochromatin protein 1) associated with chromatin. However, global phosphorylation on H3-S10 by Aurora B Kinases during mitosis induced HP1 disassociation while H3-K9me3 still exists (Fischle et al., 2005; Hirota et al., 2005). This implies that phosphorylation at

H3-S10 contributes as a switch to dispatch HP1 and give the space for other protein to attach such as spindle fiber for the next cell cycle (Hirota et al., 2005).

Binding of chromatin modifiers via the domains of PTM readers

Histone modifications serve as crucial signals for the recruitment and activation of subsequent effector molecules. A variety of chromatin modifiers engage with these histone modifications through specialized domains, in a manner akin to conventional protein-protein interactions that recognize specific motifs. Proteins that identify and bind to these histone modifications are termed 'readers (**Figure 5**)'. Consequently, these readers discern histone modifications, and enlist or stimulate downstream effectors (Hyun et al., 2017; Yun et al., 2011). Specifically, chromatin modifiers such as Chd1, Isw1 and Set3 are recruited via modification readers and the degree of their activity depends on the methylation status of H3-K4 (Sims & Reinberg, 2006).

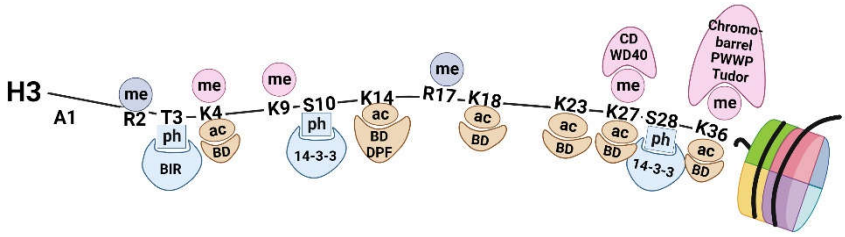


Figure 5. Reader domains capable of recognizing each histone modifications on H3 tail.
Readers of histone PTMs: Recognition of the methylated (me) lysine,

methylated (me) arginine, acetylated (ac) lysine and phosphorylated (ph) serine and threonine residues of the N-terminal histone H3 tail by indicated readers are shown. Figure modified from (Musselman et al., 2012). BD bromodomain, CD chromodomain, DCD double chromodomain, MBT malignant brain tumor, PHD plant homeodomain, PWWP Pro-Trp-Trp-Pro; TTD tandem Tudor domain, zf-CW zinc finger (Figure modified from Musselman et al., 2012).

3. STRESS RESPONSIVE HISTONE RESIDUE SCREENING

Large-scale screening methods have emerged as invaluable tools for expediting research across various fields, enhancing our comprehension of numerous factors while conserving time and resources. Yeast offers a distinct advantage for cell collection based on specific tags or conditions, facilitating high-throughput assays due to their inherent biological traits.

3.1. Histone mutant library

High-throughput studies frequently focus on the activity of histone modifiers and their cellular impact (Baas et al., 2014; Moreno-Yruela & Olsen, 2021; Rye et al., 2011). Nonetheless, this study aims to conduct a residue-focused screening using a histone mutant library to identify residues exhibited the most impact upon stress stimuli, depending on their PTM status.

Two histone mutant libraries, SHIMA H2A/H2B library (Nakanishi et al., 2008) and the H3/H4 library (Dai et al., 2008), were employed. Each library was meticulously crafted by substituting core histone residues to either negate or perpetuate their PTM status. The SHIMA library, utilized for screening H2A and

H2B, and the H3/H4 library, applied to H3 and H4, facilitated a systematic examination of each core histone residue, ranging from cellular activities to transcriptional analysis.

SHIMA, which stands for scanning histone mutagenesis with an alanine library, was developed through the comprehensive substitution of *S. cerevisiae* core histone residues with alanine, excluding those already alanine. Following site-directed mutagenesis and a rigorous selection process to eliminate wild-type histones, the library comprised 442 distinct strains, including three wild-types for H2A and H2B. Subsequent cell viability screenings identified 18 essential amino acids critical for survival under standard growth conditions, as depicted in **Figure 6. A-**

The H3/H4 mutant library comprises 486 mutants, each systematically engineered with amino acid substitutions, as illustrated in **Figure 6. C-D**. This collection extends beyond the SHIMA library by incorporating additional substitutions that reflect the chemical properties of the original residues. For instance, lysine residues are replaced not only with alanine but also with arginine and glutamine to simulate the states of constitutive deacetylation or

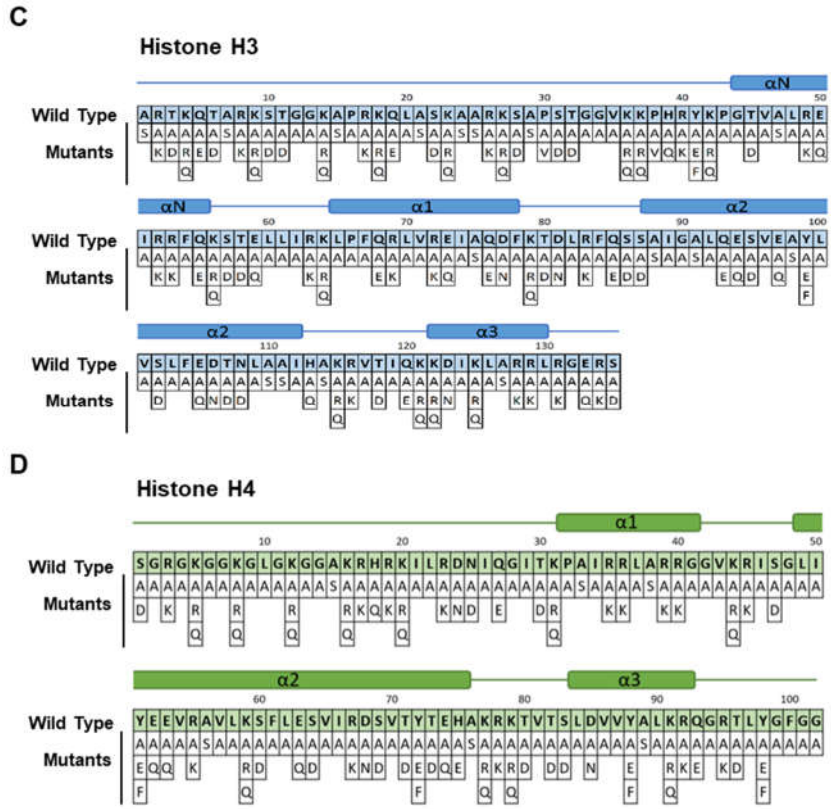


Figure 6. Histone mutant constitution of the two core histone libraries used in this thesis.
The upper colored row contains the amino acid primary sequence for the four yeast core histones. Lower rows indicate the amino acid mutants available for each position in the mutant libraries from (A-B) Nakanishi et al 2008 (H2A and H2B) and (C-D) Dai et al 2008 (H3 and H4). Above each primary sequence, schematic representation of histones' secondary structure (helix α) is shown (Luger et al. 1997)

methylation and acetylation, respectively. Furthermore, the library encompasses a range of residue deletions, allowing for the examination of specific aspects of core histone functionality.

H2A/H2B and H3/H4 were designed from two different studies, and introduced different types of mutations, such as amino acid substitutions and deletions of histone proteins. Nonetheless in the context of this thesis, the focus was solely on the amino acid substitution variants from the two libraries.

3.2. Primary screening and proof of concept

While exploration into histone tail modifications is continuing, there is growing interest in the histone residue localized at globular domains that interact with DNA within the nucleosome. The lateral surface of the nucleosome has received attention due to increasing discoveries of its significance by their capabilities to interact other effectors (Lawrence et al., 2016; Tropberger et al., 2013). A notable modification within the globular domain is H3-K79 methylation (Feng et al., 2002; van Leeuwen et al., 2002; Zhang et al., 2002), which has been shown to play a multifaceted role in transcriptional reactivation and telomeric silencing in yeast (Feng et al., 2002). These findings show the need of further exploration of the histone residues localized at non-tailed structures of the core histone.

Yeast cells undergo a rapid and massive transcriptional and cellular response upon environmental stresses (Gasch et al., 2000), which might involve histone modifications. To examine the role of histone modifications during the environmental stress responses,

yeast cells carrying different histone point mutations were stressed against two different environmental stresses. Their transcriptional responses were measured in high-throughput assay (**Figure 7**). The H2A/H2B (Nakanishi et al., 2008) and the H3/H4 libraries (Dai et al., 2008) yeast histone collections containing, in total, 549 mutants were assessed under two environmental stresses, heat- (39°C) and osmo- stress (0.4 M NaCl) (**Figure 7A**) to identify histone mutants that have impaired stress response (Viéitez et al., 2020). Three stress-responsive genes were measured, *HSP82* for heat stress, *STL1* for osmostress, and *ALD3* for both heat- and osmo-stress. The histone

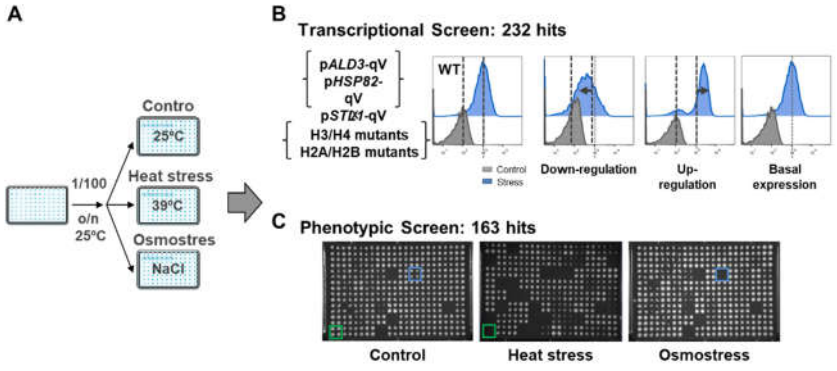


Figure 7. Schematic view of the high throughput screenings of histone libraries.
(A) Yeast cells were diluted and distributed to three different conditions, basal, heat-(39°C) and osmo- stress (0.4M NaCl). **(B)** Transcriptional screening measured 10,000 cells by flow cytometry. Down/up-regulation of indicated reporter constructs (qV: quadruple Venus) was determined in comparison with expression of wild type (WT) (dotted black line) in stress condition (blue) and control (grey). The number next to Transcriptional Screen indicates the number of histone mutants with transcriptional effects upon stress. **(C)** Phenotype screening identified thermosensitive and osmosensitive mutants by growing at indicated conditions and measured colony after size 2 (control), 3 (heat stress) and 4 (osmostress) days. A thermosensitive (green square) and osmosensitive (blue square) are shown as an example. The number next to Phenotypic Screen

indicates the number of histone mutants with phenotypic effects upon stress. Figure are modified from Viéitez et al., 2020.

library, modified to include these three reporters through mating procedures, had its signals quantified via FACS (**Figure 7B**).

The transcription induction of three representative stress-responsive promoters, each targeted by different transcription factors, were measured, using fluorescence reporter constructs. These genes include *HSP82*, for heat stress transcripts, *STL1* for osmostress, and *ALD3* for both heat and osmostress (Durchschlag et al., 2004; Ferguson et al., 2005; Proft, 2001). Cells derived from histone collection containing both point mutation and reporter, were grown for 45 minutes under basal and stress conditions, 39°C for heat stress and 0.4 M NaCl for osmostress. The expression levels of the genes were analyzed and compared with those observed in the wild type. This high-throughput analysis revealed 232 histone mutants as hits (Viéitez et al., 2020). These hits included histone mutants exhibiting either up-regulation or down-regulation of one of the three stress responsive genes under the given stress conditions (**Figure 8**).

The primary transcriptional screening provided a comprehensive overview of the core histones' responses to stress conditions (Viéitez et al., 2020). The findings revealed a novel histone modification, phosphorylation of H4-S47 and H4-T30, that were regulated by kinases, Cla4 and Ste20, by the well-defined stress response modulator HOG signaling pathway (Viéitez et al., 2020). These insights substantiate our hypothesis that histone mutants with impaired transcriptional effect from the primary

screening have higher chance of being involved in stress adaptation process.

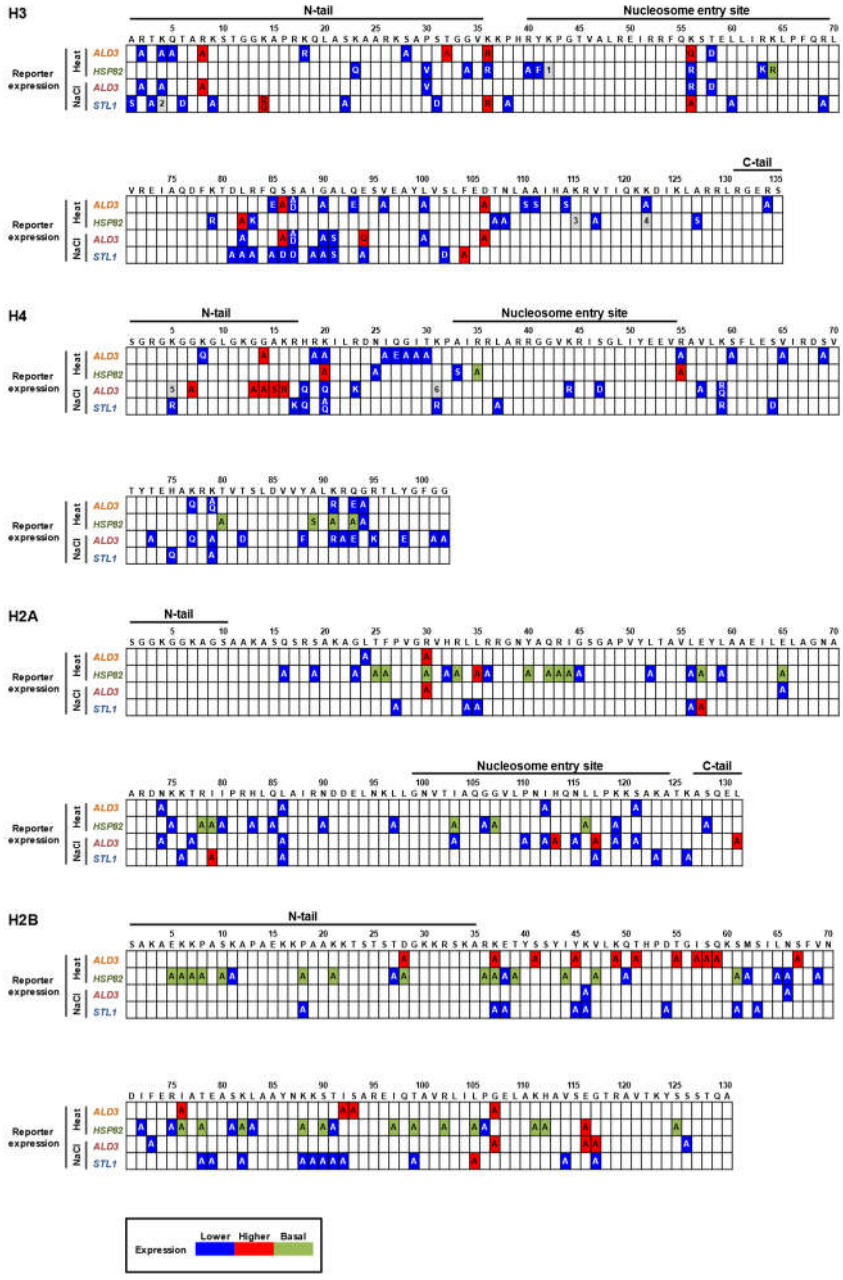


Figure 8. Comprehensive map of the histone residues required for transcriptional response upon stress.

The results from the transcriptional screenings are shown in the primary structure of each core histone for each reporter and each stress condition. Transcriptional defects upon stress in the histone mutants (indicated by the single-letter amino acid code) in comparison to the wild-type strain are shown in blue (expression down-regulated), red (expression up-regulated) or green (higher expression in basal conditions) boxes. Special grey boxes: 1: up-regulated (mutant A) and down-regulated expression (mutant R). 2: up-regulated expression (mutants A, R and Q). 3: higher expression in basal conditions (mutant A) and down-regulated expression (mutant R). 4: higher expression in basal conditions (mutant R) and down-regulated expression (mutant Q). 5: up-regulated (mutant A) and down-regulated expression (mutant Q). 6: up-regulated (mutant A) and down-regulated expression (mutant R).

In addition, a phenotypic screening was conducted to observe the changes in cellular survival and growth of the histone mutants under stress conditions (**Figure 7C**). The screening was performed by robotically placing the mutants on YPD agar plates and incubating at 30ºC as control, at 39ºC for heat stress, and on YPD plates with or without 1.2 M NaCl or 2M sorbitol for osmostress. The colonies were allowed to grow over a span of 2-4 days, the duration of which was dependent on the stress conditions. Subsequently, the size of these colonies was analyzed and compared to that of the wild type. Mutants that exhibited no growth or formed smaller colonies were classified as positive hits. We obtained 163 positive hits from the phenotypic response screening.

Following the transcriptional and phenotypic screenings, a comprehensive analysis was conducted to consolidate the findings from both high-throughput screenings. This analysis aimed to identify histone mutants that not only exhibited transcriptional reprogramming but also showed significant changes in cellular survival and growth under stress conditions. The integration of data from both screenings yielded a total of 71 mutants from 50 histone residues. They represent one or more mutants of a residue that have

altered transcriptional activity of the stress-responsive genes and exhibited cell growth or survival defect under stress conditions, compared to the wild type.

In this PhD thesis, we expanded the conceptual framework to perform a detailed analysis of the data acquired from the initial screenings. Our objective aligns with the previous findings to identify novel histone residues and their regulatory interactions that contribute to the transcriptional response elicited by environmental stressors, such as heat- and osmo- stress. Additionally, we integrated phenotypic cell survival responses observed in previous studies with the newly identified set of histone residues to further delineate those that are pivotal in the stress-response mechanism.

OBJECTIVES

Our group is interested in understanding the mechanisms of transcription regulation in response to environmental stresses. Histone proteins are known to play a key role in transcription regulation by post-translationally modified status. This project aims to identify histone residue responsible in transcriptional response upon stress conditions and expand the knowledge regarding the role of histones on stress adaptation in eukaryotes.

The main objectives of the PhD are focused on:

1. Refinement of the histone mutants selected from phenotype and transcriptional high throughput screenings.
2. Refinement of the histone mutants selected by *in vivo* phospho-proteomics analysis.
3. Validation of transcriptional and phenotypic effects of the histone mutants selected from the list refinement.
4. Transcriptome-wide analysis of histone mutant candidates
5. Identifying a mediator of the histone mutant effect on expression
6. Delineating the post-translational modification involved in the selected histone residue H3-K64.
7. Identify the writer of the modification of the selected histone residue H3-K64.

MATERIALS AND METHODS

YEAST STRAINS

Histone Libraries

Histone H3 and H4 strains are obtained from commercially available Yeast Synthetic Histone H3 and H4 Mutant Collection (Dai et al., 2008) from Open Biosystems (currently available at Horizon Discovery). All residues are mutated to alanine, alanines are mutated to serines to make it in non-modifiable status. Additional amino acid substituted mutants are also available to mimic possible modification status of the residue. 16 mutants (H3-R64A, H3-R63K, H3-K64A, H3-K64Q, H3-K64R, H4-R35A, H4-R35K, H4-R55A, H4-R55K, H4-S60A, H4-S60D, H4-S64A, H4-S64D, H4-Y88A, H4-Y88E, H4-Y88F) and 2 wild types (H3-WT, H4-WT) were utilized in this study. Histone H2A and histone H2B strains are from H2A/H2B SHIMA collection (Lelandais & Devaux, 2010; Nakanishi et al., 2008). The H2A/H2B SHIMA collection was 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. We have retrieved 8 mutant strains (H2A-R78A, H2A-S128A, H2B-S10A, H2B-T39A, H2B-S41A, H2B-S42A, H2B-T51A, H2B-S58A) and 2 wild types (H2A-WT, H2B-WT).

Yeast Knockout Collection

Yeast Knockout (YKO) Collection is a commercially available library containing over 6,000 gene deleted mutants manufactured by Dharmacon Reagents (Giaever et al., 2002; Wach et al., 1994) (currently available at Horizon Discovery). In this study, we have utilized 3 mutants from the collection (*set1*Δ, *set2*Δ, *set5*Δ).

CELL GROWTH MONITORING

Dot Assay

The wild-type and indicated strains were grown to mid-log exponential phase in YPD, and 1/5 serial dilution were spotted on agar plates according to each experimental condition (YPD, 0.4M NaCl, 0.8M NaCl, 1M NaCl, 1.4M NaCl). Cells were grown at 30ºC (osmotic stress) or 39ºC (heat stress) for 3-5 days. Images shown are a representative experiment of minimum three biological replicates.

Growth Curves

Strains were inoculated into a final volume of 200µl of YPD with or without 1.2M NaCl in 96-well plates (initial OD660 0.025). Specific wells were inoculated with medium only for background correction purposes. Cells were grown at 30ºC (osmotic stress) or 39ºC in a temperature-controlled microplate reader (Synergy H1 Multi-Mode Reader, BioTek) with double orbital shaking. Optical Density (OD) was measured at 660nm for 20-40h. Data are the mean ± standard deviation within the three technical replicates generated from an experiment. The data shown is a representative experiment of a minimum of the three biological replicates.

GENE EXPRESSION ANALYSIS

Gene expression analysis was conducted through northern blot and RT-qPCR. Histone collection mutants and the wild type strains were grown to log-phase in YPD medium and subjected to heat stress (39ºC) or osmostress (0.4M NaCl) for the indicated times. Total RNA was extracted by phenol/chloroform extraction.

Northern blot

At least 15µg of total RNA was loaded on each lane. Expression of specific genes was probed using radiolabeled PCR fragments (High Prime DNA Labeling Kit, Sigma-Aldrich) containing the indicated open reading frame region of the genes. Oligomer sequence information for primers used in probe synthesis is provided in **Table 1.** Signals were developed with a Typhoon phosphorimager and quantified by normalizing against the loading control gene, *ENO1*, using *Image J* in respect to the maximum intensity point. Images from a representative experiment of a minimum of three biological replicates are shown.

RT-qPCR

1ug of total RNA was used for cDNA synthesis with SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific). In brief, total RNA with dNTPs were incubated at 50°C for 50min with Oligo$(dT)_{20}$ for the reaction. Quantitative PCR analysis was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) using an Applied Biosystems 7700 sequence thermos cycler. Oligo sequences of primers to detect the transcripts are provided at **Table 1**. Expression level was calculated by using the ΔΔCt method with *ENO1* as a housekeeping gene. The data was presented as fold induction against the non-treated status of the strain. Data from a representative experiment of at least three biological replicates is shown.

Table 1 Oligonucleotides used to synthesize probes for northern blot and to detect gene transcripts in RT-qPCR.

Oligo name	Sequence (5'-3')

ALD3 Probe	Fw - ATGCCTACCTTGTATACTGA
	Rw - AAGTAGAGATAGAGAGGTAT
CTT1 Probe	Fw - CGGGATCCATGCCAATAAGATCAATC
	Rv - GGGGATCCTTAATTGGCACTTGCAATGGAC
HSP12 Probe	Fw - ATGTCTGACGCAGGTAGAAAAGGATTCGG
	Rw -TTACTTCTTGGTTGGGTCTTCTTCACCGTG
HSP82 Probe	Fw - AGGCAGTCGAAAAGTCTCCA
	Rw - CCAAACTGAAGCCGGAAGTC
STL1 Probe	Fw - GGCCAGTTTATCATCGGAAG
	Rw - GCGTTTGTTGATGCACGAAC
ENO1 Probe	Fw - ATGGCTGTCTCTAAAGTTTA
	Rw - AATTTGTCACCGTGGTGGAA
YDR154C Probe	Fw - TCTTCATCACCACCGTTCCA
	Rw - GTATTGTTCCAGGCAGAGCG
HPF1 Probe	Fw - AGCCAACTTACCCAGGTACC
	Rw - GCAAGAAGTGATAGTGGCGG
PEP4 Probe	Fw - ACTGAAGGTGGTCACGATGT
	Rw - AAGCCTTACGACGAACAGGT
RNR2 Probe	Fw - CGAAGCTTCTTTCTGGACCG
	Rv - ACCGAAAGCAACCAACAGTC
SSA1 Probe	Fw - TGTCTTCCTCCGCTCAAACT
	Rv - TTCTTGGTGACGGTGTCCTT
ALD3 RT	Fw - TGATAAGGCTATAGAATGGGTAGC
	Rv - GCTCCACCAATAGGAAATTCA
CTT1 RT	Fw - CAGGCTCCCACCCTGATTATAA
	Rv - TCGGGTGTCATTGTTTGCA
HSP12 RT	Fw - AGGTCGCTGGTAAGGTTCAA
	Rv - TGGACACGACCGGAAACATA
HSP82 RT	Fw - AGGTGGTGCTCAAGACAAGA
	Rv - TGGAACCTCTTCAACCGGAG
STL1 RT	Fw - GAGGATGGCACTCAACCAT
	Rv - GTCGTCATAAGAGCCCAATGC
ENO1 RT	Fw - CCAAGTGGTTGACTGGTCCT
	Rv - GCTTCCCAGTCATCTTCAGC

RNA SEQUENCING AND ANALYSIS

Two RNA-seq experiments were done with two different sets of histone mutants. Both RNA-seq libraries were prepared by the

following procedures. Yeast histone mutants and wild type were grown to mid-log phase in YPD medium and subjected or not to stress (10 minutes of 39ºC and 15minutes of 0.4 M NaCl). Three biological replicates for each condition were performed. Total RNA was extracted by phenol/chloroform method and quantified by agarose gel and BioAnalyzer (Agilent). Libraries were prepared using the Total RNA-seq Library Prep Kit (Lexogen) following the manufacturer's protocol, starting with 1µg of total RNA for poly(A)-mRNA selection (Poly(A) Selection Kit, Lexogen), Constructed libraries were first analyzed on Qubit (dsDNA HS, Thermo-Fischer Scientific) and BioAnalyzer using DNA 1000 chip to check the size distribution, and quantified by qPCR using the KAPA Library Quantification Kit (Roche) prior to sequencing with 100bp single-end reads on an Illumina HiSeq 2500 with v4 sequencing chemistry.

Sequencing analysis was conducted by IRB Biostatistics/ Bioinformatics Facility. RNA-seq reads were mapped using STAR single-end with Ensemble genome (Dobin et al., 2013). Reads were counted using Rsubread (Liao et al., 2019), and subsequent analyses were conducted using the statistical programming language R, and the DESeq2 package was used for library size normalization and differential expression testing (Love et al., 2014). To obtain stringent genes sets, we only defined genes that significantly exceeded a fold change (FC) of 2 with adjusted p-value < 0.05 for multiple testing as responsive to osmotic or heat stress, respectively. In contrast, to be able to thoroughly capture the effect of the histone mutants in basal conditions, we applied less restriction on the FC

($log_2(FC) \neq 0$, adjusted p-value < 0.05). Lastly, the effect of histone mutants on the transcriptional stress response was investigated to define the affected gene under the histone mutant compared to the WT without restrictions regarding the minimum FC ($log_2(FC) \neq 0$, adjusted p-value < 0.05). Results were visualized using the ggplot2 R-package (http://ggplot2.org).

Functional enrichment analyses for H3-K64A mutant affected genes were performed using the g:GOSt at *gProfiler* (Raudvere et al., 2019), using the commonly less-induced genes in both heat and osmotic stress. The data provided is adjusted p-value<0.05. g:GOSt performs gene set enrichment analysis, also on input gene list. It maps genes to known functional information sources and detects statistically significantly enriched terms. It regularly retrieves data from Ensembl database and fungi, plants or metazoa specific versions of Ensembl Genomes. In addition to Gene Ontology, it includes pathways from KEGG and WikiPathways; and regulatory motif matches from TRANSFAC; human phenotype ontology from Human Protein Atlas. More databases are integrated at the analysis, but only ones applied on the analysis on this thesis are introduced.

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

Short histone peptides and methyltransferase, Set1 (aa700-1080) were purified to be used as substrates and enzyme for *in vitro* methyltransferase assays. We designed three different histone

peptides, two of which were wild types and one with mutations. The wild type peptides corresponded to the H3 tail (aa 1-20) and mid region of H3 (aa 48-71). The mutant peptide covered the same mid region (aa 48-71), but with two amino acids substitution: K64A and R63A. K64 is the residue of our interest but R63A was introduced to eliminate the possible effect of R63 methylation on the K64 investigation. Methyltransferase, the Set1 region aa 700-1080 was chosen to contain the methyltransferase enzymatic domain, SET domain, instead of the full-length protein (Hsu et al., 2018; Kim et al., 2013). GST fused proteins were expressed in *E. coli* BL21 strain. The purification was done in accordance with the protocol outlined in (Viéitez et al., 2020).

WESTERN BLOT OF YEAST HISTONE MODIFICATIONS

Histone 3 wild type and mutants (H3-K64A, H3-K64Q, H3-K64R, H3-K4A) were used on following methods to detect histone modification by immune blotting method. For cellular extracts, 1ml of yeast culture at OD_{660}=0.4-0.7 was stopped growing with 100 µl of STOP solution (200mM NaF, 200mM NaN_3) and pelleted by 1minute centrifugation at 10000rpm. Cell pellets were resuspended in 50µl of 1x SDS loading buffer with 50µl of glass beads, lysed 1minute with FastPrep (MP Biomedicals) and boiled 5 minutes at 95ºC before loading. At the same time, we performed histone purification protocol for yeasts (Jourquin & Géli, 2017; Poveda et al., 2004). In brief, 100ml of yeast cultures were harvested and washed with digestion buffer (1 M sorbitol, 50 mM Tris-HCl

pH=7.5, 4 mM $MgCl_2$). 100mg cell pellets were treated with 75 mM 2-mercaptoethanol for 10 minutes on ice and centrifuged at 3000rpm. Pellets were agitated with 600ug of zymolyase 100T for 30 minutes at 30°C. Spheroplast cells were washed extensively with wash buffer (50 mM MES pH=6, 1 M Sorbitol, 0.5 mM $CaCl_2$) and protease inhibitors. The spheroplasts were pelleted and lysed in lysis buffer (50 mM MES pH=6, 75 mM KCl, 0.5 mM $CaCl_2$, 0.1% NP-40) and pellets containing crude nuclei were resuspended with and washed with lysis buffer HS (10 mM MES pH=6, 430 mM NaCl) on ice. Lysates except histone were precipitated at 4°C for 2hours with 0.25 M HCl and collected supernatant was incubated with 8 volume of acetone for overnight at -20°C. Histone extracts pelleted and resuspended with loading buffer. 16% polyacrylamide gel was used for running the samples and blotted on a PDVF membrane. Following the incubation of the blots with the indicated antibodies, signals were detected using ECL detection reagent (BioRad) and developed by Odyssey Imager (Li-Cor). Following antibodies were tested on yeast extracts and purified histones: Anti-Histone H3 (trimethyl K4) antibody (abcam, ab8580), Recombinant Anti-Histone H3 (acetyl K64) antibody (abcam, ab214808), Anti-Histone H3 (trimethyl K64) antibody (abcam, ab195483), Anti-acetyl Histone H3 Antibody (Lys64. Merck, ABE1057), H3K64me3 Antibody (diagenode, C15410211), Anti-Trimethyl-Histone H3 (Lys64) Rabbit pAb (PTM Biolabs, PTM-690), Anti-Acetyl-Histone H3 (Lys64) Rabbit pAb (PTM Biolabs, PTM-129).

IN VITRO METHYLTRANSFERASE ASSAY

Methyltransferase activity was measured by monitoring the production of S-(5'-adenyl)-L-homocystein (SAH) with MTase-Glo methyltransferase Kit (Promega) on substrates. A reaction containing 500ng of purified histone fragments, H3-WT (aa48-71), H3-R64A·K64A (aa48-71), with S-Adenosyl methionine (SAM) and 65ng of enzyme, Set1-GST, were incubated at 30°C for 30minutes for its enzymatic reaction. SAH production was measured at luminometer and calculated based on the SAH standard curve generated in parallel during the assay. All samples were performed in triplicates and data presented is the mean value of a representative experiment.

CHROMATIN IMMUNOPRECIPITATION (ChIP)

Msn2 recruitment to promoter regions of responsive genes under stress conditions were referred from previous study (Görner et al., 1998). Wild type and histone mutant strain, H3-K64A, were transformed with plasmids containing p*ADH1*-MSN2-3HA. The ChIP was done as described in Zapater et al., 2007. Briefly, transformed cells with Msn2-HA were cultured at Leu-drop out medium to mid-log phase before stress treatment. Stress treated cells (0.4 M NaCl, 5 minutes or 39°C, 10 minutes) were fixed with formaldehyde 37% and precipitated with anti-HA. DNA was purified by phenol-chloroform and subjected to a quantitative PCR analysis with a sequence detector (Applied Biosystems 7700) using oligonucleotides described in **Table 2**. Immunoprecipitation

efficiency was calculated in triplicate by diving the amount of PCR product in the immunoprecipitated sample by that in the *TEL* (telomeric region on the right arm of chromosome VI) sequence control. The binding data are presented as fold induction with respect to the non-stressed condition of each strain.

Table 2 Oligonucleotides used to detect immunoprecipitated promoter region transcripts.

Oligo name	Sequence (5'-3')
ALD3 promoter RT	Fw - CCATTATTTCAGCATGCGGA Rw - CAATCCGGTTCAAGTCTTGG
CTT1 promoter RT	Fw - ATCGGCGACGTACTCATCAT Rv - CTGCGTGCTTTCACAGAGAC
STL1 promoter RT	Fw - TAAGTGCCGTTGTCCCACTATTC Rw - GGACAAAGTCGGACCCTTCA
TEL promoter RT	Fw - ACCACTCAAAGAGAAATTTACTGGAAGA Rw - CTCGTTAGGATCACGTTCGAATC

STATISTICAL ANALYSIS

Data are reported as mean ± SD. If not declared otherwise, statistical significance was assessed using a Student's t-test for equality of means, two-tailed and equal variance were not assumed. *: $p<0.05$, **: $p<0.01$

RESULTS

1. IDENTIFICATION OF HISTONE MUTANTS WITH STRESS RESPONSE IMPAIRMENT

Upon encountering environmental stresses, yeast cells initiate a substantial transcriptional and cellular response (Gasch et al., 2000), potentially involving histone modifications. Considering previous findings from two core histone libraries, our study aimed to identify and characterize histone mutants influenced by potential stress-induced modification statuses. We subjected the initially identified 71 histone mutants to a rigorous selection process, intending to streamline the number of residues for efficient experimental validation. This process included a bibliographic analysis of the mutants, prioritizing histone residues that not only introduce novelty in stress response studies but also possess the potential for post-translational modifications implicated in transcriptional regulation.

1.1 Refinement and selection of identified histone residues from high throughput screenings

The initial selection process for the 71 mutants involved a literature review. Published results pertaining to these residues were examined to determine if they had previously been reported to have a function with a known mechanism of action in the stress response and adaptation process. From this process, the residues that were with unknown regulatory mechanism in stress signaling were considered suitable for further experimental evaluation.

Additional criteria were applied at the selection process, considering the potential for histone residues to undergo PTMs with regulatory implications. To identify such histone residues, a thorough review of relevant literature was conducted, and the HistoneDB 2.0 database (http://www.ncbi.nlm.nih.gov/projects/HistoneDB2.0) was consulted. The database provided comprehensive information on histone variants. The focus was on histone residues that could potentially be modified and were in regions of the nucleosome that are accessible to modifying enzymes.

Based on the information gathered from the literature and the database, a four-category system was employed to classify the histone residues according to their structural characteristics within the nucleosome. These categories include residues localized in the N-terminal (tail), those positioned on the lateral surface of a nucleosome in contact with DNA (lateral), those without contact with DNA (disk), and those that are buried (Draizen et al., 2016; Huang et al., 2009). Histone residues classified as 'buried' were excluded from further consideration due to their inaccessibility to modifying enzymes. Consolidating the comprehensive bibliographic analysis, the 71 histone mutants resulted in a list of 54 mutants (49 residues) for further investigation.

Prior to proceeding with their experimental validation, we decided to cross-verify the two screenings, the transcriptional and phenotypic screenings, by the 54 histone mutants. This approach aimed to enhance the likelihood of selecting histone residues with a

higher relevance to stress response and growth adaptation. We focused on histone residues that exhibited at least one mutant with transcriptional impairment and phenotypic alteration under the same stress condition. Following a rigorous analysis of those 54 histone residues, 6 histone mutants that belong to 6 residues successfully met all the selection criteria applied (**Figure 9** and **Table 3**).

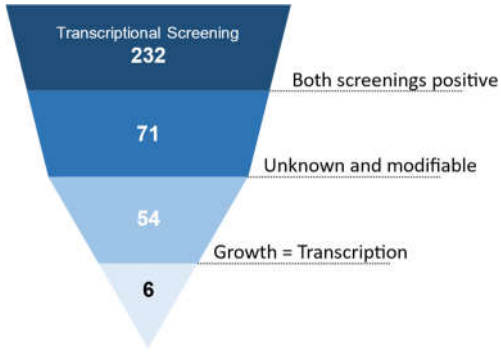


Figure 9. A funnel summarizing the selection process of the histone mutants obtained from transcriptional screening.
232 histone mutants were obtained from the transcriptional screening that had effects on one of three examined stress-responsive genes. 71 histone mutants remain after combining transcriptional and phenotypic screenings, of which 54 did not have a known regulatory mechanism on stress signaling and are located at modifying enzyme accessible region. Finally, 6 histone mutants showed a consistent phenotypic and transcriptional effect on the same stress condition. Numbers indicate the number of histone mutants left after each step.

Table 3. The transcriptional and phenotypic screening result for the selected histone mutant and other mutants affecting the same residues.

Histone Residue	Mutants	Transcriptional Screening		Phenotype Screening	
		Heat	Osmo	Heat	Osmo
H2A-R78	R78A	+	-	+	-
H3-R63	R63A	+	-	+	-
	R63K	+	-	-	-
H3-K64	K64A	-	-	+	-
	K64Q	-	-	+	-
	K64R	+	-	+	-
H4-R35	R35A	+	-	+	-
	R35K	-	-	-	-
H4-R55	R55A	+	-	+	+
	R55K	-	-	-	-

H4-Y88	Y88A	+	n/d	+	-
	Y88E	-	-	-	-
	Y88F	-	+	-	-

6 histone mutants from 6 histone residues were selected by the criteria applied on Figure 9. The histone mutant shown effects on transcriptional and phenotypic screening are indicated as + / - under the stress condition they had effect on. Grey indicates mutants fitting the selection criteria.

This strategically narrowed the list of potential candidates to ensure that the residues chosen for validation hold significant potential in the context of stress response and cellular adaptation. Thus, these 6 residues were chosen for experimental validation.

1.2 Refinement and selection of identified histone residues from phospho-proteomics analysis

Protein phosphorylation represents one of the most prevalent forms of PTM. The phosphorylation of histones is a well-documented PTM and a key regulatory mechanism of histones (Sawicka & Seiser, 2014). Our laboratory has previously identified specific phosphorylation sites on histone H4 residues T30 and S47, and their involvement in the stress signaling cascade. This was corroborated through *in vivo* phospho-proteomics analysis (Viéitez et al., 2020). In addition to these two residues, this analysis identified 11 more histone residues with phosphorylation sites under basal condition. Thus, these 11 residues were subjected to a refinement process to select those potentially involved in the regulation of transcriptional stress response.

Similar to the previous selection process, the 11 histone

residues underwent a comprehensive bibliographic analysis. The aim was to identify residues with novel functions and roles in stress response and adaptation signaling. Two residues were excluded as their functions and mechanisms of action under stress signaling were already reported (Aslam & Logie, 2010; Cerutti & Casas-Mollano, 2009). The residue localization in the nucleosome was analyzed using the HistoneDB 2.0 database. As a result, 9 residues met the criteria based on their novelty in stress signaling and accessibility to modification enzymes.

Before initiating experimental validation of the nine histone residues, we compared the results of the first two high-throughput screenings (**Table 4**). From the transcriptional screening results, eight residues showed effects in at least one of the mutants of the residue. In the phenotypic screening, only one residue, H4-S60, showed growth impairment under heat stress and none under osmostress.

Table 4. List of histone residues identified from phospho-proteomics analysis and their results from the two HTSs.

Histone Residue	Mutants	Transcriptional Screening		Phenotype Screening	
		Heat	Osmo	Heat	Osmo
H2A-S128	S128A	+	-	-	-
H2B-S10	S10A	+	-	-	-
H2B-T39	T39A	+	-	-	-
H2B-S41	S41A	+	-	-	-
H2B-S42	S42A	-	-	-	-
H2B-T51	T51A	+	-	-	-
H2B-S58	S58A	+	-	-	-
H4-S60	S60A	+	-	-	-
	S60D	-	-	+	-
H4-S64	S64A	-	+	-	-
	S64D	-	-	-	-

The histone mutants that showed transcriptional and phenotypic effects were indicated as +/- under the stress condition we observed from the screening results.

These 9 histone residues were then combined with the 6 previously selected residues for experimental validation of their stress response impairment upon heat- and osmo- stress. In total, we validated a list of 15 histone residues.

1.3 Experimental validation of selected histone residues

Experimental validation was conducted on 15 selected histone residues to assess the behavior of their mutants under stress conditions. Our objective was to identify the histone mutants that exhibited the most significant transcriptional and phenotypical changes in response to stress. To accomplish this, we evaluated the phenotypic and transcriptional responses of each histone residue, allowing us to monitor each mutant more specifically. From the two histone libraries, H2A/H2B and H3/H4, used in our high-throughput screenings, 24 histone mutants corresponding to 15 selected histone residues were subjected on the experimental validation.

For the transcriptional validation, we assessed the endogenous expression levels of stress-responsive genes. We opted to evaluate four genes for each validation: three genes that are commonly upregulated under heat- and osmo- stress, and one stress-specific gene for each stress condition. The genes *ALD3*, *CTT1*, and *HSP12* were commonly upregulated, while *HSP82* was specific to heat stress and *STL1* was specific to osmostress. We employed mainly northern blot and RT-qPCR analysis to detect the transcripts.

Among the histone residues evaluated, only the non-modifiable alanine substitution at H3-K64 (H3-K64A) demonstrated a transcriptional effect in response to both heat and osmotic stresses (**Table 5** and **Figure 10**). The H3-K64A mutation led to a significant decrease in the expression of non-stress specific genes such as *ALD3*, *CTT1*, and *HSP12*. In contrast, mutants mimicking acetylated (-K64Q) and non-acetylated or methylated (-K64R) statuses showed negligible differences compared to the wild-type (**Figure 10**).

Table 5. Experimental validation results of mutants from 15 histone residues.

Source	Residue	Mutant	Transcriptional Validation		Phenotype Validation	
			Heat	Osmo	Heat	Osmo
Two high throughput screenings	H2A-R78	R78A	+	-	+	+
	H3-R63	R63A	-	+	+	-
		R63K	-	+	-	-
	H3-K64	K64A	+	+	+	+
		K64Q	-	+	+	+
		K64R	-	+	-	-
	H4-R35	R35A	-	-	+	-
		R35K	-	-	-	-
	H4-R55	R55A	+	-	+	+
		R55K	-	+	-	-
	H4-Y88	Y88A	+	-	+	+
		Y88E	-	-	nd	nd
		Y88F	nd	nd	nd	-
Phospho-proteomics analysis	H2A-S128	S128A	nd	nd	-	-
	H2B-S10	S10A	nd	+	-	-
	H2B-T39	T39A	nd	nd	-	-
	H2B-S41	S41A	nd	nd	-	-
	H2B-S42	S42A	nd	nd	-	-
	H2B-T51	T51A	nd	nd	-	-
	H2B-S58	S58A	nd	nd	-	-
	H4-S60	S60A	-	+	-	-
		S60D	-	-	+	-
	H4-S64	S64A	-	-	-	-
		S64D	-	-	-	+

"Source of the residue" indicates how the respective residues were selected. The positive hits we obtained from the validation are colored gray. '+' in transcriptional validation signifies that the mutant strain showed transcriptional

alteration on more than 2 stress-responsive genes from the 4 genes we tested. '+' in the phenotypic validation signifies that the mutant strain showed cell growth impairment from both, growth curve and dot assay. 'nd' at the transcriptional validation signifies not decisive due to not enough of expression consistency.

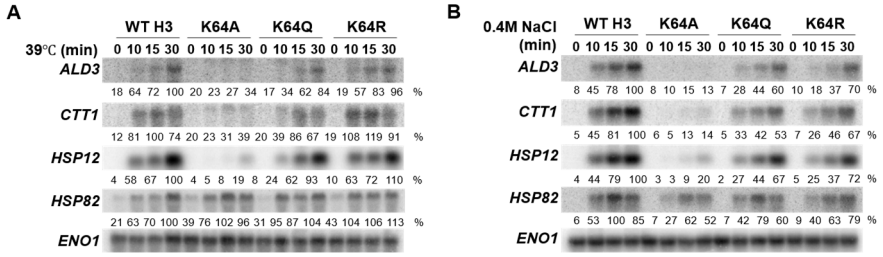


Figure 10. Transcriptional effects of H3-K64 mutants in response to stress
Quantification of endogenous expression by northern blot of H3-K64 mutants under **(A)** heat stress and **(B)** osmotic stress is shown. Wild-type (WT) and the indicated histone mutant strains were exposed to heat stress (39°C) and osmostress (0.4M NaCl) for the indicated times. Total mRNA was analyzed by northern blot using radiolabeled probes for *ALD3*, *CTT1*, *HSP12*, *HSP82*, *STL1* (stress-responsive genes), and *ENO1* (serving as a loading control). RNA quantification is expressed as a percentage, representing the ratio of mRNA levels normalized by *ENO1*, with the maximum gene expression value of the WT strain set as 100%. The images displayed are representative figures from three biological replicates.

Apart from H3-K64A that had transcriptional effect against both stress conditions, 8 other histone mutants exhibited transcriptional effect under one type of stress (see **Table 5**). We observed a case, from H4-R55 in particular where different mutants from the same histone residue were having transcriptional effects to one stress condition but not to another, vice versa (**Figure 11**). For instance, non-modifiable alanine substitution of H4-R55 (H4-R55A) showed down-regulation of the three non-stress specific genes, *ALD3*, *CTT1* and *HSP12* at 30 minutes of stress treatment, while methylation mimicking mutant, H4-R55K, showed down-regulation only at *HSP12* (**Figure 11A**). In osmotic stress, R55K exhibited up-regulation of the non-stress specific genes, while H4-R55A only showed up-regulation on *ALD3* (**Figure 11B**).

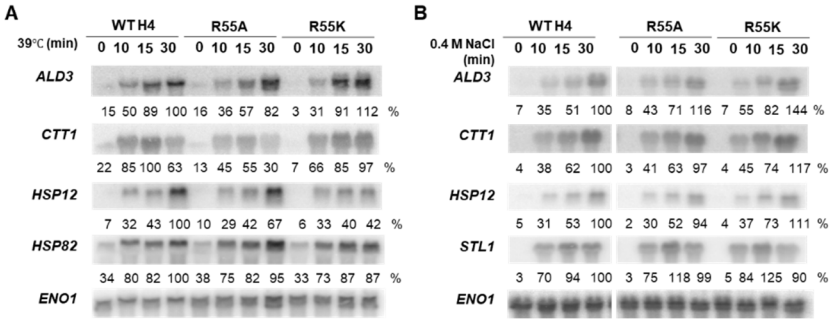


Figure 11. Transcriptional effects of H4-R55 upon heat and osmotic stress. Northern blot analyses of mutants and their wild type of H4-R55 to assess the expression levels of four stress-responsive genes under four different durations of **(A)** heat stress response (39°C) and **(B)** osmotic stress (0.4M NaCl) (Intermittent samples are removed for clarity). The methodologies employed are consistent with those detailed in Figure 2. The presented images are one representative of the three biological replicates.

In conclusion, we validated the transcriptional effects of 24 mutants across 15 histone residues. We observed that 8 of these residues had transcriptional effects on more than two of the four genes we monitored under stress conditions (**Table 5**). Notably, the non-modifiable alanine substitution of H3-K64 exhibited the most significant transcriptional impact under stress conditions. However, 7 of the 9 residues identified from the phospho-proteomics analysis did not show transcriptional effects under stress conditions.

The phenotypic effects of the selected histone residues were evaluated by growth curve analysis and dot assays. For the growth curve analysis, we monitored the cell growth rate under a fixed condition for each type of stress, 39°C for heat stress and 1.2M NaCl for osmostress. The dot assay, on the other hand, allowed us to observe cells under a wider range of conditions for osmostress. We cultured cells on YPD agar plates with varying concentrations of NaCl (0.4M, 0.8M, 1M, 1.2M) and 1M Sorbitol. The cells were

then incubated until visible growth was observed, which typically took between 2 to 4 days depending on the stress condition due to changes in growth rate.

We monitored 15 histone residues using these two methods and observed significant phenotypic effects from 8 of these residues (**Table 5**). 4 of these residues had effects against one condition, while the other 4 had effects against both stress conditions. Two positive hits were identified from the 9 residues selected from the phospho-proteomics analysis.

Among the residues exhibiting phenotypic effects, the alanine-substituted mutant of H2A-R78 displayed the most pronounced impairment under heat stress (**Figure 12A**). This mutant strain was unable to survive under the stress condition, and no visible colony was formed, suggesting that the modification plays a significant role in cell survival under stress response. We observed varying growth rates between the stress conditions (**Figure 12B-C**). The alanine-substituted mutant of H4-R55 showed severely impaired growth rate under heat stress (**Figure 12B**), but the same level of severity was not observed under osmostress (**Figure 12C**). However, the phenotypic impact that the mutant had on cell growth was consistent (**Figure 12D-E**). These findings imply that the two methodologies we employed provide insightful information on cell growth phenotype by offering both process information and outcome information, thereby facilitating a concrete conclusion.

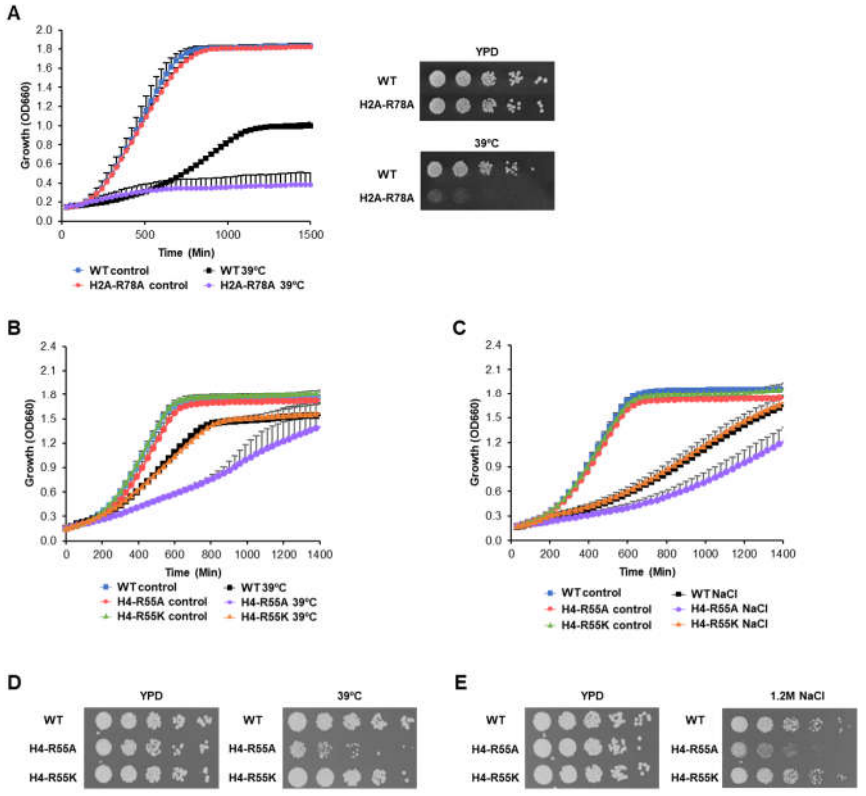


Figure 12. Phenotypic stress response analysis of H2A-R78 and H4-R55. Cell growth rate (OD660) was measured every 30 minutes where the cells were grown at 30°C (basal), 39°C (heat) in YPD (basal and heat stress) or 1.2M NaCl (osmostress) for 25-hours and 48-hours, respectively. Measurements were done in three technical triplicates and graphs shown are representative one of the three biological replicates. For dot assay, cells cultured on different concentration of NaCl at YPD-agar plates, and the plates were scanned when the size of the colony stopped growing. Pictures shown at the figure are one representative of the three biological replicates. **(A)** (Left) Growth curve analysis of mutant of H2A-R78, R78A on heat stress. (Right) Dot assay of H2A-R78A upon heat stress. Growth curve analysis of mutant of H2A-R78, R78A on heat stress **(B)** heat and **(C)** osmotic stress. Dot assay of mutant of H4-R55, R55A and R55K on heat stress **(D)** heat and **(E)** osmotic stress.

By integrating both transcriptional and phenotypic response validations, we compared the results obtained from screenings and validations (**Table 6**). The initial screening results identified 16 transcriptional hits and 10 phenotypic hits. Our experimental

validation detected 3 mutants from the transcriptional validations (19% detection rate) and 8 mutants from the phenotypic validation (80% detection rate). While the percentage of transcriptional hit detection may seem low, we identified new mutants with transcriptional and phenotypic effects that were not detected during the screenings. For instance, 9 mutants were newly identified to have transcriptional effects on different stress types that were not detected during the screenings, including the mutant of H3-R63 on osmostress. Additionally, 5 mutants exhibited phenotypic effects that were not detected during the screening, such as H3-K64 on osmostress. However, there were mutants that were not detected in our validation but were identified during the screening, 13 in transcription validation and 1 in phenotypic validation.

The consolidated results of the screenings and validation helped us identify which histone mutant consistently exhibited effects both transcriptionally and phenotypically. Furthermore, the newly discovered effects provided insights into the potential impact of the selected residues' modifications. We comprehensively applied both results to decide which histone residues to further characterize for their impact on the observed stress effects. As a result, we selected 8 residues. 6 residues from the two HTS were selected due to their consistent effects transcriptionally and phenotypically upon the stress condition, leading us to conclude that H4-R35 does not have any effects on osmostress. 2 residues were selected from the phospho-proteomics as they exhibited transcriptional or phenotypic effects in one consistent stress condition. Consequently, we proceeded with a genome-wide

understanding of these 8 histone residues.

Table 6. Summary of the two screenings and experimental validation results of the 15 selected histone residues.

Source	Histone Residue	Transcription Screening		Phenotype Screening		Transcription Validation		Phenotype Validation		Genome-wide RNA-seq	
		Heat	Osmo	Heat	Osmo	Heat	Osmo	Heat	Osmo	Heat	Osmo
Two high throughput screenings	H2A-R78	+	-	+	-	+	-	+	+	Yes	Yes
	H3-R63	+	-	+	-	-	+	+	-	Yes	Yes
	H3-K64	+	-	+	-	+	+	+	+	Yes	Yes
	H4-R35	+	-	+	-	-	-	+	-	Yes	No
	H4-R55	+	-	+	+	+	+	+	+	Yes	Yes
	H4-Y88	+	+	+	-	+	-	+	+	Yes	Yes
Phospho-proteomics analysis	H2A-S128	+	-	-	-	-	-	-	-	No	No
	H2B-S10	+	-	-	-	-	+	-	-	No	No
	H2B-T39	+	-	-	-	-	-	-	-	No	No
	H2B-S41	+	-	-	-	-	-	-	-	No	No
	H2B-S42	-	-	-	-	-	-	-	-	No	No
	H2B-T51	+	-	-	-	-	-	-	-	No	No
	H2B-S58	+	-	-	-	-	-	-	-	No	No
	H4-S60	+	-	+	-	-	+	+	-	Yes	Yes
	H4-S64	-	+	-	-	-	-	-	+	Yes	Yes

6 residues, derived from two HTS analyses, were chosen for further investigation of their genome-wide impact using RNA-seq. Additionally, 2 residues from phospho-proteomics analysis were selected for a genome-wide impact study using RNA-seq. The presence or absence of effects from each screening and validation method is represented by '+' and '-' symbols, respectively. The last two columns with genome-wide RNA-seq indicate the stress condition that the selected residues were analyzed. The colored cells represent the histone residues and the stress conditions under which they were analyzed in RNA-Seq.

2. GENOME-WIDE EFFECTS OF SELECTED HISTONE MUTANTS

Throughout the experimental validation of 24 mutants in 15 selected histone residues, 17 mutant strains from 8 histone residues showed an impaired response of stress-responsive genes. Our previous assessments, however, were constrained as we only monitored the transcriptional effects on a select set of stress-responsive genes. To overcome this limitation, we analyzed the genome-wide impact of these histone mutants on transcription by RNA-seq.

We divided our genome-wide analysis of the 8 histone residues into two distinct datasets: RNA-seq 1 and RNA-seq 2. RNA-seq 1 is comprised of six residues that originated from two high-throughput screenings, along with two unique wild-type (WT) strains depending on which histone library they were retrieved from: H2A-WT for strains derived from H2A/H2B and H4-WT selected to represent strains derived from H3/H4. RNA-seq 2 includes two residues of histone H4 that were obtained through phospho-proteomics analysis, in addition to their wild type, H4-WT (**Figure 13)**

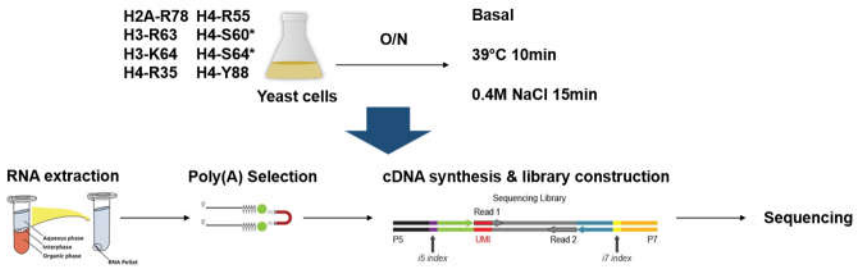


Figure 13. RNA-seq sample preparation for two datasets, RNA-seq 1 and RNA-seq 2.
Histone mutant strains, in triplicate, along with their corresponding wild-type strains (H2A-WT for the H2A strain, H4-WT for the H3/H4 strains), were cultured overnight to reach exponential phase. Following dilution to achieve an OD660 between 0.4 and 0.6, the cultures were subjected to stress (39'C for heatstress and 0.4M NaCl for osmostress). Total RNAs were then extracted and processed for poly(A) selection and library construction in a single batch, following the manufacturer's instructions provided with the CORALL Poly(A) Selection Kit and CORALL Total RNA-seq Kit. *Samples sequenced in RNA-seq 2

The two datasets generated from our RNA-seq analyses offered a comprehensive view on the selected histone mutants under three distinct conditions: basal, heat stress, and osmotic stress. This analysis has enabled us to successfully identify the histone residue

that significantly influences transcriptional stress response. Furthermore, it has offered additional insights into the residues that did not show large transcriptional effects upon stress but in basal condition.

2.1. Identification of histone mutant with transcriptional genome-wide effect in response to stress conditions

The 8 selected residues were divided into two datasets by the sequencing batch we performed: RNA-seq 1 and RNA-seq 2. The primary objective of our RNA-seq analysis was to identify the number of genes that exhibited changes in histone mutants compared to their wild-type under stress. This necessitated the crucial step of selecting stress-responsive genes for our data analysis. For this analysis, we defined a set of genes exhibited an expression level with an absolute log2-fold change (FC) ≥ 1 and an adjusted p-value ≤ 0.05 under each stress condition compared to its basal status.

Of the 6,500 transcripts analyzed, the two datasets yielded a comparable number of stress-responsive genes in WT (**Figure 14**). Specifically, in H4-WT, 29% and 33% of the total genes analyzed from RNA-seq 1 and RNA-seq 2, respectively, were identified as heat stress-responsive genes. 16% of the genes from both RNA-seq 1 and 2 were classified as osmostress-responsive genes. These stress-responsive transcriptional response is in concordance with

previously reported studeis (Latorre et al., 2022; Viéitez et al., 2020).

A

Dataset	Condition	Stress-repressed	Stress-induced	Total (% of genome)
RNA-Seq 1	Heat	951	1,072	2,023 (29%)
	NaCl	477	616	1,093 (16%)
RNA-Seq 2	Heat	1,120	1,129	2,249 (33%)
	NaCl	612	516	1,128 (16%)

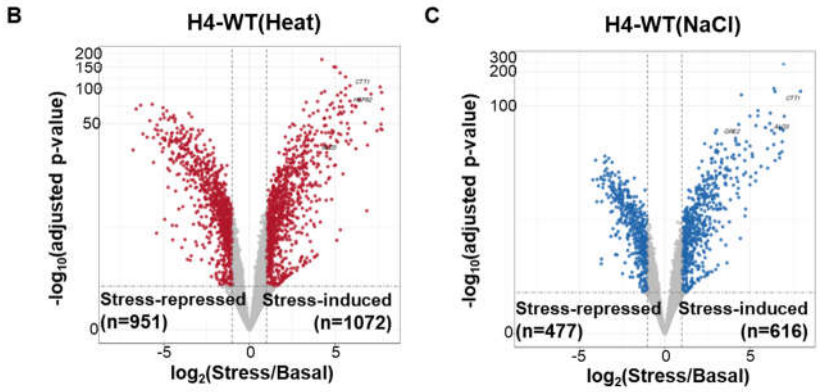


Figure 14. Stress-responsive gene identification and their expression distribution of H4-WT.

(A) A table showing the number of stress-responsive genes identified from H4-WT of the two RNA-seq datasets under the different stress condition by repressed (log2(FC)≤-1, adjusted p-value≤0.05) and induced (log2(FC)≥1, adjusted p-value≤0.05). Volcano plots showing the distribution of the selected stress-responsive genes defined in H4-WT are colored **(B)** in red as heat stress responsive genes and **(C)** in blue as osmostress-responsive genes.

Next, we conducted a Principal Component Analysis (PCA) with all measured genes to ensure that the samples were appropriately clustered according to the experimental conditions (**Figure 15**, RNA-seq 2 not shown). 59% of the total variance was accounted for by the first and second principal components (PC1 and PC2). The PCA plot exhibited a well-defined clustering that was consistent with each of the conditions except for the mutant,

K64A, displaying a significant differential expression pattern compared to the WT. Importantly the position of this mutant between the basal and induced clusters suggested a partially repressed response.

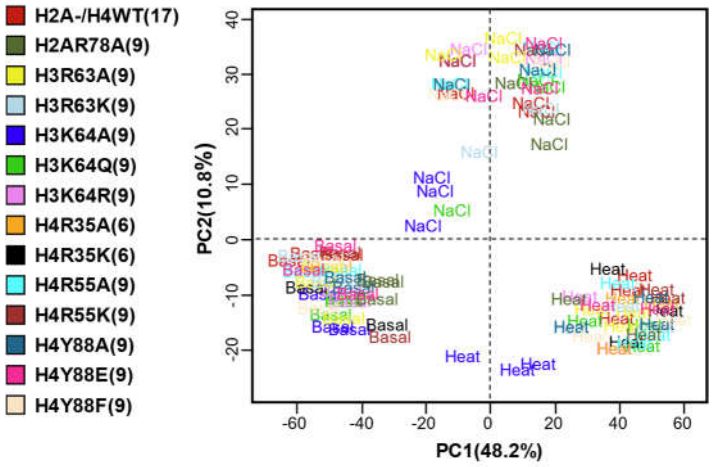


Figure 15. Principal component analysis (PCA) visualization of the H4-WT and strains from H3/H4 histone library.
The first two principal components plotted with all measured genes from triplicated samples of WT (one sample of H4-WT is excluded at QC before sequencing) and 13 different histone mutants treated in three different experimental conditions: basal, heat and osmotic stress. Yeast strains indicated by color and stress by text. Number in parentheses shows total number of samples across all conditions.

Then, based on the stress-responsive genes we selected from WT, we assessed the expression of those genes in the histone mutants by each experimental condition ($\log2(FC)\neq0$, adjusted p-value≤0.05). In addition, we independently analyzed genes that are significantly altered their expression in basal condition between WT and mutants ($FC\neq1$, adjusted p-value≤0.05). From the 24 finally chosen histone mutants in 8 residues with transcriptional or phenotypic effects, we observed one residue was highly affected in

stress condition, and three residues in basal condition in the RNA-seq analysis (**Figure 16**).

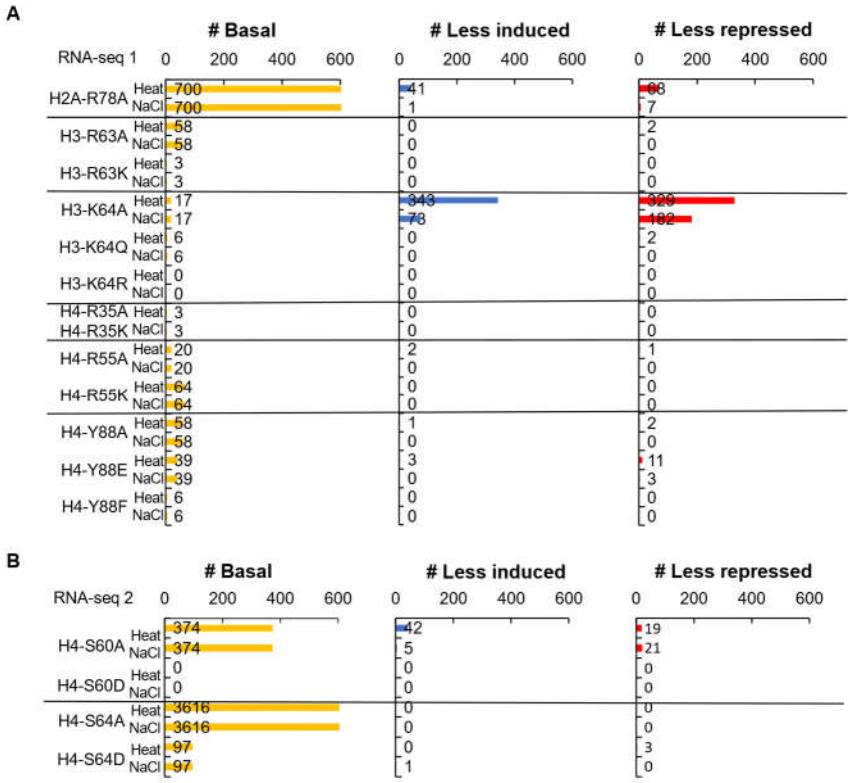


Figure 16. Comprehensive analysis of gene expression of the histone mutants compared to the wild-type.
Genes exhibiting significant alterations in expression in the wild-type (WT) strain under each condition (heat and NaCl) were selected based on an adjusted p-value ≤0.05 and absolute abs(log2(FC)) ≥2. Differentially expressed genes in each histone mutant were identified through comparative analysis against its WT strain, defined as the interaction term in the analysis. Genes that were less-induced or -repressed in mutant strains than in the WT under different conditions (FC≥2) were defined. For basal condition, p-value of ≤0.05 and a FC ≠1, was applied. The number of genes that significantly altered their expression under the given condition in the indicated mutants selected from **(A)** two HTS analyses and **(B)** phosphor-proteomics analysis, are presented.

Three residues, H2A-R78A, H4-S60A, and H4-S64A, exhibited significant transcriptional alterations under basal conditions compared to wild-type. These effects were unexpected from the

experimental validation of these residues. H2A-R78A exhibited a strong phenotypic impairment in stress conditions (**Figure 12A-B**, osmostress not shown). In the growth analysis of H4-S60 and H4-S64, we observed growth impairment in D-mutants in heat for H4-S60 and osmostress for H4-S64 (**Figure 17A-B**). However, the growth of the wild-type and mutants of the three residues did not exhibit a significant difference under basal conditions (YPD) (**Figure 17A-B**).

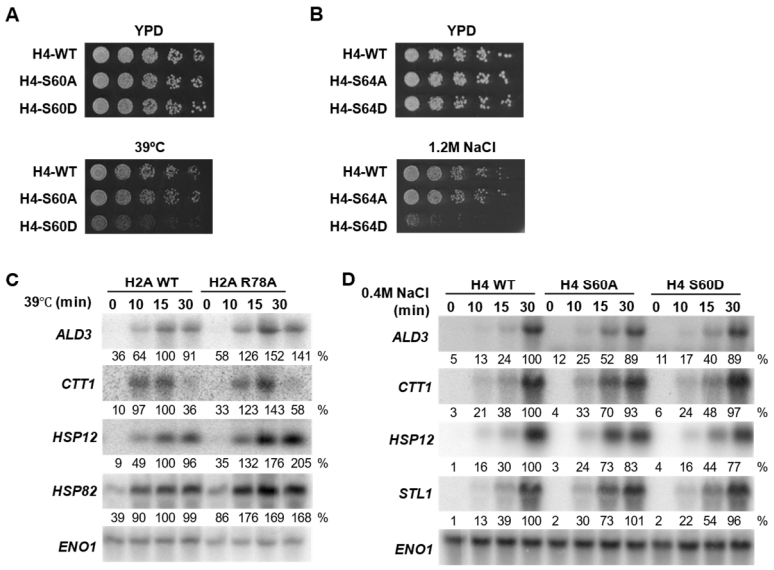


Figure 17. Phenotypic and transcriptional validation results of the histone residues show genome-wide effect under basal condition.
Phenotypic effects were validated by dot assay of **(A)** H4-S60 upon heat stress (39°C) and **(B)** H4-S64 upon osmostress (1.2M NaCl). The dot assay was performed the same method as did in Figure 5. **(C)-(D)** Northern blot analyses histone mutants and their wild type of H2A-R78 and H4-S604 to assess the expression levels of four stress-responsive genes under four different durations of stress. **(C)** Heat stress response (39°C) of H2A-R78A mutant and its WT, H2A-WT. **(D)** Osmostress response (0.4M NaCl) of mutants of H4-S60, S60A and S60D. The methodologies employed are consistent with those detailed in Figure 3.

We noted from the previous transcriptional validation (**Table 6, Figure 17C-D**), H2A-R78A exhibited an increase in the selected stress-responsive genes, but no difference was observed under basal conditions for these genes (**Figure 17C**). In the case of H4-S60, a smaller than H2A-R78A but consistent increase in selected stress-responsive genes was observed at 15-minute osmostress treatment in the A-mutant (**Figure 17D**). Nonetheless, this transcriptional effect offers a limited perspective as we only assessed a few stress-responsive genes. To address this, we decided to confirm the endogenous expression of genes shown to be upregulated in basal conditions. To do this, we selected genes that were highly expressed from the RNA-seq (**Figure 18**).

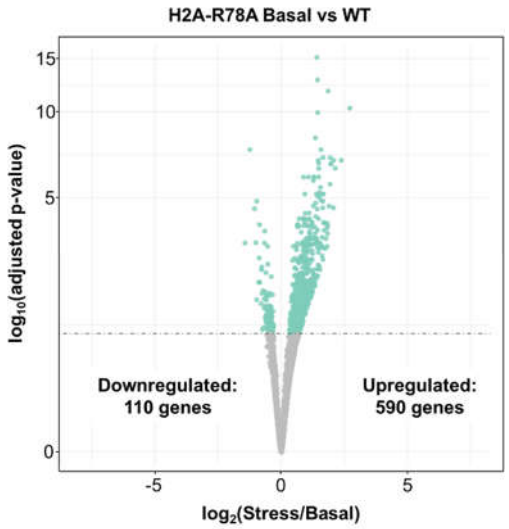


Figure 18. Volcano plot showing significantly altered gene expression in H2A-R78A strain on basal condition.
The dashed horizontal line indicates an adjusted p-value of 0.05. Colored dots are genes within the p-value (<=0.05) and log2 fold change threshold of 0 (up-/down-regulation)

We chose the RNA-seq result of H2A-R78 as representative for the three histone mutants (H2A-R78A, H4-S60A, and H4-S64A) that exhibited significant genome alterations under basal conditions. In the non-modifiable mutant H2A-R78A, 700 genes showed affected expression (**Figure 18**). Among these, 84% (590 genes) were upregulated and 16% (110 genes) were downregulated compared to H2A-WT. From the 590

upregulated genes, we selected three that were highly induced in H2A-R78A for detection by northern blot: *HPF1*, *PEP4*, and *SSA1* (**Figure 19A**). Those selected genes showed a significant level of increase in basal condition by the mutant (**Figure 19B**). This shows

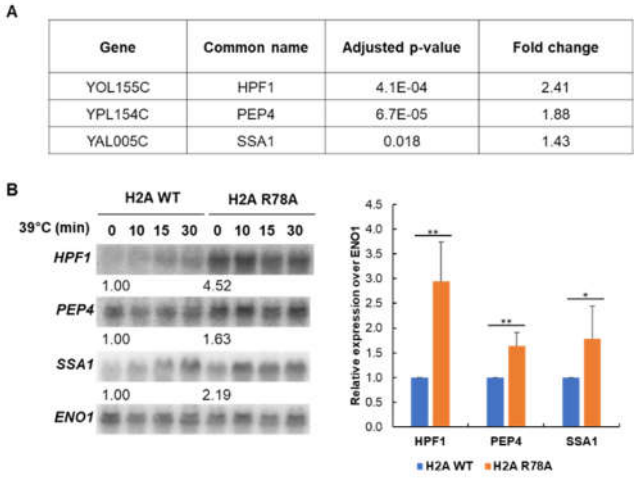


Figure 19. Endogenous expression level of up-regulated genes in H2A-R78 in basal condition.
(A) Adjusted p-value and the fold-change of the three selected genes from RNA-seq that are the most up-regulated and assessed in northern blots. **(B)** H2A-WT and H2A-R27A cells exposed to heat stressed and total RNA extracted at 0, 10, 15, 30 minutes were northern blotted for the three selected genes. One house keeping control ENO1 was performed. Shown is one representative experiment (left) and mean normalized relative expression levels (n=6, right). *p ≤ 0.05, **p ≤ 0.001

the difference seen in the RNA-seq data are reliable and that these three genes were indeed upregulated in the histone mutant H2A-R78A under basal conditions. This suggests that the transcript assessment we conducted on certain stress-responsive genes by Northern blot may provide a limited perspective for understanding the function and role of the histone mutants.

In addition to the H2A-R78A, H4-S64A mutant exhibited a more severe transcriptional effect under basal conditions, with alterations observed in 3,616 genes representing 52% of all genes. Importantly, for both, H4-S60 and H4-S64, this was only observed in the non-modifiable mutant but not in the phosphomimetic D mutant (**Figure 16B**), suggesting that modification of these histone residues is critical for basal transcription.

2.2. H3-K64A transcriptional effects under stress conditions

The non-modifiable mutant of H3-K64A exhibited the most pronounced transcriptional effects in response to stress treatments among the 24 mutants for 8 histone residues analyzed via RNA-seq (**Figure 16A**). A severe transcriptional defect was already observed in the representative stress-responsive genes, namely *ALD3*, *CTT1*, and *HSP12*, in response to both heat and osmotic stress (**Figure 10**). Including the three genes observed in northern blots, a significant number of genes displayed transcriptional alterations by RNA-seq analysis (**Figure 20**).

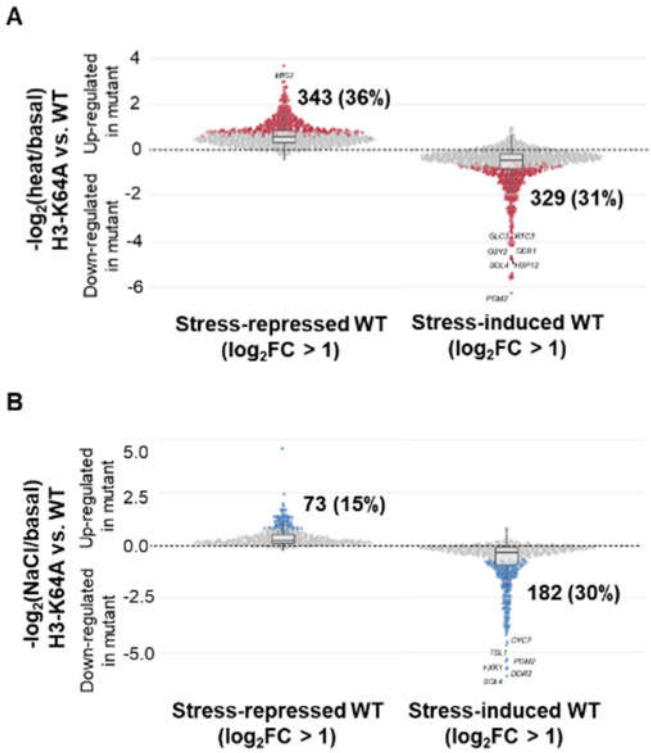


Figure 20. The distribution of stress-responsive genes affected in H3-K64A upon stress treatments.
(A-B) Differences in the stress-activated and stress-repressed transcriptional responses defined in Figure 14 between H3-K64A and WT strains upon heat and osmotic stress. Horizontal lines in the boxplots indicate the median, and dashed gray lines indicate a relative absolute fold change of 1. The highlighted numbers represent genes significantly (abs(log2(FC))>1, p-value≤0.05) altered by the mutant in response to **(A)** heat stress and **(B)** osmostress compared to non-stress conditions.

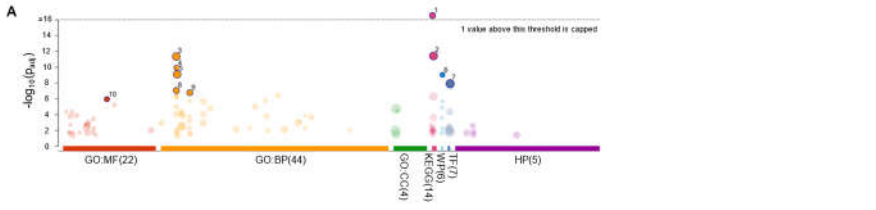
The initial analysis of the RNA-seq involved defining the stress-responsive genes in the wild-type (WT) under stress conditions. In the heat stress condition, we identified 951 repressed and 1,072 induced genes (**Figure 14B**). In the H3-K64A mutant, 36% of the repressed genes and 31% of the induced genes upon heat stress exhibited a weaker repression/induction than in the WT (**Figure 20A**). Under osmotic stress, we identified 477 repressed and 616

induced genes (**Figure 14C**), and the H3-K64A mutant showed a weaker response in 15% and 30%, respectively, of those genes (**Figure 20B**). The exclusivity of this effect to the non-modifiable alanine mutant but not the modification mimicking mutants suggests that a total of 33.2% and 23.3% from the stress-responsive genes in heat and osmotic stress were impaired in their proper regulation without a potential H3-K64 modification.

In addition to the genome-wide impact that H3-K64 exerts under stress, the mutant also showed a growth defect under stress (**Table 6**). We identified 143 genes that consistently showed less induction in the histone mutant under both heat and osmotic stress conditions. We utilized the web-based gene set enrichment analysis tool, *g:Profiler* (biit.cs.ut.ee/gprofiler/gost) (Raudvere et al., 2019) to analyze these 143 genes. This yielded a total of 102 significantly enriched Gene Ontology (GO) terms and other gene groups (adjusted p-value ≤0.05) (**Figure 21B**). The top 10 groups with the most significant p-values were distributed across different databases, with the majority being biological processes. These top 10 groups also indicated that the 143 genes were significantly enriched in metabolism processes, particularly those related to carbohydrates (**Figure 21C**).

Furthermore, we conducted an in-depth examination of the six groups identified from the regulatory motif analysis (**Figure 21D**). We aimed to discover a potential transcription factor associated with the H3-K64A mutant-induced effect. Although Tda9 had the most significant p-values, we placed greater emphasis on the

proportion of motif-containing genes covered by the 143 genes. We observed the highest rate in STRE (Stress Response Element) and Msn2. The STRE is present in 166 genes, 16 of which were less induced in H3K64A mutants. Moreover, five genes from our input intersected with the 18 genes from Msn2. Msn2 is as a well-established stress response regulator that binds to DNA via STREs, and largely modulates carbohydrate metabolism along with Msn4 in response to environmental stresses, thereby enhancing cell survival and adaptation (Ferguson et al., 2005; Kuang et al., 2017; Mat Nanyan et al., 2019; Schmitt & McEntee, 1996) (see introduction).



B

ID	Source	Term Name	P_{adj}
1	KEGG	Starch and sucrose metabolism	$9.633X10^{-19}$
2	KEGG	Biosynthesis of secondary metabolites	$4.138X10^{-12}$
3	GO:BP	Carbohydrate metabolic process	$4.921X10^{-12}$
4	GO:BP	Trehalose metabolic process	$1.265X10^{-10}$
5	GO:BP	Generation of precursor metabolites and energy	$8.625X10^{-10}$
6	WP	Trehalose anabolism	$9.967X10^{-10}$
7	TF	Factor: Yml091	$1.937X10^{-8}$
8	GO:BP	Glycogen metabolic process	$9.489X10^{-8}$
9	GO:BP	Oligosaccharide metabolic process	$1.831X10^{-7}$
10	GO:MF	UDP-glycosyltransferase activity	$1.200X10^{-6}$

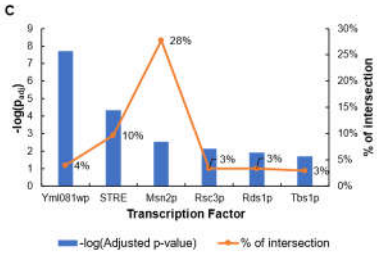


Figure 21. Gene enrichment analysis of common less-induced genes in H4-K64A by heat and osmotic stressed.
(A) Manhattan plot showing each term enrichment by data source. Each circle on the plot represents a single functional term. The term location on the x-axis corresponds to the GO subtree. The circles are color-coded by database and size scaled according to the number of annotated genes in that term. The number behind each database corresponds to the number of enriched terms per catefory. Numbered circles are the top 10 GO terms with the lowest adjusted p-values and described in **(B)**. Database: MF (Molecular Functions), BP (Biological Process), CC (Cellular Component), KEGG (KEGG Pathways), WP(WikiPathways), TF(TRANSFAC), HP (Human Protein Atlas). **(C)** Transcription factors with most significantly matched regulatory motif from TRANSFAC database in gene promoters are presented with their p-values and the % of down-regulated genes

from Msn2 targets.

In our exploration of the potential involvement of Msn2 in regulating the transcriptional defects observed in H3-K64A, we first sought to determine whether the binding of Msn2 to stress-responsive genes was influenced by the PTM status of H3-K64. Msn2-HA tagged recruitment to osmostress-responsive *CTT1* promoter was performed in wild-type and H3-K64A mutant strains (**Figure 22**).

We performed a ChIP with 3HA tagged Msn2 in the histone collection strains, H3-WT and H3-K64A from the H3/H4 library. This was done to ascertain whether Msn2 recruitment upon stress treatment is affected by the H3-K64 modification. Our subsequent qPCR analysis revealed a differential recruitment level of Msn2 to the CTT1 promoter upon osmostress (**Figure 22A**). It's known that Msn2 occupancy increases on the CTT1 promoter region upon osmotic stress (Edmunds & Mahadevan, 2004; Gorner et al., 1998). In the H3-K64A mutant, we observed an impairment of Msn2 binding on the CTT1 promoter under osmotic stress, which coincided with the transcriptional defect we observed in H3-K64A. Despite the limitation of monitoring Msn2 activity on a single gene, we obtained a promising signal indicating that Msn2 binding to target promoters under osmotic stress depends on the presence of H3-K64 PTM. This suggests a possible pathway through which K64A prevents the upregulation of stress-responsive promoters.

Consequently, we conducted a series of experiments using commercially available antibodies designed to detect reported

modifications of H3-K64me3 and H3-K64ac from various organisms (Daujat et al., 2009; Di Cerbo et al., 2014; Jabeena et al., 2021). Initially, we attempted to use yeast total protein extract with the commercial antibodies, but we were unable to observe the antibody specificity (**Figure 22B-C**). We then attempted to use a yeast histone extraction protocol (Jourquin & Géli, 2017) to obtain a sample

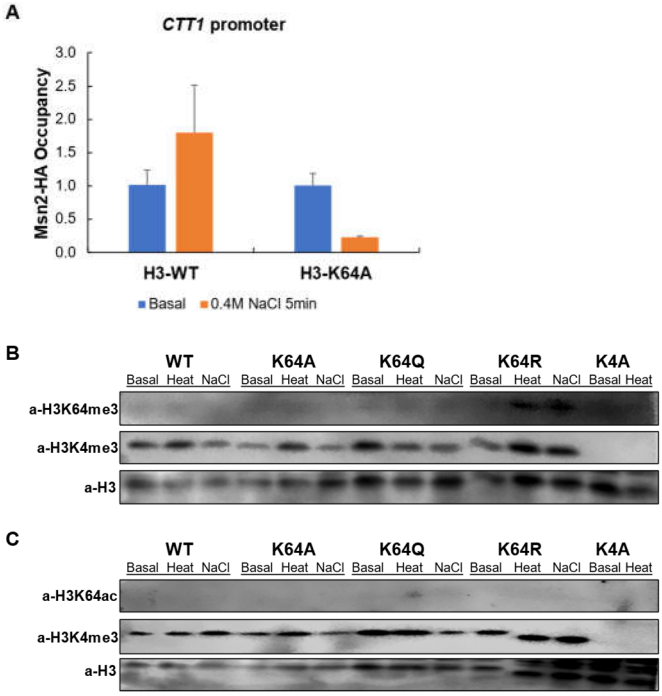


Figure 22. Msn2 recruitment monitoring on stress-responsive genes in H3-K64 mutants upon stress treatment.
(A) Msn2-HA recruitment to promoters of stress responsive gene, *CTT1* in H3-K64 mutant upon osmotic stress. Cells transformed with HA-tagged Msn2 were exposed to osmotic stress for 5min, crosslinked and chromatin extracted. ChIP against HA was performed to analyze binding of Msn2 to promoters of CTT1. Real-time PCR results are shown as the fold induction relative to the WT in basal condition normalized to a telomere internal control. Data is reported as mean ±SD from technical repeats of qPCR (n=1). **(B-C)** Yeast total protein was extracted from indicated histone strain with or without stress (heat 39°C 10min, osmostress 0.4M NaCl 15min). Samples were run in two

separate 16% SDS-acrylamide gel to detect epitopes independently from each other for **(B)** anti-Tri-methyl-Histone H3 (Lys64) Rabbit pAb (PTM Biolabs, PTM-690) and **(C)** anti-Acetyl-Histone H3 (Lys64) Rabbit pAb (PTM Biolabs, PTM-129). Shown images are representative of the trials with yeast total protein. An antibody that detects the PTM on yeasts was not obtained.

enriched with histone proteins. Unfortunately, we were unable to obtain an antibody specific to the PTMs or purified yeast histone extracts (Data not shown).

Despite the limitation of monitoring Msn2 activity on a single gene, we obtained a promising signal indicating that Msn2 binding to target promoters under osmotic stress depends on the presence of H3-K64 PTM. Suggesting a possible pathway through which K64A prevents the upregulation of stress-responsive promoters.

3. H3-K64 POST-TRANSLATIONAL MODIFICATION IN YEAST

The H3-K64A mutant emerged as the most consequential variant in our histone mutant selection process, exhibiting a pronounced transcriptional defect in the expression of stress-responsive genes (**Figure 16**) and severe consequences on growth (**Table 6**). Among the impacted genes we observed from RNA-seq, *CTT1* has shown a differentially binding by Msn2 (**Figure 22A**). Our observations suggest a potential dependency of Msn2 binding activity on H3-K64 modifications. Prior studies have identified trimethylation and acetylation as modifications occurring on this residue (Di Cerbo et al., 2014; Jabeena et al., 2021; X.-F. Wang et al., 2020). Consequently, our investigation focused on determining

which of these two modifications might play a role in yeast stress response signaling.

Phenotypic effects varied among the H3-K64 mutants under stress conditions, as observed in our experimental validation (**Figure 23**). The histone mutant library contains three distinct H3-K64 mutants: K64A, K64Q, and K64R, representing non-modifiable, acetylated, and potentially methylated or non-acetylated states, respectively. From the transcriptional analysis, we observed non-modifiable mutant, K64A, had showed the most significant stress effects. To identify which modification is more involved on this regulation, we analyzed the phenotypic behavior of the modification-mimicking mutants under the stress condition (**Figure 23**).

The phenotypic behavior of the acetylation-mimicking mutant (K64Q) suggests that acetylation is unlikely to play a role in the stress response (**Figure 23A-B**). No significant behavioral change under stress was observed between mutants with acetylation (H3-K64Q) and without acetylation (H3-K64A). Both strains were unable to adapt to the stress treatment as effectively as the wild type. Furthermore, dot assays provided additional clarity on the phenotypic behavior of these mutants, with the methylation/non-acetylated mimicking mutant, K64R, exhibiting the least severe defect under stress conditions (**Figure 23C-D**). This phenotypic effect of H3-K64R suggests that methylation or deacetylation may be the necessary modification for cell survival and adaptation under stress conditions. Consequently, we inferred that methylation of H3-

K64 is more likely to be involved in stress response generation than acetylation, and we proceeded to identify the enzyme responsible for modifying H3-K64 in yeast.

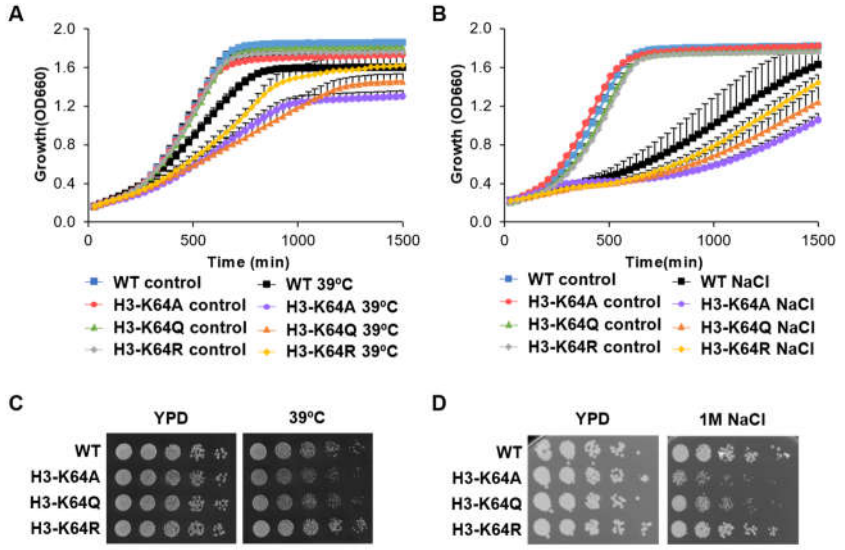


Figure 23. Phenotypic effects of H3-K64 mutants upon heat and osmotic stress.
(A, B) Cell growth rate (OD660) was measured every 30 minutes where the cells were grown at 30°C (basal), 39°C (heat) in YPD (basal and heat stress) or 1.2M NaCl (osmostress) for 24 hours and 48 hours, respectively. Measurements were done in three technical triplicates and graphs shown are representative one of the three biological replicates. Plotted growth curve of WT and mutants of H3-K64 upon **(A)** heat stress, **(B)** osmotic stress. **(C, D)** Cells were cultured at different concentrations of NaCl and temperatures on YPD-agar plates. Plates were scanned when the size of the colony stopped growing. Pictures shown at the figure are one representative of the three biological replicates. Dot assay of WT and mutants of H3-K64 upon **(C)** heat and **(D)** osmotic stress.

3.1. Identification of SET-domain containing methyltransferase in yeast.

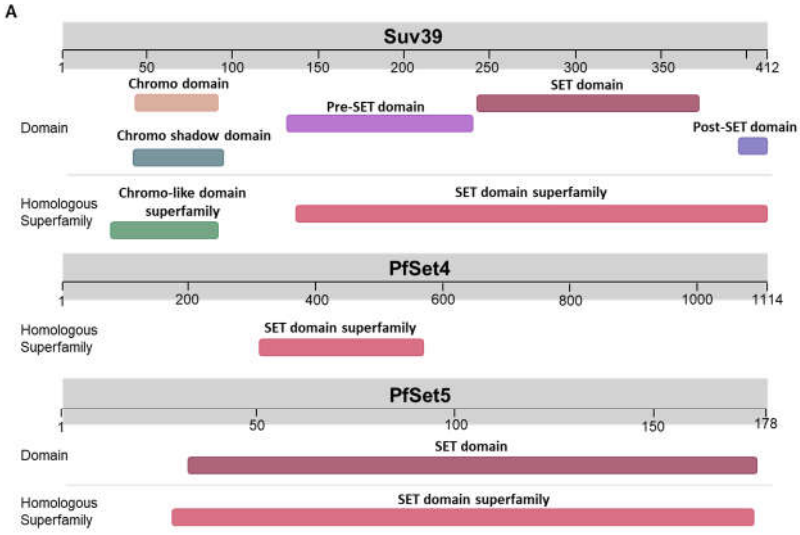
K64 of histone H3 is highly conserved in eukaryotes. Studies conducted in various organisms have reported the acetylation and

trimethylation of H3-K64 and their PTM writers in mammalian species and *Plasmodium falciparum*(Daujat et al., 2009; Di Cerbo et al., 2014; Jabeena et al., 2021; Pradeepa et al., 2016; Tessarz & Kouzarides, 2014). Based on the phenotypic screening conducted in this study, we hypothesized that methylation, rather than acetylation, is the active PTM under stress conditions, given the higher stress response resilience of H3-K64R. We thus aim to identify the potential methyltransferase responsible for H3-K64 methylation in yeast.

Three enzymes, namely Suv39 from mammals and PfSet4/PfSet5 from *P. falciparum*, have been reported as methyltransferases (MTases) responsible for the methylation of H3-K64 in other species (Daujat et al., 2009; Jabeena et al., 2021). Our initial step involved conducting a literature search to identify homologous proteins of these three enzymes in yeasts. We also referred to databases such as the PANTHER Classification System (https://www.pantherdb.org) and STRING DB (https://string-db.org) to determine if there were any reported homologs of these three proteins in yeast. However, we did not identify any reported homologous proteins for Suv39 and PfSet4/PfSet5 in yeast. Consequently, we decided to analyze the protein domains, as our goal was to identify an enzyme with a specific function for histone protein methylation.

We utilized the InterPro database (https://www.ebi.ac.uk/interpro), which provides functional analysis of proteins by classifying them into families and predicting domains

and important sites (Paysan-Lafosse et al., 2023). We input the protein sequences into InterPro and obtained a list of families and predicted domains that the three proteins might possess (**Figure 24**). Seven domains were identified from Suv39, one domain from PfSet4, and two domains from PfSet5 (**Figure 24A**). From these domains, we inferred that the SET domain might be the key regulatory domain functioning as a histone methyltransferase.



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Methyltransferase	InterPro Accession Number	Name	Source Database	Sequence matches
Suv39	IPR023780	Chromo domain	Pfam	43-91
	IPR000953	Chromo/chromo shadow domain	SMART	42-94
	IPR016197	Chromo-like domain superfamily	SUPERFAMILY	30-92
	IPR007728	Pre-SET domain	Pfam	132-240
	IPR001214	SET domain	SMART	243-372
	IPR046341	SET domain superfamily	Gene3D	137-412
	IPR003616	Post-SET domain	InterPro	396-412
PfSet4	IPR046341	SET domain superfamily	Gene3D	311-572
PfSet5	IPR001214	SET domain	SMART	34-176
	IPR046341	SET domain superfamily	Gene3D	30-175

Figure 24. Protein domain analysis of H3-K64 methyltransferases from different organisms.
Domain analysis provided from InterPro of Suv39 from *Mus musculus*, and PfSet4/PfSet5 from *P. falciparum*. **(A)** Domain composition of each protein. The scale under the protein name indicates protein length in amino acids. Each colored bar represents the position of different domains identified from the protein sequence analysis with different InterPro accession numbers **(B)** Accession number and detail annotation obtained from each methyltransferase.

Source data indicates where the domain is modeled based on the input sequence. Sequence match is position of the match in the AA sequence.

The database provided accession numbers for the predicted domains (**Figure 24B**) and a list of proteins that could potentially be modeled with the domain. We retrieved the commonly annotated accession number from the three proteins, IPR046341 SET domain superfamily, and selected the candidates that are reported to have a function as a histone methyltransferase (HMTase). As a result of this process, we identified three yeast SET domain containing HMTases, Set1, Set2 and Set5. The 3 selected HMTases have distinct enzymatic activity in histone methylation and function in different phases of transcription (Green et al., 2012; Krogan et al., 2003; K. Y. Lee et al., 2018) (**Table 7**).

Table 7. Histone methyltransferases containing SET domain in *S. cerevisiae*. Three proteins identified from *InterPro* to have SET domain and reported to have histone methyltransferase activities. Aliases refer to alternative names of the protein.

Protein name	Description	Aliases
Set1	Histone methyltransferase of histone H3 Lys4, subunit of the COMPASS (Set1C) complex	YTX1, KMT2
Set2	Histone methyltransferase of histone H3 Lys36 with a role in transcriptional elongation	EZL1, KMT3
Set5	Methyltransferase involved in methylation of histone H4 Lys5, -8, -12	-

3.2. Identification of Histone Methyltransferase Responsible for H3-K64 Methylation

Set1, Set2, and Set5 were further examined to investigate the potential role of the enzyme in the methylation of H3-K64. As the first assessment of the HMTases, knock-out strains of HMTase were procured from the Yeast Knockout Collection (YKO, Dharmacon™ Reagents) and their cell growth and survival were monitored under osmostress conditions. Our objective was to identify an HMTase mutant that exhibits a phenotypic effect similar to H3-K64A, assuming that the modification is functional and necessary under stress conditions.

Among the three HMTases tested, *set1*Δ exhibited the most significant phenotypic effect (**Figure 25**). In basal conditions, early

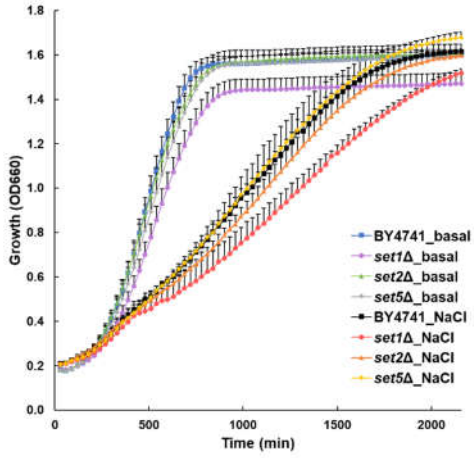

Figure 25. Cell growth monitoring of SET domain containing MTases knock-out strains upon osmotic stress.
The knockout strains of Set1, Set2 and Set5 are from Yeast Knockout Collection (Dharmacon™ Reagents). The WT strain of YKC is BY4741 which is the background strain used for gene deletion. Stress treatment and growth monitoring was conducted under the same conditions as other growth monitoring experiments throughout this study (Figure 5).

cell growth saturation and plateauing of *set1*Δ were considered a technical error, as the same strain demonstrated reaching higher levels under stress conditions. Among the three HMTase candidates, *set1*Δ grew significantly slower than the BY4741 (wild type) under stress conditions. Based on this analysis, we considered the phenotypic effect of *set1*Δ under stress closely aligns with the trend observed from H3-K64A, suggesting a higher likelihood of its association with H4-K64 function as the HMTase.

In previous attempts, we were unable to obtain the modification-specific antibody for yeasts (**Figure 22A**). To circumvent this limitation, we designed an *in vitro* methyltransferase assay with purified enzymes and histone fragments as substrates to observe the enzymatic reaction of the selected HMTases with H3-K64 (Promega MTase-Glo^{TM} Methyltransferase Assay). In brief, the assay is a bioluminescence-based method that measures the final product of the methyltransferase reaction, S-adenosyl homocysteine (SAH) generated from S-adenosyl methionine (SAM), by converting it to a flureoscent substrate and quantified in reference to the SAH standard curve drawn on each experiment.

The necessary materials for the reaction, such as HMTase and histone fragments as substrates, were expressed in *E. coli* and purified by the GST-tag purification system. We designed the assay with Set1 and the histone fragments, as we expected Set1 to be active under stress conditions with higher chances than the other HMTases. Set1 is a relatively large protein composed of 1,080 amino acids in full length, and after a few attempts to obtain the full-length protein, we decided to express and purify part of the

protein. Residue 938-1080 of Set1 is reported to be the catalytic SET core domain and the n-SET domain (residues 762-937) is reported to function on the trimethylation of H3-K4. We choose residues 700-1080 of Set1 to ensure it correctly forms the structure to have enzymatic activity with SAM (Hsu et al., 2018; Kim et al., 2013) (**Figure 26A**).

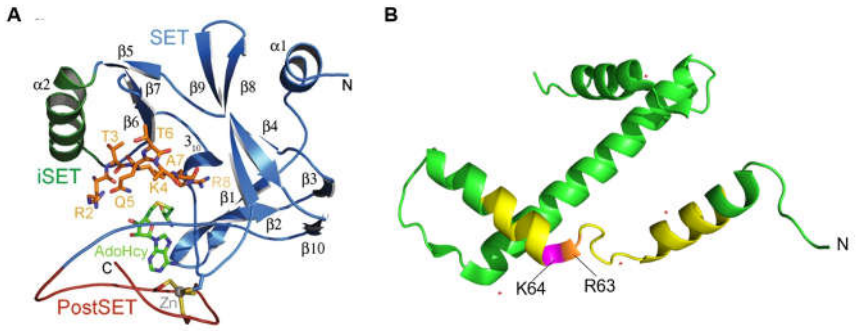

Figure 26. Structural information of the materials used for in vitro methyltransferase, Set1 and histone H3 fragments.
(A) Ribbon representation of the secondary structure of the Set1 catalytic domain illustrating the SET domain (blue) and PostSET motif (magenta). A histone H3 substrate peptide spanning residues 1–6 (orange carbon atoms) and the product SAH are docked in the active site (Figure modified from Takahashi & Shilatifard, 2010). **(B)** Ribbon representation of yeast histone H3. H3 residues used on *in vitro* methyltransferase assay. Fragment (aa48-71) colored in yellow, and residues substituted to alanine are R63 (orange) and K64 (magenta). 3D structure modified from (White et al., 2001).

For substrates, we initially designed different histone fragments. Histone H3 residues 48-71 served as the wild type, the same residue fragment with R63 and K64 substituted with alanine (A) and only K64 substituted with A. K64A mutant was not successfully purified. Another H3 tail residues 1-21 containing K4 was designed additionally, as a positive control (Deshpande & Bryk, 2023). When designing the fragments, we ensured the K64 containing fragment

had as much surrounding structure as possible, so the structural change of the histone does not affect the enzymatic reaction (**Figure 26B**) In addition, there was no case reported on H3-R63 methylation, but we attempted to avoid the possible false signal of methylation that arginine can generate. Thus, three histone fragments were successfully purified and used on the MTase-Glo™ Methyltransferase Assay.

HMTase, Set1 aa700-1080, and the three histone fragments were purified through GST-purification. Set1 and histone fragments were incubated for the enzymatic reaction at 30°C for 30 minutes, and the generated SAH concentration was measured (**Figure 27**). A SAH standard curve was generated for each assay to calculate the SAH concentration of the Set1 enzymatic reaction with the histone fragments (**Figure 27A**). We obtained a linear progression of

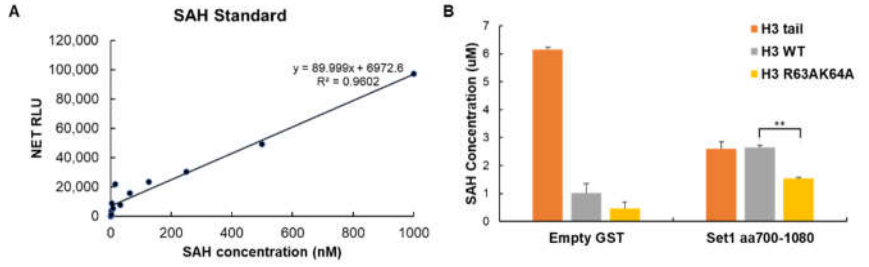

Figure 27. MTase-Glo™ Methyltransferase Assay to assess the enzymatic activity of Set1 towards selected histone fragments.
The assay measures the amount of SAH generated from the enzymatic reaction by converting SAH to ADP and detected through luciferase reaction of ADP to ATP. Luminescence signal is converted to SAH concentration using an SAH standard curve generated with 12 known concentrations of SAH **(A)**. NET RLU represents the relative luminescence unit measured on a luminometer. **(B)** SAH concentration calculated from reaction between Set1 aa700-1080 and the three histone fragments. H3 tail (residue 1-21), H3 WT (residue 48-71), H3 R63AK64A (residue 48-71 with R63 and K64 substitution to Ala) (**: p-value < 0.01).

relative light units (RLU) of the standard curve of SAH and calculated the amount generated from our enzymatic reaction (**Figure 27B**). A high background signal was read from the emptyGST sample reacted with the H3 tail. However, we inferred that the assay for our wild type (WT) and mutant fragments were correctly measured, as their signals with emptyGST were not elevated and a significant signal was only observed in the reaction with the enzyme. In final calculation, we observed a significant level of SAH generation was observed on H3 tail and the WT fragment showed a similar level of SAH concentration. Moreover, a significant decrease in Set1 activity on the H3-R63AK64A fragment was observed compared to the activity against WT.

We speculated methylation of H3-K64 is the active PTM involved in the stress response and adaptation. In addition, the findings support the idea that Set1 would be the responsible PTM writer of H3-K64 methylation. In conclusion, Set1 may play a key role in stress response gene transcription and stress adaptation of the cells by modulating the methylation of H3-K64 in yeast.

DISCUSSION

Post-translational modifications (PTM) of histones, a form of epigenetic modification, have been implicated in the regulation of gene expression and are frequently associated to stress responses, aiding cellular adaptation to environmental changes (Mushtaq et al., 2021; Weiner et al., 2015). The numerous modifications at the histone residues can act directly (i.e., changing nucleosome structure) or indirectly (i.e., binding of PTM reader proteins) to alter gene expression or chromatin structure (Millán-Zambrano et al., 2022). This thesis focuses on the identification of histone residues in the eukaryotic model *S. cerevisiae* that induce significant transcriptional changes in response to environmental stress through histone residue modification.

Systemic analysis of histone proteins in yeast

A comprehensive analysis of core histone proteins was conducted prior to this study, yielding 232 histone mutants that elicited a transcriptional response upon stress exposure (Viéitez et al., 2020). An additional 13 histone mutants were obtained from the phospho-proteomics analyses of histone proteins from the same previous study. A systematic approach was employed on these 245 histone mutants to reduce this number to a manageable size for in depth evaluation. The selection criteria for potential histone mutants of interest were based on bibliographic data, structural information, and consistency of the mutant-effect under stress conditions as indicated by previous screening results. This analysis allowed us to significantly reduce the initial list from 232 to 7 mutants

corresponding to 6 histone residues from the two HTSs. In parallel, out of the 13 mutants from the phospho-proteomics analysis, we selected 11 mutants located in 9 histone residues. Therefore, we ended up with a list of 15 residues consisting of 24 different mutants. The 24 mutants included some other mutants not identified as hits but under the selected residues. In total, 24 mutants were subjected for further experimental validation.

The selection process took into account the consistency in growth and transcriptional effects under the same stress conditions. We anticipated the possibility of false positives arising from large-scale screenings, see below, and therefore placed greater emphasis on the results of histone mutants that had two positive hits. Prior to this, we considered the localization of a given residue and the novelty of the potential modification to conduct the investigation of its function in stress signaling. Residues that were buried within the nucleosome which are not easily accessible by modifying enzyme represented 23% of the candidate mutants and were discarded because of their difficulty to be modified in principle.

The list was further refined by comparing the screening results with the experimental validation for 24 selected mutants. To our surprise, we observed a significant discrepancy between the results of the screening and our experimental validation. From the screening, there were 18 mutants with transcriptional effect under stress. We were able to only validate three of them. 9 mutants with phenotypic effect under stress, and we validated 8 of them.

The discrepancy between transcriptional and phenotypic results

offers three key insights. Firstly, the phenotypic effects tend to be highly reproducible, yielding more consistent results devoid of significant batch effects. We reasoned that we could reproduce the data because the same measurement method between screening and validation was used. Both assessed colony growth. In addition, validation involved an extra measurement to assess quantitative growth which in the end increased the credibility of the results. This reliability was a primary reason for our decision to place greater emphasis on phenotypic results when selecting histone mutants for genome-wide analysis in the final phase of our study.

Of note, in some cases we identified mutants with both transcriptional and phenotypic effects that were not initially identified in the large-scale screenings. Specifically, our transcriptional validation detected eight additional mutants exhibiting transcriptional effects under osmostress, which were not initially identified in the HTS. The transcriptional screening results were less reproducible in osmotic stress. For instance, in the HTS, we did not detect the transcriptional defects of 6 mutants upon osmostress that were later observed in the validation. Particularly, H3-K64A, which we found the most dramatic transcriptional effect, was not detected either in heat or osmostress. A possible explanation for this is a less sensitivity or variability in the HTS in comparison with the direct detection by specific radioactivity-labeled probe detection system (Chalfie et Howal., 1994). An additional explanation for this observation is the use of a reporter that only includes the promoter of a stress-responsive gene in the HTS whereas we monitored the expression of the full-length gene in

the validation.

From the phospho-proteomics analyses of the histone mutants, 11 mutants corresponding to 9 residues were chosen for further experimental validation. We included these mutants despite not showing an effect in the HTS as they are phosphorylated *in vivo* providing a strong starting point for a molecular mechanism. From the validation, we successfully selected two mutants from two residues. Compared to the six residues from the HTS for which at least one mutant could be validated, the validation rate for the residues from the phospho-proteomics list was relatively low. One possible explanation for this could be that the phospho-proteomic analysis was performed under basal conditions and the identified modification and residues may thus not be involved in the stress response. In addition, compared to histone methylation which is often directly linked to transcriptional regulation (Kouzarides, 2002), histone phosphorylation plays a role in broader cellular processes that include but are not limited to transcription regulation (Nowak & Corces, 2004; Rossetto et al., 2012; Zhao & Malik, 2022).

Despite certain discrepancies encountered during the validation phase, we found the results from the two screenings and the phospho-proteomics analysis to be valuable resources for initiating our investigation into core histone proteins. Furthermore, we successfully validated at least 8 histone residues from H2A, H3, and H4 for subsequent genome-wide effect analysis. We anticipated those residues may harbor PTMs relevant to the regulation of stress

response.

Five selected histone residues of arginine/lysine residues are associated with histone chaperone FACT

We employed a combinatorial approach encompassing cellular and transcriptional screening, which led to the selection of eight histone residues for in depth genome-wide analysis. These eight histone residues could be classified into two groups based on their amino acid, reflecting their potential modifications. Three of the residues were phosphorylatable serine and tyrosine, while the remaining five were either arginine or lysine.

The three phosphorylatable histone residues are potentially a direct outcome of activated cellular signaling pathways and their function can be to translate signals to biological outputs (Nowak & Corces, 2004; Rossetto et al., 2012; Zhao & Malik, 2022). By contrast, the remaining five, arginine or lysine, are the most abundant residues in histone proteins and subject to a variety of post-translational modifications that in certain cases affect various cellular processes such as DNA replication, repair, and transcription (Green et al., 2012; Husmann & Gozani, 2019; Martin & Zhang, 2005; Suka et al., 2002; Williams et al., 2008). These findings indicate that while all eight histone residues promote stress responses and adaptation, they might act using different mechanisms.

The three phosphorylatable residues were H4-S60, H4-S64 and

H4-Y88. Histone phosphorylation reduces the positive charge of histones and thus has severe structural consequences. Phosphorylation of H4-Y88, for example, likely disrupts a spring like structure formed with H2B-Y86 stacking structure (Dai et al., 2008). This suggests the possible function of H4-Y88 phosphorylation affecting physical accessibility of DNA. In human, H4-Y88 is reported to be phosphorylated by the kinase ACK1 (activated CDC42 kinase 1), which subsequently promotes androgen receptor (AR) transcription in castration-resistant prostate cancer (CRPC) (Mahajan et al., 2017).

While phosphorylation of H4-S60 and H4-S64 was reported earlier in a systemic analysis (Albuquerque et al., 2008; Chen et al., 2010; Holt et al., 2009; Zhou et al., 2021), no function has been reported so far. These two residues are localized at disk of the nucleosome, likewise Y88 of H4, where they can be involved in histone-histone interaction (**Figure 28**). This may suggest that modifications on these residues possibly alter chromatin structure through the interaction with DNA or other histones which may affect chromatin condensation (Small & Osley, 2023).

Two of the arginine residues, H2A-R78 and H3-R63 were described as sprocket arginine residues inserted into the DNA minor groove and engaged in DNA like the teeth of a bicycle sprocket (Hodges et al., 2015). Global histone mutant studies had reported that H2-R78A mutant increased sensitivity against stress treatments (Dai et al., 2010; Huang et al., 2009), and functions in DNA repair of UV-induced DNA lesions (Hodges et al., 2015). Moreover, these

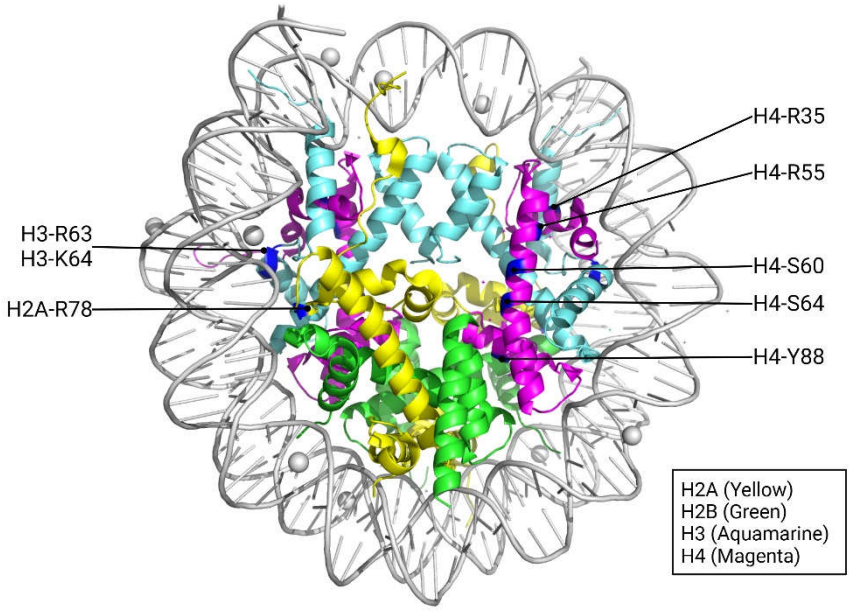

Figure 28 Yeast nucleosome structure highlighting the 8 histone residues analyzed by RNA-seq in this study.
Eight histone residues selected for experimental validation and analyzed their genome-wide effect via RNA-seq are depicted in blue in each histone protein. The DNA structure surrounding the core histones is colored grey and the core histones are colored differently. Color annotation is written on the figure (bottom right). (Protein Data Bank, I1D3 (White et al., 2001), images rendered in Pymol)

two sites and the three histone residues, H3-K64, H4-R35, and H4-R55, were reported to interplay with a highly conserved histone chaperone complex, known as the FACT complex (Facilitates Chromatin Transactions) (Bondarenko et al., 2015; Kemble et al., 2015; McCullough et al., 2013; Nyamugenda et al., 2018).

FACT is an essential heterodimer protein complex composed of Spt16 and Pob3 in yeasts. The FACT complex is reported to have high affinity binding towards the tail of the H2A-H2B dimer (Kemble et al., 2015), and also with the complete nucleosome (Y. Liu et al., 2020; Winkler et al., 2011). The direct binding between

H2A-R78 and CTD of Spt16 was observed under the non-modified status of the histone, H2A-R78A, while the constitutively active mutant, H2A-R78E, did not show significant binding with Spt16 (Kemble et al., 2015). The three residues, H3-R63, H3-K64 and H4-R35, were shown to act together on their interaction with Spt16. This section of the nucleosome containing these residues is known as the ISGI (Influences Spt16-Gene Interactions) based on the contribution of the region with Spt16 (Nyamugenda et al., 2018). 8 residues are included in the ISGI, and the three arginine/lysine residues H3-R63, H3-K64 and H4-R35, are reported to have a moderate effect on Spt16-gene interaction (Nyamugenda et al., 2018). Spt16 has been reported to play a significant role in heat and osmotic stress, as evidenced by phenotypic impairment upon its depletion. It also promotes the expression of stress-responsive genes induced by Hsf1 through the process of nucleosome reassembly or relocation (Erkina & Erkine, 2015). Moreover, another important subunit of FACT, Pob3, has been reported to be association with one of the selected residues, H4-R55 (McCullough et al., 2013). A proper H4-R55 interaction with Pob3 revived the loss of heat stress adaptation generated by Pob3 mutation (McCullough et al., 2011, 2013).

While it may be apparent that histones interact with histone chaperone, our study provides specific evidence that the residues involved in stress adaptation are associated with the activity of FACT. FACT is recognized for its role in nucleosome reorganization through interactions with various factors. Alterations of the specific amino acids have a substantial impact in stress

adaptation. For instance, the loss of interaction observed in H2A-R78E or the effect of the mutation in H4-R55G are indicative of their potential contributions these residues could make on the FACT function to induce cellular survival upon stress and expression of stress responsive genes (Kemble et al., 2015; McCullough et al., 2013).

Unexpected transcriptional impact of certain mutation in basal conditions

To elucidate the functional role of the identified residues during stress, we profiled the transcriptome of all selected mutants upon stress. This approach allowed us to define the landscape of the transcriptional effects that the selected histone mutants generate under basal and stress conditions.

Our RNA-seq analysis, conducted with the eight selected residues comprised of 24 different mutants (including several modifications for each residue), revealed a complex landscape of transcriptional changes. While our primary focus was on stress signaling, the results suggested a broader involvement of these residues in gene expression. Three histone mutants, H2A-R78A, H4-S60A, and H4-S64A, exhibited substantial alterations in transcription under basal conditions. While H2A-R78A mutant demonstrated a strong stress phenotypical defect, also showed about 10% of the genome with altered expression in basal conditions. In addition, 41 genes were found to be less induced in the mutant under heat stress, including *CTT1*, which showed 2.3-fold reduced

induction than wild-type.

Previously, it was reported that the R78A mutant was sensitive to various stresses, including hydroxyurea, heat, and the DNA-damage reagent MMS (Dai et al., 2008, 2010; Huang et al., 2009). Our observations of sensitivity in heat and osmotic stress conditions align with these reports. In addition, a microarray of H2A-R78A mutant strain showed up-regulation of 290 genes while 8 genes down-regulation (Hodges et al., 2015). This aligns with the expression trend of our H2A-R78A RNA-seq results that 84% of the significantly altered genes were up-regulated and 16% were down-regulated. The histone occupancy analysis of the 290 up-regulated genes upon H2A-R78A indicated higher histone occupancy in their promoters in wild-type which suggests a repression role of H2A-R78 in transcription (Hodges et al., 2015).

We discussed the binding activity of H2A-R78 and FACT. The H2A-R78A mutant lost binding with FACT subunit, Spt16, and their suggested model showed disruption of H2A-H2B and DNA contact (Kemble et al., 2015). This is consistent with the FACT activity competing with DNA for binding to H2A-H2B (Hsieh et al., 2013), indicating FACT binding with H2A-H2B induces open and accessible nucleosome status (Formosa, 2012). By this structural confirmation, we could speculate the repressive role of H2A-R78 in basal condition, however, further evaluations of the FACT activity in combination with the R78 mutations are necessary to fully elucidate the role of this residue upon growth defects we observed from the phenotypic response assessments.

Acetylation and methylation, the two possible modifications of H3-K64

A part of this study was dedicated to the analysis of the residue H3-K64, which exhibited the most significant effects in our genome-wide RNA-seq analysis. HTS results indicated that H3-K64 was affected solely by heat stress. However, experimental validation revealed a pronounced transcriptional defect in H3-K64A under both heat and osmotic stress conditions. Furthermore, the phenotypic impact of the residue suggested a greater possibility of methylation involvement under stress conditions, as evidenced by the differential phenotypic behavior of the two mutants. The acetylation-mimicking mutant, K64Q, demonstrated a growth trend similar to that of the non-modifiable mutant, K64A, under stress conditions. Interestingly, the mutant mimicking methylation or a non-acetylated state, K64R, exhibited growth behavior comparable to the wild-type under stress.

H3-K64 is the first amino acid of the alpha 1 helix of histone 3 and it is at the lateral surface of the nucleosome that contacts with DNA. To decipher the regulatory mechanism behind the transcriptional role of this amino acid we performed RNA-seq analysis. The function of several PTMs on the residues located at the lateral surface of the histone octamer have been reported to alter transcription by altering the efficiency of histone binding with DNA by unwrapping the DNA near the entry-exit region (Neumann et al., 2009; Williams et al., 2008), H3-K64 is located at the DNA entry-

exit point (Tropberger & Schneider, 2013).

Acetylation and trimethylation are the reported modifications of H3-K64 in mammals and *P. falciparum* (Di Cerbo et al., 2014; Pradeepa et al., 2016; X.-F. Wang et al., 2020). Our study suggests that methylation could be involved in yeast stress response, based on the differential phenotypic effects observed in mutants. The phenotypic effects suggested a role for methylation in cell growth under stress conditions. However, the regulation of transcriptional stress response appears to involve a more complex mechanism, with more than one modification on H3-K64. In our RNA-seq analysis, neither of the two modification status-mimicking mutants showed a significant genome-wide effect, with only the non-modifiable mutant, H3-K64A, exhibiting a significant transcriptional effect. This suggests that maintaining a proper balance between the two potential post-translational modifications (PTMs) may be critical for stress-responsive gene expression.

It has been described that the H3-K64ac is associated with active chromatin regions and found in active gene promoters of developing cells that stimulate gene transcription (Di Cerbo et al., 2014; Pradeepa et al., 2016; Wang et al., 2020). A previous study on H3-K64ac identified the p300/CBP as the enzyme responsible for the acetylation of K64, among different HAT families, suggesting the possible mechanism to activate genes and to promote transcription (Di Cerbo et al., 2014). In contrary, trimethylation of H3-K64 (H3-K64me3) is localized to inactive chromatin regions and it is associated to a repressive mark at embryonic stem cells

(Jabeena et al., 2021; Lange et al., 2013).

Our phenotypic observations from the H3-K64A mutant suggested that the associated modification may play a role in transcription, acting as an activating mark similar to what has been described for H3-K64ac. However, the genome-wide effect observed in the non-acetylated mutant (H4-K64R) did not fully support this hypothesis. Consequently, it is likely that methylation might be involved in growth rate control whereas a broader effect on transcription could be dependent on the acetylation. Nevertheless, we acknowledge that the regulation of the transcriptional stress response likely involves more complex mechanisms, potentially involving both modifications of H3-K64.

A functional role of K64 on the general stress response mediated by the transcription factor, Msn2

Our RNA-seq analysis led us to conclude that H3-K64 exhibits the most significant transcriptional defect among our selected histone residues. To further understand its functional role under stress, we performed a gene enrichment analysis of the subset of genes that showed reduced induction upon stress in the K64A mutant.

The transcription factors Msn2 and Msn4 mediated the general stress response, they are activated by multiple types of stress and specifically target genes with stress response elements (STRE) (Jacquet et al., 2003; Martínez-Pastor et al., 1996). In the context of

heat stress, Hsf1 is a key transcription factor that mediates the induction of heat stress-responsive genes through heat shock elements (HSE) (Pincus, 2020). During osmotic stress, the MAP kinase Hog1 is recruited along with the transcription machinery, promoting the expression of stress-responsive genes (Alepuz et al., 2001).

In the functional enrichment analysis, a set of 143 genes were consistently less induced in response to the two stress conditions in the H3-K64A mutant. These genes served as the input for the enrichment analysis. The analysis yielded Msn2 and the STRE motif among the top three categories with the lowest p-values and the highest number of genes. Notably, 21 out of the 143 genes, representing 15% of the total, intersected with these two categories. This led us to investigate the possibility that the H3-K64A mutation might influence Msn2 activity over its target element, STRE.

To assess Msn2 activity, we examined the binding of Msn2 to the promoter regions of target genes in wild type or the K64A mutant using ChIP-qPCR. We observed a decrease in Msn2 binding to the promoter of the stress-responsive gene, *CTT1*, in the K64A mutant.

Our data is consistent with the binding of Msn2 via K64, however, only provides a partial understanding of the complex regulatory mechanisms governing stress-responsive gene transcription. Given that only 15% of our input genes intersected with *Msn2* and STRE, it is likely that K64 modifications would alter the binding of other transcription factors.

The Set1 histone methyltransferase is an upstream regulator of K64 methylation

Our results suggested a stronger association between the methylation of H3-K64 rather than acetylation in stress adaptation. Specifically, WT and K64R exhibited similar stress adaptation, while K64A and K64Q showed stress sensitivity.

Histone methyltransferases are divided into two groups based on their target residue and the structural domain they possess, namely the SET domain. One group, which includes the SET domain, targets lysine residues (Husmann & Gozani, 2019; Nimura et al., 2010), while the other group, which lacks the SET domain, methylates arginine residues (Nimura et al., 2010). Given that the K64 histone residue of interest is lysine, we anticipate that a SET domain-containing methyltransferase would be responsible for H3-K64 methylation.

It has been reported that the methyltransferases identified to target H3-K64 were PfSET4/PfSET5 and Suv39 in *P. falciparum* and mammalian models, respectively (Daujat et al., 2009; Jabeena et al., 2021; Lange et al., 2013). As expected, all three enzymes contained SET domains.

In yeasts, we have several SET domain histone methyltransferases, and Set1 displays a similar growth defect to H3-K64A. We examined whether Set1 could target K64 by an *in vitro* methyltransferase assay. Indeed, we observed a significant level of

methylation by Set1 over different histone fragments containing K64 in contrast to the K64A mutant.

The assay was conducted *in vitro,* and we assayed different substrates for histone methylation, ranging from small peptides of H3 tail, a fragment of H3 containing H3-K64 and mutant fragments with H3-R63A and -K64A. This allowed us to measure the relative Set1 activity using purified histone fragments as substrates, circumventing the need for histone proteins, which we were unable to obtain from yeast extracts using antibodies. Nonetheless, the assay had limit of detecting the depletion of SAM as it was unable to differentiate between the number of methyl groups added on H3-K64 by Set1. Furthermore, *in vivo* demonstration of methylation remains a gap in these experiments. Mass spectrometry (MS), a frequently used tool for histone modification analysis with an ability to detect histone PTMs *in vivo* (Lu et al., 2021; Scheid et al., 2022; M. Zhou et al., 2020), could be applied to validate Set1 activity on H3-K64. In conclusion, although we have not a direct proof in vivo of the modification, our findings highlight the potential role of Set1 as a PTM writer for H3-K64 methylation in the stress response.

Our study elucidated the function of a histone residue located on the lateral surface of a nucleosome (**Figure 29** illustrates this working model). H3-K64 can undergo multiple modifications, and changes in charge on the lateral surface can affect histone-DNA or histone-histone interactions, potentially leading to structural changes in the nucleosome. Previous studies observed such structural changes in mammalian cells,

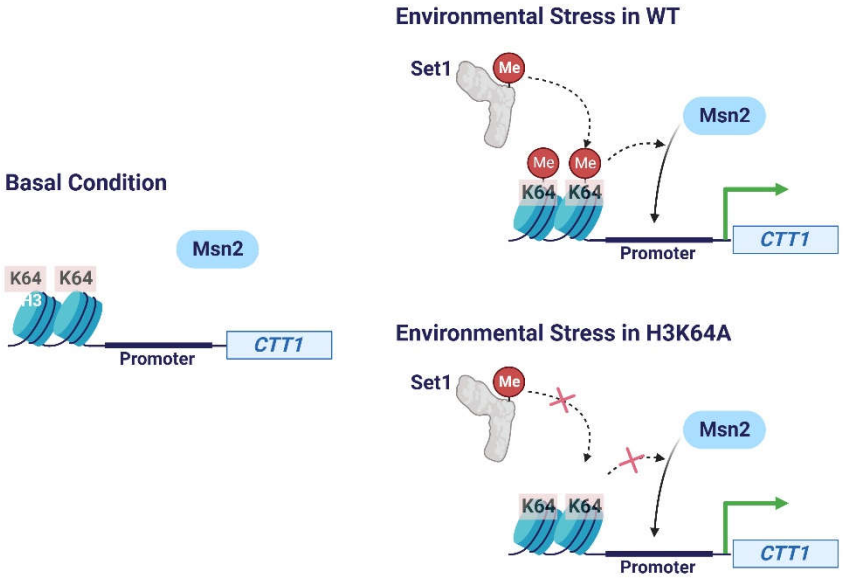

Figure 29 Schematic view of the working model for H3-K64 upon stress. Stress responsive genes are highly induced under stress conditions, such as *CTT1*, but not in basal condition. The H3-K64 methylation is required for transcription of *CTT1* upon stress, which is then targeted by the transcription factor, Msn2. In contrast, in the non-modifiable mutant, H3-K64A, transcription was not induced properly upon stress.

where H3-K64 methylation induced nucleosome instability by preventing acetylation (Tropberger & Schneider, 2013).

Our study has limitations. We are still uncertain about how methylation is involved in the induction of stress-responsive genes. Despite these uncertainties, we have successfully identified a histone residue with significant function in the stress response and adaptation from our screening data. We have also proposed that the K64 site is modulated by Set1 to control the transcriptional response to environmental stress.

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CONCLUSIONS

This PhD thesis aimed to identify histone residues required for cellular adaptation to stress and elucidate the mechanisms that mediate transcription regulation through those histone residues.

More specifically, the results obtained during this PhD thesis led to the following conclusions:

1. 24 histone mutants corresponding to 15 histone residues selected from screenings and phospho-proteomics analysis were experimentally validated by phenotypic and transcriptional analysis.
2. Comprehensive analysis of screening data and experimental validation results concluded 8 histone residues to be subjected to a genome-wide RNA-seq analysis.
3. By RNA-seq analyses 3 histone mutants showed significant defects under basal conditions, and one mutant, the H3-K64A, showed significant defects on stress conditions, showing alteration in 33% and 23% of heat stress and osmotic stress responsive gene expression respectively.
4. H3-K64A, was associated to Msn2-mediated transcription which depended on the PTM status of H3-K64.
5. H3-K64 was likely modified by the Set1 methyltransferase.

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