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Cellular Response to External Signals in *S. cerevisiae*

Trabajo Realizado en el Departament de Bioquímica i Biología Molecular de la Universitat de València por

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Para optar al grado de Doctor por la Universitat de València

Trabajo dirigido por

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PAULA ALEPUZ, Doctora en Ciencias Químicas y Profesora Titular del Departamento de Bioquímica y Biología Molecular de la Universitat de València, informa:

Tianlu Li, licenciada en Biomedicina por la Universidad de Melbourne, Australia, ha realizado bajo mi dirección el trabajo bajo el título "Cellular Response to External Signals in *S. cerevisiae*", el cual presenta para optar al grado de Doctor por la Universitat de València.

Valencia, Julio del 2015

Fdo. Dra. Paula Alepuz

Your average budding yeast may not live extraordinarily exciting lives, and you might think this little single-celled organism is unable to protect itself from the dangerous world. You are wrong. This thesis will cover a large aspect of the yeast´s life and give insights to how he is able to detect external signals, respond, adapt and prosper in an ever-changing environment.

Acknowledgements

En primer lugar quiero agradecerle a Paula, que sin ella nada hubiera sido posible. Gracias por haberme enseñado absolutamente todo y por haberme ayudado siempre cuando lo necesité. Lo más importante, gracias por todas las risas que compartimos y por haber sido paciente conmigo durante los tiempos difíciles. No solo has sido una jefa sino una amiga. A José Enrique, gracias por darme la oportunidad de adentrarme en el mundo de la investigación y también por haber compartido tanto, tanto dentro como fuera del laboratorio.

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To my parents and Sky: this thesis is for you. Thank you for absolutely everything, not just in my thesis, but in my life.

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Abstract

In this thesis, we utilised the model organism the budding yeast *Saccharomyces cerevisiae* to gain fundamental knowledge on a variety of molecular mechanisms employed by the cell to respond and adapt to external stimuli. In response to increased external osmolarity, the yeast high osmolarity glycerol (HOG) MAPK pathway becomes activated to mediate changes to various cellular functions, including changes in glycerol accumulation, cell-cycle arrest, re-establishment of ion homeostasis and global reprogrammation of transcription and translation of the whole genome, in order to achieve adaptation. Upon detection of high external osmolarity, signal is transduced via two functionally redundant but mechanistically distinct branches, SLN1 and SHO1, to finally activate the effector MAPK Hog1. Although multiple interactions between osmosensors, adaptor proteins and signalling components of the SHO1 branch have been previously described, this thesis further characterises the complexity of their interaction profiles to elucidate how signal is effectively propagated and how signal fidelity is achieved. Here, we utilised the novel M-track method, which detects both short-lived and stable protein interactions, and made several interesting observations. First, interaction signals between Ste11 and many of its signalling partners can already be detected under basal conditions, in which the signals increase after stress exposure. Second, all Ste11 interactions require the presence of the Ste11-adaptor protein Ste50. Third, the presence of the osmosensors Msb2 and Hkr1 is necessary for the interaction of Ste11 to Sho1 and Pbs2. Fourth, our results detect, for the first time, an interaction between osmosensor Msb2 and Ste11, and that Msb2 may function as an individual Ste11 concentrator at the plasma membrane. Finally, we observed the existence of negative feedback systems targeting the protein levels of Ste11 and Msb2 and also hint at changes in the dissociation rates of intermediate signalling complexes.

Once the osmostress signal is transduced, activated Hog1 enters the cell nucleus and, amongst its most prominent downstream functions, modulates global gene expression changes. Within minutes of shock, global transcription rate rapidly decreases by 50 %, however the transcription machinery is reallocated to specific genes implicated in osmostress cellular protection and their transcription is strongly and rapidly induced. The cap-binding complex (CBC), consisting of mRNA-binding proteins Cbc1 and Cbc2, associates with the 5´ mRNA cap co-transcriptionally and has been described to function in various aspects of the mRNA life, including transcription. Utilising both genomic and molecular biology techniques, we describe Cbc1 as a global transcription regulator, both under stress and non-stress conditions, to mediate high and timely gene expression. First, our Genomic Run-on (GRO) data suggest that Cbc1 is required for the global reprogrammation of transcription following osmotic stress. More specifically, Cbc1 is required for the rapid and high induction of osmostress genes during osmotic stress stimulation. Second, Cbc1 mediates the recruitment of the Hot1/Hog1 complex to osmostress promoters by interacting with Hog1, which in turn regulates the

formation of the pre-initiation complex (PIC) and RNAPII recruitment on the same genes. Third, the transcriptional role of Cbc1 also extends to the regulation of ribosomal protein (RP) gene expression both during the recovery of RP transcription following osmotic stress and for high transcription under normal conditions, where Cbc1 modulates the recruitment of Rap1 to RP promoters, which subsequently induces PIC formation. Fourth, utilising genomic data, Cbc1 appears to specifically drive the transcription of highly induced genes in contrast to lowly expressed genes. Finally, our results also hint at a possible role for Cbc1 in the regulation of signalling shutoff as well as protein stability of transcription factors (TFs).

In response to pheromones of the opposite mating type, the yeast pheromone MAPK pathway is activated to mediate a series of physiological changes in preparation for mating; including reprogrammation of gene expression, cell-cycle arrest, formation of a sexual projection termed ¨shmoo¨, and ultimately cell fusion of mating partners. The evolutionarily conserved and essential translation factor eIF5A has recently been described to promote translation of proteins containing three or more consecutive proline residues (polyPro) by binding to ribosomes and alleviating ribosome stalling during the formation of the Pro-Pro peptide bond. The activation of eIF5A requires the addition of a unique post-translational modification, a hypusine residue, which is derived from spermidine, an essential factor for mammalian fertility and required for yeast mating. Here we investigated eIF5A as a regulator of the pheromone response through the translation of polyPro proteins with roles in mating. In this thesis, we first show that in response to pheromone, hypusinated eIF5A is required for shmoo formation, localisation of polarisome components, induction of cell fusion proteins, and assembly of actin cables for polarized cell growth. We also show that eIF5A is required for the translation of Bni1, a proline-rich formin involved in actin polymerisation during shmoo formation. Our data indicate that translation of the polyPro motifs in Bni1 is eIF5A-dependent and this translation dependency is lost upon deletion of the proline residues. Moreover, an exogenous increase in Bni1 protein levels partially restores the defect in shmoo formation seen in eIF5A mutants.

In conclusion, our work provides several interesting observations to the yeast cellular response to external stimuli. In response to osmotic stress, we have characterised the protein interaction profiles of several signalling components of the HOG pathway and provided more insights in how signal is rapidly and effectively transduced. Furthermore, we have identified Cbc1 as a transcriptional regulator of the osmotic stress response to induce high and timely expressions of osmostress-protective genes through the recruitment of TFs and PIC components. Additionally, Cbc1 also has more general functions to drive the expression of highly transcribed genes under normal non-stress

conditions. Finally, our results identify eIF5A as a novel and essential regulator of yeast mating through formin translation. Since eIF5A and polyPro formins are conserved across species, our results also suggest that eIF5A-dependent translation of formins could regulate polarised growth in such processes as fertility and cancer in higher eukaryotes.

Resumen

En esta tesis, se utilizó como organismo modelo la levadura *Saccharomyces cerevisiae* para obtener conocimiento fundamental sobre una variedad de mecanismos moleculares utilizados por la célula para responder y adaptarse a los estímulos externos. En respuesta al aumento de la osmolaridad externa, la ruta de señalización MAPK High Osmolarity Glycerol (HOG) se activa para mediar cambios en diversas funciones celulares, incluyendo cambios en la acumulación de glicerol, la parada del ciclo celular, el restablecimiento de la homeostasis de iones y la reprogramación global de la transcripción y traducción, con el fin último de lograr la adaptación. Tras la detección de alta osmolaridad externa, la señal se transduce a través de dos ramas funcionalmente redundantes pero mecanísticamente distintas, SLN1 y SHO1, para finalmente activar el efector MAPK Hog1. Aunque múltiples interacciones entre sensores, proteínas adaptadoras y los componentes de señalización de la rama SHO1 se han descrito anteriormente, esta tesis caracteriza aún más la complejidad de los perfiles de las interacciones para dilucidar cómo se propaga la señal con eficacia y cómo se logra la fidelidad de la señal. En este sentido, hemos utilizado el nuevo método M-track que detecta tanto las interacciones entre proteínas de corta duración como las estables y hemos realizado varias observaciones interesantes. En primer lugar, las señales de interacción entre Ste11 y muchos de los componentes de señalización se pueden detectar incluso en condiciones basales, y las señales aumentan después de la exposición al estrés. En segundo lugar, todas las interacciones de Ste11 requieren la presencia de la proteína-adaptadora Ste50. En tercer lugar, la presencia de los osmosensores Msb2 y Hkr1 es necesaria para la interacción de Ste11 a Sho1 y a Pbs2. En cuarto lugar, nuestros resultados detectan, por primera vez, una interacción entre el osmosensor Msb2 y Ste11, y además hemos encontrado que Msb2 puede funcionar como un concentrador adicional de Ste11 en la membrana plasmática. Por último, se observó la existencia de sistemas de retroalimentación negativos dirigidos a los niveles de las proteínas Ste11 y Msb2 y también hacia los cambios en las tasas de disociación de complejos de señalización intermedios.

Una vez que se transduce la señal de respuesta a estrés, Hog1 activado entra en el núcleo de la célula y, entre sus más destacados funciones aguas abajo, modula los cambios de expresión génica global. A los pocos minutos de choque osmótico, la tasa de transcripción global disminuye rápidamente en un 50%, sin embargo la maquinaria de transcripción se reasigna a genes específicos implicados en la protección celular frente al estrés y su transcripción se induce rápida y altamente. El complejo de unión a la caperuza CBC (por cap-binding complex), que está formado por las proteínas de unión a RNA Cbc1 y Cbc2, se asocia con la caperuza en 5' del mensajero co-transcripcionalmente y se ha descrito su función en diversos aspectos de la vida de mensajero, incluyendo la transcripción. Utilizando técnicas de biología molecular y genómica, describimos Cbc1 como regulador de la transcripción global, tanto en condiciones de estrés y no estrés, para mediar la expresión génica de

manera que se consigan niveles altos de una forma rápida. En primer lugar, nuestros datos de Genomic Run-On (GRO) sugieren que Cbc1 se requiere para la reprogramación global de la transcripción tras el estrés osmótico. Específicamente, Cbc1 se requiere para la inducción rápida y alta de los genes de protección frente al estrés osmótico. En segundo lugar, Cbc1 media el reclutamiento del complejo activador de la transcripción Hot1-Hog1 a los promotores de estrés, a través de una interacción con Hog1, que a su vez regula la formación del complejo de pre-iniciación (PIC, por pre-initiation complex) y el reclutamiento de la RNAPII en los mismos genes. En tercer lugar, la función transcripcional de Cbc1 se extiende también a la regulación de la expresión génica de las proteínas ribosomales (RP), tanto durante la recuperación de la transcripción de los genes RP durante el estrés osmótico como para conseguir una alta transcripción en condiciones normales, donde Cbc1 modula el reclutamiento de Rap1 a los promotores RP y por lo tanto induce la formación del PIC. En cuarto lugar, y utilizando los datos genómicos, Cbc1 parece contribuir específicamente a la transcripción de genes altamente expresados, en contraste con genes con bajos niveles de expresión. Por último, nuestros resultados también apuntan a un posible papel de Cbc1 en la regulación del apagado de la señalización de estrés así como en la regulación de la estabilidad de los factores de transcripción.

En respuesta a feromonas del tipo sexual contrario, la ruta de MAPK de feromonas de células de levadura haploides se activa para mediar una serie de cambios fisiológicos en preparación para el apareamiento, que incluyen la reprogramación de la expresión génica, la parada del ciclo celular, la formación de una proyección sexual denomina ¨shmoo¨, y en última instancia la fusión celular de los tipos de células de apareamiento contrario. El factor de traducción eIF5A, esencial y conservado evolutivamente, se ha descrito recientemente que funciona en la traducción de proteínas que contienen tres o más residuos de prolina consecutivos (polyPro), a través de su unión a ribosomas para aliviar el estancamiento del ribosoma durante la formación del enlace péptidico entre prolinas. La activación de eIF5A requiere una modificación post-traduccional única, la hipusinación, donde el residuo de hipusina se deriva de la espermidina, un factor esencial para la fertilidad de los mamíferos y que se requiere para el apareamiento de la levadura. Aquí se investigó eIF5A como regulador de la respuesta a feromonas a través de la traducción de proteínas polyPro con funciones en el apareamiento. En esta tesis, en primer lugar se demostró que en respuesta a feromonas se requiere eIF5A hipusinado para la formación del shmoo, la localización de componentes del polarisoma, la inducción de proteínas de fusión celular y para la nucleación de los cables de actina durante el crecimiento polarizado. También mostramos que eIF5A se requiere para la traducción de Bni1, una formina rica en prolinas implicada en la polimerización de actina durante la formación del shmoo. Nuestros datos indican que la traducción de los motivos polyPro de Bni1 es eIF5A-

dependiente y esta dependencia se pierde cuando se delecionan las poliprolinas. Además, un aumento en los niveles de proteína Bni1 exógena restaura parcialmente el defecto en la formación del shmoo visto en mutantes de eIF5A.

En conclusión, nuestro trabajo ofrece varias observaciones interesantes sobre la respuesta celular de la levadura a estímulos externos. En respuesta a estrés osmótico, hemos caracterizado los perfiles de interacción de proteínas de varios componentes de señalización de la vía de HOG y proporcionado más conocimiento sobre cómo se transduce con rapidez y eficacia la señal. Por otra parte, hemos identificado Cbc1 como un regulador transcripcional de la respuesta al estrés osmótico que permite expresiones altas y rápidas de los genes implicados en la supervivencia al estrés a través del reclutamiento de los factores transcriptionales específicos y la consiguiente formación del PIC. Además, Cbc1 también es necesario para mediar niveles de expresión génica altos en condiciones normales sin estrés. Por último, nuestros resultados identifican eIF5A como un nuevo regulador esencial del apareamiento de levadura mediante la traducción de las forminas. Dado que eIF5A y las forminas ricas en polyPro se conservan a través de las especies, nuestros resultados también sugieren que la traducción de las forminas, dependiente de eIF5A, podría regular el crecimiento polarizado en procesos tales como la fertilidad y el cáncer en eucariotas superiores.

Contents

Glossary

aa – Amino acid

ABA – Abscisic acid

Ac – Acetylation

ATP – Adenosine triphosphate

bp – Base pair

CBC – Cap-binding complex

CDK – Cyclin-dependent kinase

ChIP – Chromatin immunoprecipitation

CK2 – Casein kinase II

CPE – Core promoter element

CPSF – Cleavage and polyadenylation specificity factor

CR – Conserved region

CstF – Cleavage stimulatory factor

CTD – Carboxy-terminal domain of RNA polymerase II

CTIF – CBC-dependent translation initiation factor

DHS – Deoxyhypusine synthase

DOHH – deoxyhypusine hydroxylase

eEF – Eukaryotic elongation factor

eIF – Eukaryotic initiation factor

FG – Filamentous growth pathway

FH – Formin homology domain

G_1 phase – Growth 1 phase of cell cycle

GDP – Guanosine diphosphate

GEF – Guanine nucleotide exchange factor

GMP – Guanine monophosphate

GO – Gene ontology

GRO – Genomic Run-on

GTF – General transcription factor

GTP – Guanosine triphosphate

H – Histone

HMH – Hkr1-Msb2 homology domain

HOG – High osmolarity glycerol pathway

IRES – Internal ribosome entry site

K – Lysine

kDa – Kilodalton

Lys – Lysine

m^7GpppN – N^7-methyl guanosine mRNA cap

MAPK – Mitogen-activated protein kinase

MAPKK – MAPK kinase

MAPKKK – MAPK kinase kinase

MAT – Mating-type locus of *Saccharomyces cerevisiae*

me – Methylation

Met – Methionine

min – Minutes

miRNA – MicroRNA

Mot1 – Modifier of transcription 1

mRNA – Messenger RNA

mRNP – Messenger ribonucleoprotein complex

NMD – Nonsense-mediated decay

NPC – Nuclear pore complex

N-terminus – Amino-terminus

OD – Optic density

ORF – Open reading frame

PABP – Poly(A)-binding protein

P-body – Processing body

PCR – Polymerase chain reaction

PHAX – Phosphorylated adaptor of RNA export

PIC – Pre-initiation complex

PKA –Protein kinase A pathway

poly(A) – Polyadenosine tail

polyPro – Poly-Proline residues

Pro – Proline

p-value – Significance level of a statistical test to reject the null hypothesis

RA – mRNA amount

RA – Ras association domain

Rap1 – Repressor activator protein 1

RNAP – RNA polymerase

RP – Ribosomal protein

rRNA – Ribosomal RNA

RT-qPCR – Reverse transcription real-time PCR

S phase – Synthesis phase of cell cycle

SAM – Steril-α domain

Ser – Serine

SG – Stress granule

SH3 – SRC homology 3 domain

snRNA – Small nuclear RNA

STRE – Stress response element

TAF – TATA-associated factors

TBP – TATA-binding protein

TF – Transcription factor

TM – Transmembrane

TOR – Target of rapamycin pathway

TR – Transcription rate

tRNA – Transfer RNA

TSS – Transcription start site

UAS – Upstream activation sequence

URS – Upstream repression sequence

UTR – Untranslated region

Chapter 1

Introduction

1.1 *Saccharomyces cerevisiae* as a model organism

The use of model organisms for research to gain better understanding of particular biological phenomena is a hallmark of scientific endeavour. The attractiveness of the application of such organisms stem from several reasons, including: facilitating the optimisation of techniques; providing insights into the biological functions of other organisms; and overcoming ethical or experimental constraints associated with more complex organisms (Fields and Johnston, 2005). To date, the extensive list of model organisms studied ranges from the single-celled prokaryotic *Escherichia coli* and single-celled eukaryotic yeasts to more complex eukaryotic organisms such as the roundworm *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster* and the vertebrate mouse *Mus musculus* (Nussbaum-Krammer and Morimoto, 2014, Guisbert *et al.*, 2008, Abmayr and Keller, 1998). Several yeasts, including the fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae* comprise some of the most studied model organisms today, largely due to their rapid growths and that many of their biological processes are regulated by proteins that have homologs in humans (Botstein *et al.*, 1997).

S. cerevisiae, also known as the baker´s yeast or the budding yeast, is eukaryotic, unicellular, spore-forming budding yeast belonging to the phylum *Ascomycota*. Believed to be originally isolated from the skin of grapes, it is widely used for fermentation in such alimentation processes as bread baking and alcohol brewing (Maraz, 1999). Due to its fermentative properties, early geneticists utilised the budding yeast to study underlying mechanisms of metabolism, and simultaneously developed many genetic methodologies that were applicable to a wide variety of biological process (Mell, 2002). In 1996, the entire genome of *S. cerevisae*, containing more than 6000 genes on 16 chromosomes, was sequenced (Cherry *et al.*, 1998). Surprisingly, around 30 per cent of the genes implicated in human diseases have functional homologs in yeasts. Yeast genes have very few introns and the intergenic regions are relatively short, which facilitates the analyses of gene function (Cherry *et al.*, 1998). Overall, in modern day research, *S. cerevisiae* is utilised as a fundamental tool to study a variety of processes: from gene expression to protein interactions in such biological systems as yeast mating and responses to environmental stresses.

In this thesis, we utilise *S. cerevisiae* as a model organism to study a variety of molecular mechanisms, including signal propagation and changes in gene and protein expressions, employed by the cell in response to environmental stimuli.

1.1.1 Life cycle of *S. cerevisiae*

The life cycle of *S. cerevisiae* can be both asexual and sexual. A haploid yeast cell can reproduce by simple mitosis, termed ¨budding¨, where a progenitor or ¨mother¨ cell pinches off a portion of itself to yield a genetically identical progeny or ¨daughter¨ cell (Zorgo *et al.*, 2013). Haploid yeast cells can be one of two mating types: a or α, in which the mating type is encoded by two different alleles of the mating-type (*MAT*) locus, *MATa* and *MATα*, located on chromosome III, and the definitive mating type of the cell is dependent on which allele is actively transcribed (Haber, 2012). Non-laboratory yeast cells are able to switch between the two mating types in alternating mitotic divisions, where the mother cell switches during G1 after budding. This process is mediated by the enzyme HO endonuclease, where it cleaves the genetic information of a or α from the *MAT* locus, leaving only DNA coding for the opposite mating type to the daughter cell (Mell, 2002, Haber, 2012). Most laboratory yeast strains harbour a mutation in the HO endonuclease in order to obtain stable cell cultures, and these strains are termed *MATa* and *MATα*.

MATa and *MATα* cells release pheromones a- and α-factor respectively, which bind to either Ste3 receptor (in *MATα* cells) or Ste2 receptor (in *MATa* cells) expressed in cells of the opposite mating type (see Figure 1.1) (Bardwell, 2005).When cells of opposite mating types are in close proximity, pheromones produced by the other mating type bind to its receptor, and this initiates a biological process called ¨mating¨ (see Figure 1.1). This yields transient cell cycle arrest and the formation of a projection called the ¨shmoo¨ towards the pheromone gradient, which results in cell fusion to form a diploid. The resulting diploid cell (*MATa/α*) contains two of each chromosome and can undergo mitotic reproduction (see section 1.4 and Figure 1.1) (Herskowitz, 1988). However, during nutrient deprivation, the diploid cell is able to reproduce sexually by meiosis through the formation of spores, which, upon the reintroduction of nutrients, will germinate to yield four haploid cells (Figure 1.1) (Herskowitz, 1988).

Although termed the ¨budding¨ yeast, both haploid and diploid strains of *S. cerevisiae* can undergo another form of cellular growth: filamentous growth. The filamentous growth pathway is activated upon nitrogen starvation and stresses, leading to the formation of pseudohyphaes, which are chains of elongated cells formed by unipolar budding where the buds do not separate (Gancedo, 2001). Pseudohyphaes are able to penetrate agar in the laboratory, and this ability has been hypothesised to be important for yeasts in the wild to facilitate foraging for scarce nutrients or escape from harmful environments (Gancedo, 2001).

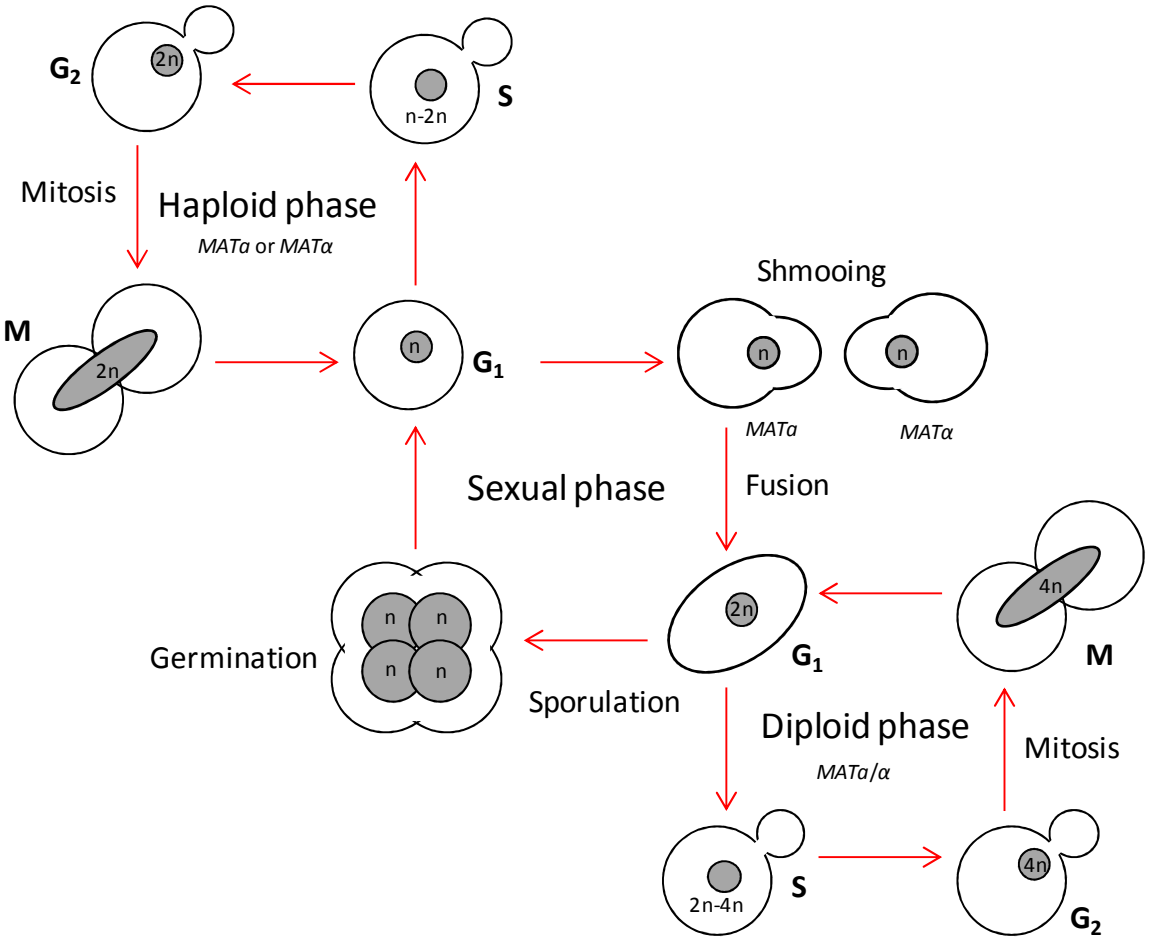


Figure 1.1 Schematic representation of the *S. cerevisiae* sexual and asexual life cycle. Yeasts undergo both mitotic and meiotic cellular divisions. Under nutrient-rich conditions, cells, which can be *MATa* or *MATα*, undergo mitotic divisions, where a haploid cell form a bud upon entering S phase followed by migration of the nucleus, and finally the complete formation of a daughter cell with identical genetic material as the mother cell. During the sexual phase, cells of opposite mating types secrete pheromones that arrest each other at G1 phase. Cells elongate towards the pheromone gradient to form a sexual projection termed shmoo, and eventually fuse to produce a diploid (*MATa/α*) containing genetic material from both cells. Under nutrient-rich conditions, the diploid cell undergoes mitotic divisions, and under nutrient starvation, the diploid cell sporulates to germinate four daughter cells. The annotated n indicates the number of genetic material the cell contains. Yeast mating type is denoted as *MATa* or *MATα* for haploid cells, and *MATa/α* for diploid cells.

1.1.2 Response to external signals

Yeast cells have evolved to efficiently detect and respond to external signals. In the wild, yeast cells must contend with fluctuations in temperature, acidity, osmolarity, oxygen and nutrient availability,

and the presence of mating pheromones. Growth under these varying conditions involves changes in cellular processes such as signalling, gene expression and protein interactions in order to mediate adaptation (Posas *et al.*, 1998a, Gasch *et al.*, 2000). Mechanisms employed by *S. cerevisae* in response to changes in the environment have been subjected to extensive research, as many of these processes are also shared in high eukaryotes (Chen and Thorner, 2007, Hohmann and Mager, 2003). Environmental stimuli, such as pheromones or osmotic stress, are detected by sensors or receptors located on the cell surface, which generate specific intracellular signals that are then amplified and transmitted to effector molecules. The propagation of signal is carried out by dedicated signal transduction pathways, and amongst the most prominent signalling pathways are the mitogen-activated protein kinase (MAPK) cascades [reviewed in (Hohmann and Mager, 2003, Saito and Posas, 2012, Hohmann, 2009)]. MAPK signalling modules are crucial for many aspects of cell physiology and are central elements of phosphorylation cascades that are evolutionarily conserved from single-celled yeast to vertebrates. Each MAPK module is composed of three sequentially activating kinases: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK, in which the MAPKKK can be activated either by phosphorylation by an upstream kinase or by the binding of an activator protein (see Figure 1.2) [reviewed in (Hohmann and Mager, 2003, Saito and Posas, 2012, Hohmann, 2009)].

In *S. cerevisiae*, five functionally distinct MAPK cascades have been identified to date, and these cascades function in the pheromone response, filamentous growth, HOG (**H**igh **O**smolarity **G**lycerol), cell integrity, and spore wall assembly pathways (Chen and Thorner, 2007, Furukawa and Hohmann, 2013, Saito, 2010). Several common signalling components are shared by two or more of these MAPK pathways, and, upon a particular external stimulus, various mechanisms are employed to ensure that, in addition to efficient activation of the corresponding pathway, there is no leakage of signal to incorrectly activate other pathways (Saito, 2010, Saito and Posas, 2012, O'Rourke and Herskowitz, 1998, Posas *et al.*, 1998a). Hence, the budding yeast *S. cerevisiae* serves as an excellent model to study cellular response to environmental stimuli, and in this thesis, we discuss the mechanisms by which these response pathways are activated and how they regulate downstream molecular and cellular processes. Briefly, in Chapter 3 we characterise the specific protein-protein interactions that are required for effective signal propagation through the HOG pathway, Chapter 4 describes the role of cap-binding protein Cbc1 in downstream gene expression during hyperosmotic shock, and Chapter 5 analyses the functional role of the translation factor eIF5A in yeast mating during pheromone response.

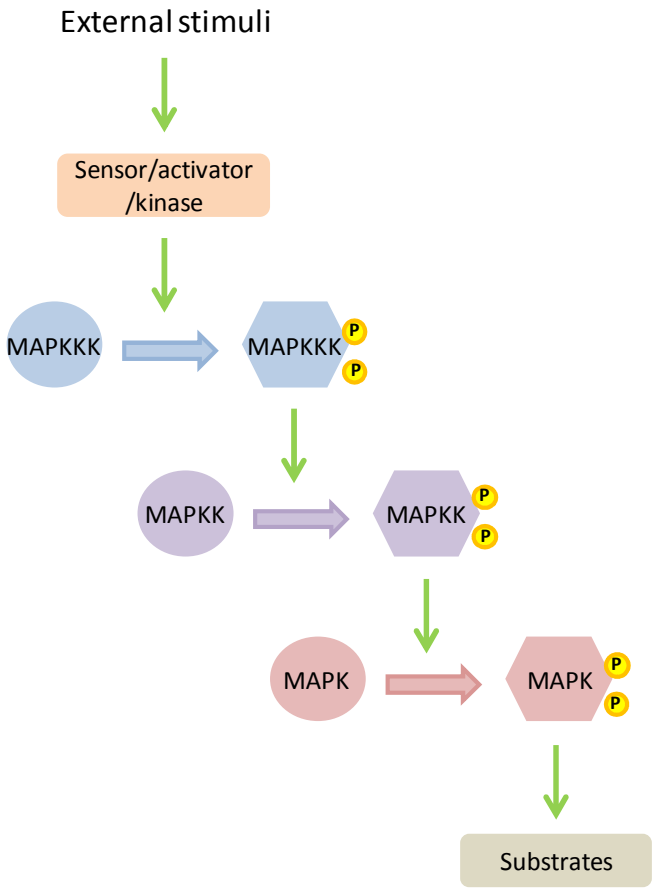


Figure 1.2 Schematic representation of a MAPK signalling module (modified from Saito and Posas, 2012). Circles and hexagons represent inactive and active forms of kinases respectively. Yellow circles with annotated P represent phosphorylation residues. Green arrows indicate sequential activation of MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), MAP kinase (MAPK), and downstream substrates (Saito and Posas, 2012).

1.2 Regulation of gene expression in *S. cerevisiae*

Regulation of gene expression is fundamental in many biological processes, including cellular growth, asexual and sexual reproduction, response to environmental changes, and disease. Control of the information flow from DNA to functional proteins encompasses many stages, including regulation of transcription, post-transcriptional processing and export of mRNA, and translation.

1.2.1 RNAPII transcription cycle

Transcription of DNA is the first stage of many in the process of gene expression, and it constitutes as the most important stage that is subjected to control to regulate gene expression (Jovanovic *et al.*, 2015, Battle *et al.*, 2015, Li *et al.*, 2014a). Many components of the transcriptional machinery are highly conserved amongst the eukaryotic kingdom, and in some cases are functionally interchangeable (Struhl, 1995). In eukaryotes, transcription is carried out by three distinct RNA polymerases (RNAP): RNAPI transcribes the large ribosomal RNA (rRNA) precursor (Zylber and Penman, 1971), RNAPII transcribes protein-coding messenger RNAs (mRNAs) (Zylber and Penman, 1971), most small nuclear RNA (snRNA) and microRNA (Sims *et al.*, 2004), and RNAPIII transcribes transfer RNAs (tRNAs) and the 5s rRNA (Weinmann and Roeder, 1974). The three RNA polymerases are structurally similar and share five of the ten core subunits; however RNAPII contains a very distinct C-terminal domain (CTD) on its large subunit Rpb1, which is subjected to post-translational modifications including phosphorylation, glycosylation and isomerisation, and this has been termed the ¨CTD code¨ [reviewed in (Egloff and Murphy, 2008, Liu *et al.*, 2013)]. Post-translational modifications of the CTD play an essential role in dictating the coordinated binding of different proteins to impose order on the transcription cycle of initiation, elongation and termination, as well as presenting a dynamic surface to proteins that carry out co-transcriptional mRNA processing and chromatin modifications (Kim *et al.*, 2010, Buratowski, 2003, Zhang *et al.*, 2012). Phosphorylations of residues Ser2, Ser5 and Ser7 of the CTD are the best-characterised CTD modifications, and are principally catalysed by members of the family of cyclin-dependent kinases (CDK). In *S. cerevisiae*, at least four CDK complexes are found to phosphorylate the CTD: Kin28/Ccl1/Tfb3, Srb10/Srb11, Ctk1/Ctk2/Ctk3, and Bur1/Bur2, and the delicate coordination between phosphorylation by kinases and dephosphorylation by phosphatases of these CTD serine residues dictate the different stages of the transcription cycle, as well as influencing transcription regulation [reviewed in (Egloff and Murphy, 2008, Phatnani and Greenleaf, 2006)].

Transcription by RNAPII is a precisely timed event that is tightly regulated at many steps, and transcriptional regulation becomes fundamental in the maintenance of cellular homeostasis. The RNAPII transcription cycle initiates with the recognition of upstream activation sequences (UASs) by activated transcription factors, which then serves as a nucleation point for co-regulators and general transcription factors (GTFs) to mediate the assembly of the pre-initiation complex (PIC), containing RNAPII, at the promoters (see Figure 1.3). Conversely, transcription repression initiates with the recognition of upstream repression sequences (URSs) by transcription repressors (Struhl, 1995, Hahn and Young, 2011).

Generally, genes are categorised based on the presence of specific DNA sequences in its promoter termed core promoter elements (CPEs), which is located 50-120 bp upstream of the transcription start site (TSS) in yeasts (Zhang and Dietrich, 2005). CPEs can be divided into two main groups: promoters that contain a canonical TATA box sequence TATAWAWR (W is A/T, R is A/G) and promoters that contain up to two mismatches to the TATA box sequence that is termed ¨TATA-like¨ (Rhee and Pugh, 2012, Basehoar *et al.*, 2004, Zhang and Dietrich, 2005). Genome-wide analyses revealed that only 10-20 % of total yeast promoters contain the canonical TATA box sequence, and these genes are highly regulated and many of them are implicated in the stress response (Huisinga and Pugh, 2004, Basehoar *et al.*, 2004). In contrast, majority of yeast promoters (~90 %) are TATA-like and more constitutively active (Bryant and Ptashne, 2003). Differences in the two groups of promoters dictate PIC assembly via two distinct and independent pathways: either via the SAGA coactivator complex which recognises the canonical TATA sequence or via the TFIID coactivator complex which recognise TATA-like promoters (Qiu *et al.*, 2004, Bryant and Ptashne, 2003, Grunberg and Hahn, 2013). Although mechanistically independent, the SAGA and TFIID complexes share several common TBP-associated factors (TAFs); however, each complex also contain distinct subunits, including Gcn5 in SAGA and TAF145 in TFIID, which dictate their promoter specificity (Han *et al.*, 2014, Lee *et al.*, 2000, Grunberg and Hahn, 2013). The binding of coactivators mediates the recruitment of the GTF TATA-binding protein (TBP) to all yeast promoters, which exist in the TFIID complex or can be recruited independently (Rhee and Pugh, 2012). TBP recruitment is crucial for PIC formation and therefore it is tightly regulated, either positively through activators during transcriptional activation, or negatively through repressors to decrease TBP-DNA binding ability, and many activators and repressors have multiple functions that are gene-specific (Kuras and Struhl, 1999, Cang *et al.*, 1999, Chymkowitch *et al.*, 2015, Moyle-Heyrman *et al.*, 2012).

The GTFs TFIIA and TFIIB are then recruited to assist and stabilise TBP binding to the DNA, and in turn, TFIIB interacts with and recruits RNAPII, bound to TFIIF (Imbalzano *et al.*, 1994, Buratowski and Zhou, 1993, Barberis *et al.*, 1993, Conaway *et al.*, 1991, Fishburn and Hahn, 2012). TFIIE and TFIIH join the emerging PIC and mediate promoter opening to create a single-stranded region, allowing access of RNAPII to the template strand (Conaway *et al.*, 1991, Fishburn and Hahn, 2012). The Mediator is a large multiprotein complex that is subsequently recruited to the PIC, and it interacts with the CTD of RNAPII, effectively forming a bridge between the general transcription machinery and upstream transcription factors (TFs) (see Figure 1.3) (Hahn and Young, 2011, Bjorklund and Gustafsson, 2005, Reeves and Hahn, 2003).

The organisation of eukaryotic chromatin poses a significant obstacle for the binding of TFs and PIC components to their cognate sequences. During transcription initiation, histone modifying enzymes, such as acetyltransferases and methyltransferease are recruited by the gene-specific activator to the chromatin, and there, they work in combination with nucleosome remodelers to reorganise the chromatin architecture to allow efficient and adequate assembly of the PIC (see Figure 1.3) (Venters and Pugh, 2009, Shandilya and Roberts, 2012).

Once bound to the open promoter, RNAPII scans downstream sequences for a suitable TSS, and once found, the CTD is phosphorylated at Ser5 by the TFIIH-associated cyclin-dependent kinase Cdk7 (Kin28 in yeasts), which mediates the dissociation of RNAPII from PIC to enhance promoter escape (Figure 1.3) (Hahn and Young, 2011, Kim *et al.*, 2010). Initially, abortive transcription of short RNAs occurs until the transcription of 12-13 bp of nascent RNA, which clashes and releases TFIIB from RNAPII, enabling the formation of a stable elongation complex (Sainsbury *et al.*, 2013). Meanwhile, Ser5 phosphorylation leads to the recruitment of both Set1, which trimethylates histone H3 at lysine 4 (H3K4), and SAGA, which acetylates the N-terminal tails of histones H3 and H4, and together, they give rise to the histone modification marks of active transcription (Figure 1.3) (Kuo *et al.*, 1996, Venters and Pugh, 2009, Ng *et al.*, 2003). Importantly, Ser5 phosphorylation mediates the recruitment of the Ceg1 and Abd1 subunits of the mRNA capping enzyme complex, which leads to the attachment of the N^7-methyl guanosine cap structure to the 5´ end of the nascent RNA (Schroeder *et al.*, 2000, Cho *et al.*, 1997). The cap structure, bound by the cap-binding complex (CBC), protects the nascent RNA from degradation, and marks the mRNA for nuclear export to be ultimately translated in the cytoplasm (Proudfoot *et al.*, 2002, Fortes *et al.*, 2000).

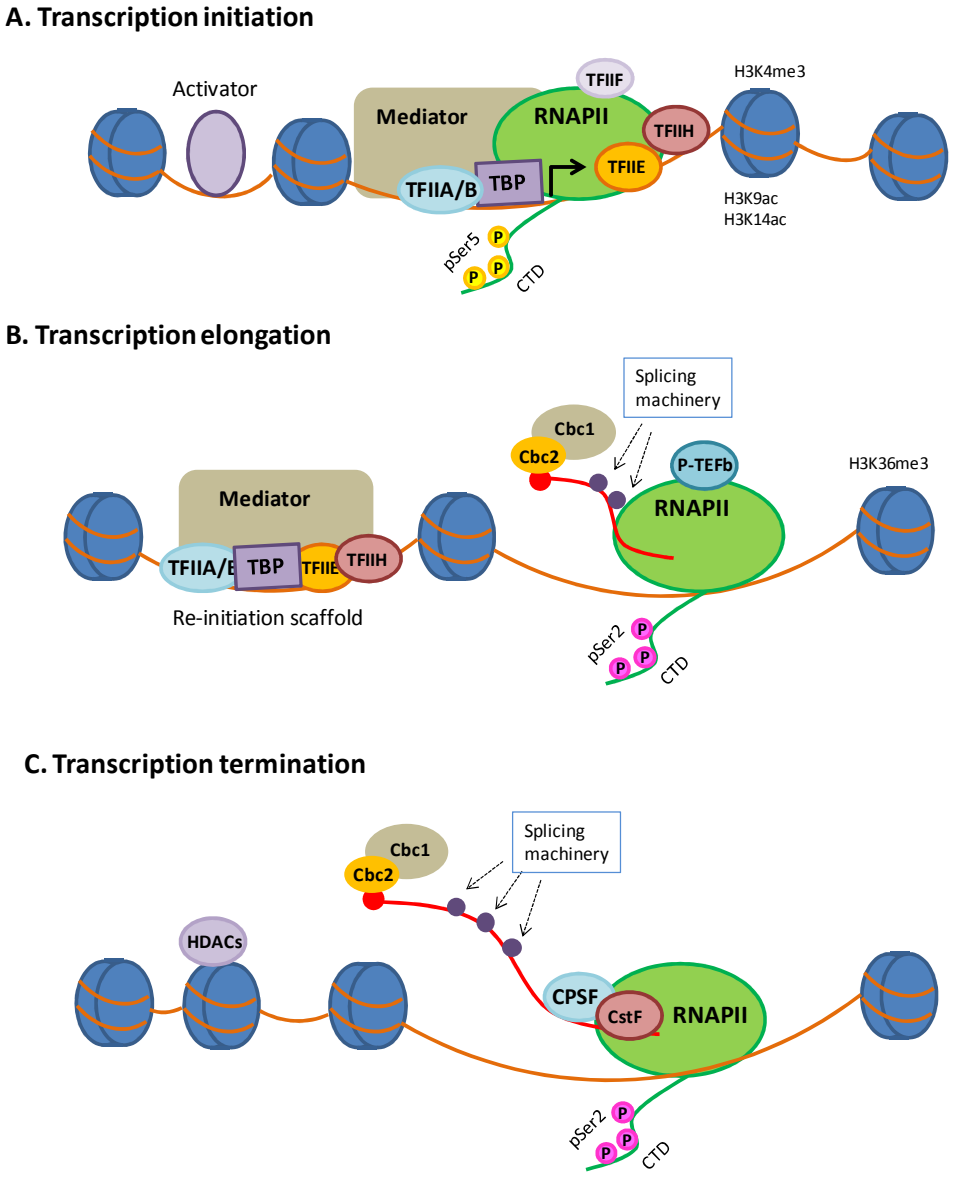


Figure 1.3 Transcription cycle (modified from Shandilya and Roberts, 2012). (A) Transcription initiates with the binding of an activator to the promoter, which promotes the binding of TBP along with the general transcription factors (GTFs) TFIIA, TFIIB, TFIIE and TFIIH, the mediator complex, and RNAPII bound to TFIIF. C-terminal domain (CTD) of RNAPII is phosphorylated at Ser5, which recruits histone remodellers to catalyse H3K4 methylation and H3 acetylation. **(B)** Following promoter clearance, RNAPII proceeds to elongate the transcript (red line) aided by P-TEFb, while a part of the PIC components remains associated at the promoter to form a reinitiation scaffold. The phosphorylation state of RNAPII CTD gradually changes from Ser5 to Ser2 phosphorylation during elongation, and Set2 is recruited to trimethylate H3K36. Splicing machinery and mRNA capping enzymes associate with the mRNA co-transcriptionally and mediate processing. The cap-binding complex (Cbc1/Cbc2) binds the cap during productive elongation. **(C)** Once RNAPII reaches the termination signal, 3´ end processing and termination-specific complexes such as CPSF and CstF are recruited. Histone deacetylases (HDACs) are also recruited to deacetylate histones. RNAPII dissociates from the gene and this marks the end of the transcription cycle (Shandilya and Roberts, 2012).

As RNAPII transcription proceeds towards the 3´ end of genes, Ser2-phosphorylated CTD recruits the 3´ end RNA processing machinery, including cleavage and polyadenylation specificity factor (CPSF), cleavage stimulatory factor (CstF) and Poly (A) polymerase, to cleave the newly synthesised RNA and to add a polyadenosine (poly(A)) tail to the 3´ end of the transcript (Figure 1.3). Upon completion of mRNA synthesis (transcription termination), RNAPII becomes dephosphorylated and dissociates from the gene (Grzechnik *et al.*, 2014, Kuehner *et al.*, 2011, Mischo and Proudfoot, 2013). The released, hypo-phosphorylated RNAPII is competent to initiate a new round of transcription. It has been previously described that following promoter clearance, some of the GTFs remain at the promoter to form the reinitiation scaffold that allows reinitiation by RNAPII during successive rounds of transcription, and therefore enabling RNAPII recycling events (Shandilya and Roberts, 2012). In yeast, TFIIB, a GTF that forms part of the PIC, is found to interact with CPSF and CstF complexes in order to contribute to the juxtaposition of promoter and terminator DNA through a process termed ¨gene looping¨ (Hampsey *et al.*, 2011, Kuehner *et al.*, 2011). This process aids the close contact between a terminating RNAPII and the promoter, hence enables RNAPII recycling and the reinitation of a new transcription cycle.

1.2.2 Transcription factors

All stages of the transcription cycle are tightly regulated, and this regulation initiates with the recruitment of transcription factors (TFs) to the gene promoters. In *S. cerevisiae*, a wide variety of transcription activators exists to specifically recognise and bind upstream promoter elements to promote transcription (Struhl, 1995). Most transcription activators are present at low intracellular concentrations and interact with relatively few promoters. Furthermore, some activators function only in a specific cellular process, such as the osmotic stress-specific TF Hot1 which will be discussed in detail in 1.3.3, whereas others are more abundant and have more general activities, such as Rap1 (Struhl, 1995, Gomar-Alba *et al.*, 2013, Knight *et al.*, 2014). Generally, transcription activators enhance transcription by direct contact with the basal transcription machinery or by interaction with chromatin modification complexes, which results in enhanced PIC formation (Hahn and Young, 2011). However, many transcription activators require the activity of co-activators, which are proteins that do not directly bind promoter elements and are not part of the basal transcription machinery but have a specific effect on transcription. In theory, co-activators can stimulate transcription by modulating the activities of GTFs and/or RNAPII, or by altering chromatin structure (Struhl, 1995, Hahn and Young, 2011, Danino *et al.*, 2015).

In contrast, transcription repressors regulate transcription by the prevention of the general transcription machinery to initiate transcription, and their activities initiate with the recognition and binding to specific DNA sequences in the gene promoter. Repressors can compete with activators by binding to overlapping sites and block transcription initiation by steric hindrance, or they can modify chromatin by such mechanisms as nucleosome coating of DNA to restrict access of the transcription machinery (Struhl, 1995, Gaston and Jayaraman, 2003, Thiel *et al.*, 2004). Moreover, many TFs have been described both as transcription activator and repressor with gene-specific activities, and in this thesis we have studied some of these TFs, including Rap1 and Mot1.

In *S. cerevisiae*, Rap1 (**R**epressor **A**ctivator **P**rotein 1), with a human homolog hRap1, is a key multifunctional regulator of transcription and chromosomal integrity and has been described to bind approximately 5 % of all yeast genes and participates in the activation of a remarkable ~40 % of total downstream mRNA transcripts (Lieb *et al.*, 2001). Rap1 has been shown to directly interact with TFIID and has been proposed to bind ~90 % of all yeast ribosomal protein (RP) gene promoters (Knight *et al.*, 2014, Lascaris *et al.*, 1999). Expression of RP genes are co-ordinately regulated in response to growth stimuli and environmental stress as RP gene transcription is very costly given that it occupies approximately 10 % of RNAPII activity (Perez-Ortin *et al.*, 2011), and in response to stress, such as amino acid starvation and high osmolarity, RP genes are rapidly down-regulated (Wade *et al.*, 2004, Warner, 1999, Canadell *et al.*, 2015). Rap1 plays a crucial role in the global transcriptional regulation of RP genes through its association with TFs Hmo1 and Fhl1/Ifh1 to enhance PIC formation and transcription initiation, and has been described to bind RP promoter 150bp to 400bp upstream of transcription start site (TSS) (Knight *et al.*, 2014). In Chapter 5 of this thesis, we study the transcriptional regulation of RP genes during osmotic stress and under normal conditions utilising both genomic and molecular tools.

Mot1 (**M**odifier **o**f **T**ranscription 1), the yeast homolog of the human BTAF1, is an essential yeast protein that is part of the evolutionarily conserved family of enzymes Snf2/Swi2 ATPases, whose members use ATP hydrolysis to alter protein-DNA interactions (Viswanathan and Auble, 2011, Chicca *et al.*, 1998, Auble *et al.*, 1994, Davis *et al.*, 1992). The molecular function of Mot1 is complex as it has been described that Mot1 acts both as a repressor and an activator of transcription through its function to displace TBP from DNA using ATP hydrolysis, where Mot1-mediated displacement of TBP from DNA both increases and decreases gene expression (Viswanathan and Auble, 2011, Pereira *et al.*, 2003, Geisberg and Struhl, 2004, Venters *et al.*, 2011, Zentner and Henikoff, 2013). Furthermore, depletion of Mot1 has also been observed to reduce TBP association with some promoters (Choukrallah *et al.*, 2012, Venters *et al.*, 2011, Zentner and Henikoff, 2013). A recent study by

Zentner *et al.* has elucidated that Mot1 functions to displace TBP from TATA-containing genes, through synergic interactions with SAGA, and redistributes TBP to stably associate with TATA-like promoters (Zentner and Henikoff, 2013). Furthermore, Mot1 has been found to occupy stress promoter with RNAPII only under stress conditions (Geisberg and Struhl, 2004). Collectively, all these results seem to suggest that Mot1 drives transcription of RP and stress genes under normal and stress conditions, respectively, and in contrast, is able to act as a repressor of stress genes under non-stress conditions.

1.2.3 The mRNA life

During and after synthesis, mRNA undergoes several stages of processing in which at each stage, specific proteins associate with the mRNA to form distinct and dynamic ribonucleoprotein (mRNP) complexes. mRNA processing initiates co-transcriptionally in the nucleus with capping, splicing, and polyadenylation. mRNA can also be targeted for nuclear degradation. Once exported from the nucleus to the cytoplasm, the mRNA can undergo translation, stored at specialised cytoplasmic foci, including P-bodies and stress granules, or targeted for decapping and cytoplasmic degradation (see Figure 1.4). Regulation of gene expression can be realised by regulating mRNA fate, in which each of these processes are subjected to elaborate control.

During nascent RNA synthesis, the N^7-methyl guanosine (m^7GpppN) cap structure is attached to the 5′ end of the nascent RNA when it is ~25 nucleotides in length, and this is a signal for productive transcription (Shandilya and Roberts, 2012). The capping event occurs in three steps: first, the 5′-triphosphate of the newly synthesised mRNA is hydrolysed by a triphosphatase, encoded by *CET1* in *S. cerevisiae*, to produce a diphosphate terminus; second, the diphosphate end of the nascent mRNA is capped with guanine monophosphate (GMP) via 5′-5′ triphosphate linkage, catalysed by a guanylyltransferase, which is encoded by *CEG1*; finally, a methyl group is transferred to the cap by a methyltransferase encoded by *ABD1*, thus generating the m^7GpppN cap [reviewed by (Cowling, 2010)]. The capping enzymes are some of the first processing factors to act on the transcript during RNAPII transcription, and their recruitment to the mRNA is mediated by interactions with Ser5-phosphorylated CTD, however they have also been described to bind Ser2-phosphorylated CTD (Figure 1.4) (Cho *et al.*, 1997, McCracken *et al.*, 1997, Yue *et al.*, 1997, Ursic *et al.*, 2008). CTD-mediated recruitment of capping enzymes may explain why only transcripts transcribed by RNAPII, and not RNAPI or RNAPIII, become capped. Furthermore, the relationship between capping enzymes and RNAPII has been described as reciprocal, where yeast triphosphatase Cet1 has been found to

decrease the accumulation of RNAPII towards the 5′ end of the coding sequence to enhance transcription, and the methyltransferase Abd1 is found to contribute to the recruitment of CTD kinases Bur1 and Ctk1 to mediate Ser2-phosphorylation of RNAPII during transcription elongation (Lahudkar *et al.*, 2014, Lidschreiber *et al.*, 2013).

Once N^7-methyl guanosine cap is attached to the 5′ end of the newly synthesised mRNAs, the cap-binding complex (CBC), composed of Cbc1 and Cbc2 (Cbp80 and Cbp20, respectively, in humans), binds to the cap and protects the mRNA from exonucleases (Figure 1.4) (Gonatopoulos-Pournatzis and Cowling, 2014, Topisirovic *et al.*, 2011). It has been suggested that the capping process acts as a check-point for transcription elongation, where failure will lead to termination of RNAPII transcription and degradation of the nascent RNA by the nuclear exosome (Buratowski, 2009, Seila *et al.*, 2009). Later, the cap and the cap-binding complex serves as a binding site for proteins involved in the export of mature mRNA into the cytoplasm (Proudfoot *et al.*, 2002). Details of the role of CBC in the regulation of mRNA fate and gene expression are described in section 1.5. Once the mRNA exits the nucleus and enters the cytoplasm, CBC is replaced by the cap-binding complex eIF4F, in which the subunit eIF4E binds the cap, and, through its interaction with scaffold eIF4G, in turn recruits translation initiation factors to promote the engagement of ribosomal subunits with the mRNA (Figure 1.4) (Sachs *et al.*, 1997, Proudfoot *et al.*, 2002).

Control of pre-mRNA splicing is a critical part of the eukaryotic gene expression, and extensive studies indicate that transcription and splicing are spatiotemporally coordinated [reviewed in (Moehle *et al.*, 2014)]. Most splicing events occur co-transcriptionally, where pre-mRNAs are first committed to splicing by binding of splicing factors to 5′ and 3′ splice sites (Figure 1.4). In yeast, introns are spliced to generate the mature mRNA by the spliceosome, which consists of five small nuclear RNAs (snRNAs) (U1, U2, U4, U5 and U6) in association with proteins to form snRNA-protein (snRNP) complexes (Cramer *et al.*, 1997, Proudfoot *et al.*, 2002). The functional connection between RNAPII transcription and splicing are starting to be elucidated, where one study observed that splicing efficiency is sensitive to RNAPII elongation rate with an anti-correlated relationship (Pleiss *et al.*, 2007).

Once the stop codon is reached, the pre-mRNA is cleaved at a specific site by CstFs. The cleavage site is then bound by the protein complex CPSF which recruits poly(A) polymerase and together they direct the addition of ~200 adenosine residues to the 3′ end of the mRNA, termed the poly(A) tail (Figure 1.4) (Murthy and Manley, 1995, Mandel *et al.*, 2006). It has been established that 3′ end processing is absolutely required for transcription termination, where binding of CPSF and CstFs

displaces elongation factors and consequently rendering RNAPII less processive (Figure 1.4) (Buratowski, 2005, Proudfoot, 2004, Richard and Manley, 2009).

Following mRNA processing, mRNAs existing in correct mRNPs exit the nucleus through the nuclear pore complex (NPC) with the support of the TREX (**TR**anscription-**EX**port) multiprotein complex (Figure 1.4) [reviewed in (Nino *et al.*, 2013, Garcia-Oliver *et al.*, 2012)]. Furthermore, nuclear export factors are found to have roles in transcription, as the coactivator SAGA interacts directly with the TREX complex to promote transcriptional activation and to ensure efficient mRNA elongation and nuclear export through the NPC (Figure 1.4) (Rodriguez-Navarro *et al.*, 2004, Pascual-Garcia *et al.*, 2008, Faza *et al.*, 2009).

In the nucleus, defects in nuclear mRNA processing and in the formation of export-competent mRNA trigger nuclear mRNA degradation, which is predominatantly exercised by the nuclear exosome containing the evolutionary conserved 3′-5′ exonuclease Rrp6 (Figure 1.4) [reviewed in (Perez-Ortin *et al.*, 2013)]. In the cytoplasm, several decay pathways of mature mRNAs have been identified to date (Coller and Parker, 2004, Garneau *et al.*, 2007, Perez-Ortin *et al.*, 2013). Majority of mRNA decay is deadenylation-dependent and initiates with deadenylation to shorten the poly(A) tail, which is catalysed by deadenylases. A subset of mRNAs then undergo decapping to remove the m^7GpppN cap, mediated by decapping enzymes Dcp1 and Dcp2. Decapping stimulates the recruitment of the 5′-3′ exonuclease Xrn1 to degrade the RNA. mRNAs that do not undergo decapping are degraded by the exosome in the direction of 3′ to 5′, and the m^7GpppN cap on the remaining oligomer is metabolised by the scavenger decapping enzyme DcpS (Figure 1.4) [reviewed in (Garneau *et al.*, 2007, Coller and Parker, 2004, Perez-Ortin *et al.*, 2013)]. Mechanisms to detect and eliminate faulty transcripts also exist in the cytoplasm in order to preserve translational fidelity. The most well-known pathway, the nonsense-mediated decay (NMD), detects and degrades transcripts that contain premature termination codons to avoid translation of proteins with aberrant functions, although correct mRNAs can also be degraded via this pathway (Garre *et al.*, 2013). NMD is mainly mediated by the highly conserved factors Upf1-3, which triggers, predominantly, deadenylation-dependent decapping and 5′-3′ decay by Xrn1 (Lejeune *et al.*, 2003, Garneau *et al.*, 2007, Perez-Ortin *et al.*, 2013).

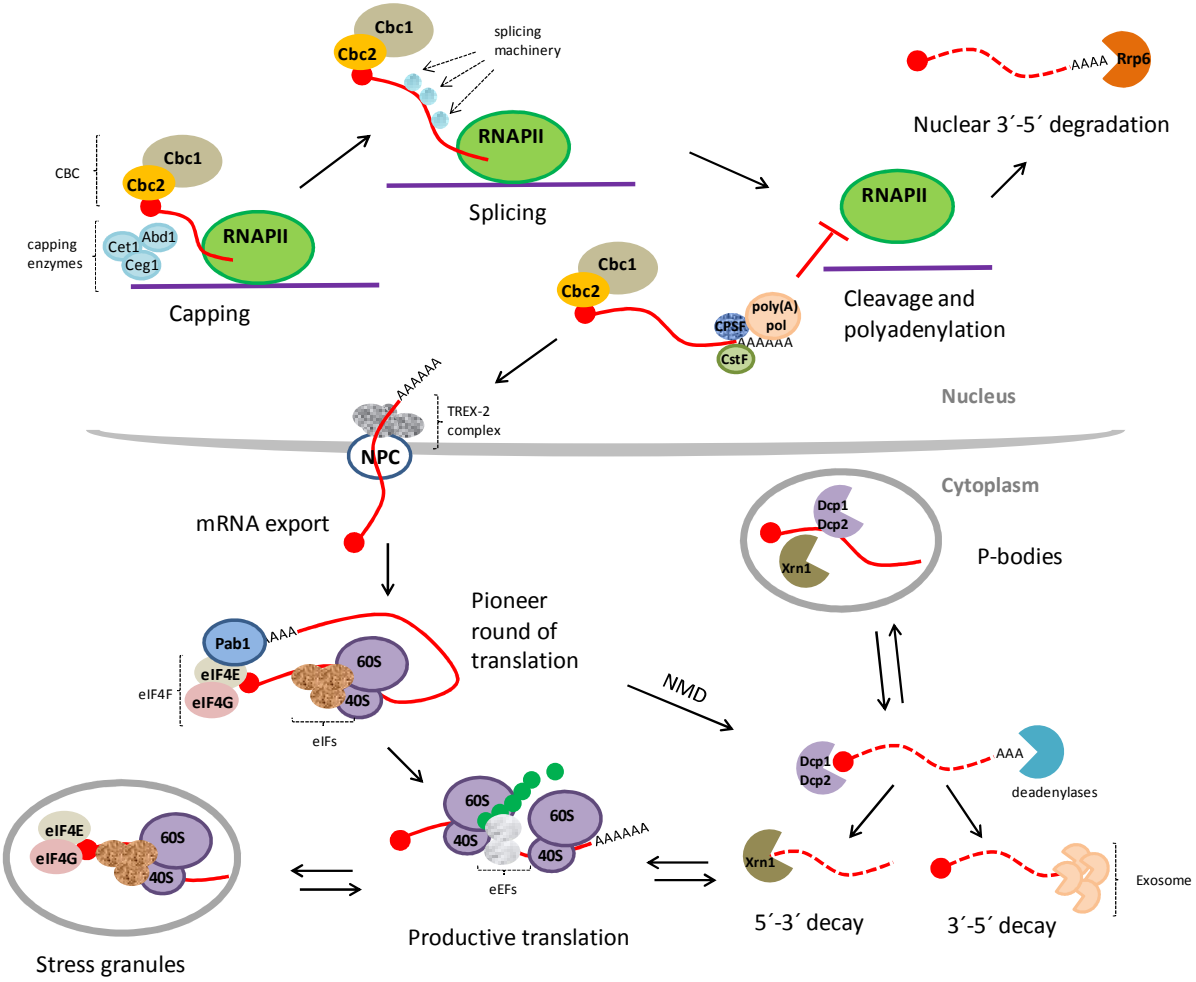


Figure 1.4 Overview of mRNA life in *S. cerevisiae*. In the nucleus, a 5´ cap is added to the mRNA and is bound by the cap-binding complex (CBC), Cbc1/Cbc2, co-transcriptionally. Introns of pre-mRNA are co-transcriptionally spliced by the spliceosome. Upon transcription termination, mRNA is cleaved by CstF and CPSF, which then recruit the poly(A) polymerase that adds the poly(A) tail. Nuclear degradation is exercised by the nuclear exosome containing the exonuclease Rrp6. Mature mRNA is exported through the nuclear pore complex (NPC) aided by the TREX complex. In the cytoplasm, the mRNA cap is bound by the eIF4F complex, eIF4E/eIF4G, and the poly(A) tail is bound by poly(A)-binding protein (PABP), which interacts with eIF4F. Translation initiates with the assembly of the 80S ribosome composed of 40S and 60S subunits, along with $tRNA_i^{Met}$ ternary complex and eIFs. To promote elongation, eIFs dissociate and eEFs bind to the ribosome. mRNA undergoes a pioneer round of translation to ensure correct protein synthesis, and aberrant mRNA is sent for degradation via the nonsense-mediated decay (NMD) pathway. Other cytoplasmic mRNA degradation pathways include deadenylation-dependent pathways, where deadenylated decapped mRNA is degraded from 5´ to 3´ by the Xrn1, and deadenylated capped mRNA is degraded from 3´ to 5´ by the exosome. Finally, mRNA and associated proteins can accumulate in specialised cytoplasmic foci, including stress granules and P-bodies (see text for details). Black solid arrows indicate sequential events in the mRNA life.

mRNP composition controls every aspect of mRNA life, from pre-mRNA processing to translation and mRNA turnover, and transition between these events is accompanied by major mRNP remodelling. (Erickson and Lykke-Andersen, 2011, Xing and Bassell, 2013, Decker and Parker, 2012). mRNPs can be packaged and stored in distinct classes of RNA granules that ensure regulated translation and decay at different phases of the mRNA life cycle (Anderson and Kedersha, 2009, Sunnerhagen, 2007). Two well-described and highly conserved RNA granules, P-bodies and stress granules (SGs), are both formed in response to conditions that result in translation repression, including many types of environmental stresses, however, they differ in their mRNP compositions. P-bodies, which are present in low numbers under normal conditions, contain translationally-repressed mRNA with various proteins of the mRNA decay machinery, whereas stress granules (SGs), which are only induced under stress conditions, contain mRNAs that are stalled at translation initiation, along with translation initiation factors and ribosomal subunits (see Figure 1.4) (Anderson and Kedersha, 2009, Olszewska *et al.*, 2012, Decker and Parker, 2012, Gimenez-Barcons and Diez, 2011, Sunnerhagen, 2007).

1.2.4 Translation

Regulation of translation plays an essential role in the mechanisms of controlling gene expression, as translational control of existing mRNAs allows for more rapid changes in cellular concentrations of proteins in order to modulate transient and permanent responses in cell physiology [reviewed in (Sonenberg and Hinnebusch, 2009)]. In eukaryotes, majority of mRNAs are translated in a cap-dependent manner, although a subset of mRNAs have been described to be translated via an internal ribosome entry site (IRES)-dependent manner (Sonenberg and Hinnebusch, 2009, Huch and Nissan, 2014, Topisirovic *et al.*, 2011). The translation initiation process starts with the assembly of the eIF4F complex on the cap structure, which consists of the initiation factors eIF4E (Cdc33 in yeast), eIF4G (Tif4631 and Tif4632) and eIF4A (Tif1 and Tif2). The 43S preinitiation complex, composed of the small 40S ribosomal subunit, the eIF2-GTP-methionyl-$tRNA_i^{Met}$ ternary complex and additional translation initiation factors eIF3, eIF1, eIF1A and eIF5, binds the eIF4F cap-binding complex at the 5´ cap of the mRNA, forming the 48S initiation complex. The 43S complex then scans the 5´ untranslated region (5´ UTR), inspecting successive triplets as they enter the P site, to locate the initiation codon. Upon the location of the start codon, hydrolysis of GTP-eIF2 occurs to yield eIF2-GDP-methionyl-$tRNA_i^{Met}$ ternary complex, which triggers the dissociation of other initiation factors, and at the same time, the 60S ribosomal subunit joins the 43S preinitiation complex to form

the 80S ribosome (see Figure 1.4) (Sonenberg and Hinnebusch, 2009, Huch and Nissan, 2014, Topisirovic *et al.*, 2011, Jackson *et al.*, 2010).

During translation elongation, cognate aminoacyl-tRNAs are delivered to the A site of the ribosome by eEF1A, and once codon/anti-codon match is detected, eEF1A is released from the ribosome. A peptide bond is made to elongate the growing polypeptide. eEF2 then catalyses the movement of peptidyl-tRNA-mRNA complex from the A site to P site of the ribosome, leaving the A site vacant to accept the next aminoacyl-tRNA and the process repeats (Figure 1.4) [reviewed in (Schmeing and Ramakrishnan, 2009, Mateyak and Kinzy, 2010)]. However, not all peptide bonds between the donor peptidyl-tRNA and the acceptor aminoacyl tRNA are formed with equal efficiency, as certain amino acids are poor donors or acceptors. In particular, proline is both a poor acceptor and a poor donor in the peptidyl transferase reaction, which causes ribosomes to stall when two or more consecutive prolines are encountered (Wohlgemuth *et al.*, 2008, Pavlov *et al.*, 2009).

More recently, eIF5A (detailed in section 1.6) has been identified as a translation elongation factor, as its depletion reduces ribosome transit time, amongst other phenotypes (Saini *et al.*, 2009, Gregio *et al.*, 2009, Henderson and Hershey, 2011). In Chapter 5 of this thesis, we will discuss in detail the molecular functions of eIF5A, as well as providing insights to its biological role.

Translational control has been described to occur at several stages in order to preserve translation fidelity. Mechanisms regulating initiation fall under two broad categories: regulation of eIFs or ribosomes, and regulation of the mRNA itself. First, several eIFs and eIF-binding proteins are subjected to direct phosphorylation which may increase or decrease translation efficiency (Ueda *et al.*, 2004, Jackson *et al.*, 2010, Dever *et al.*, 2007). An example for eIF phosphorylation-dependent translational control is the phosphorylation of yeast eIF2α by Gcn2 in yeast primarily during amino acid starvation, in which phosphorylated eIF2α inhibits eIF2B leading to reduced global translation (Castilho *et al.*, 2014). Second, RNA-binding proteins may also regulate translation, for example, poly(A)-binding protein (PABP) is essential for translation initiation as it interacts with the eIF4F complex via eIF4G subunit in a ¨closed loop¨ configuration (Kahvejian *et al.*, 2005, Kessler and Sachs, 1998, Imataka *et al.*, 1998). Finally, microRNAs (miRNAs), which are short oligonucleotides, are described to either directly inhibit translation or destabilise mRNA or both [reviewed in (Sonenberg and Hinnebusch, 2009)].

1.2.5 Coordination of mRNA metabolism

Although individual events of transcription, mRNA processing, decay and translation were discovered independently, emerging evidence has since established that these seemingly separate events closely influence one another, and many stages of gene expression are regulated by common factors which allow cross-talk to occur between processes. The complexity of cross-talk mechanisms utilised by the cell is beginning to be visualised, and their regulation forms a crucial aspect of gene expression control. In the nucleus, cross-talk between transcription and mRNA processing constitutes one of the most described communication networks. Firstly, the phosphorylation state of the CTD is crucial in regulating the association of mRNA processing factors to mRNA, for example, the switch from Ser5 to Ser2 phosphorylation serves as a key event in the recruitment of capping machinery during transcription elongation (Komarnitsky *et al.*, 2000, Cho *et al.*, 2001). Second, several factors of the transcription machinery also function in the correct recruitment and assembly of mRNA processing complex, for example the transcription elongation factor TAT-SF1 is able to directly recruit factors of the splicing machinery and regulate assembly of splicing complexes (Fong and Zhou, 2001, Kim *et al.*, 1999). Furthermore, several mRNA processing steps also loop back and regulate transcription, one such example consists of the splicing machinery, where snRNPs are able to interact with elongation factors to strongly stimulate RNAPII elongation (Fong and Zhou, 2001).

In the cytoplasm, cross-talk between translation and mRNA degradation constitutes a crucial aspect of gene expression regulation. It has always been evident that both the 5´ mRNA cap and the poly(A) tail play essential roles in mRNA fate, however it was later described that the poly(A) tail and Pab1, the poly(A) binding protein, serve to enhance translation and inhibit decay (Wilusz *et al.*, 2001, Mangus *et al.*, 2003). Furthermore, decay factors can assemble on mRNAs and mediate translational repression, for example proteins of the decapping complex Dhh1, Pat1 and Dcp1 have been shown to facilitate translational repression in response to various environmental stresses (Coller and Parker, 2005, Decourty *et al.*, 2008, Nissan *et al.*, 2010, Holmes *et al.*, 2004)

Cross-compartmental coordination occurs when a cross-talk factor has roles in more than one cellular compartment. Two of the most well-defined cross-compartmental coordinators are the RNAPII subunits Rbp4 and Rbp7, which are known to regulate all aspects of transcription, as well as being involved in several stages of post-transcriptional processes, including mRNA export, translation and mRNA decay (Edwards *et al.*, 1991, Lotan *et al.*, 2005, Farago *et al.*, 2003, Harel-Sharvit *et al.*, 2010, Dahan and Choder, 2013). Even more recently, a new group of coordinators termed synthegradases has been described to uniquely mediate cross-talk between transcription and decay. Recent studies have provided novel insights to the functions of the exoribonuclease Xrn1

in transcription, where the originally-described cytoplasmic decay factor is shown to bind transcribed genes in the nucleus and its deletion causes a marked decrease in the transcription of highly induced genes (Haimovich *et al.*, 2013, Medina *et al.*, 2014).

Chapter 4 of this thesis will provide insights to the relevance of the cap-binding protein Cbc1 as a cross-talk factor that functions not only in mRNA fate, but also regulates transcription.

1.3 Response to hyperosmotic stress

In their natural environment, *S. cerevisiae* thrive on decomposing fruits where sugar concentration, including glucose, fructose and sucrose, is high. At certain stages of decomposition, the concentration of sugars may bring unfavourable osmotic conditions, where increased external osmolarity induces water efflux, increases internal ion concentration and cell shrinkage, leading to cell growth arrest and cell death. Therefore, the solution to prosper in the changing osmotic environment is to maintain an appropriate cell volume and a favorable water and ion concentration to carry out biochemical reactions. To achieve successful adaption to unfavorable osmotic environment, yeast cells undergo a number of biochemical changes in various aspects of cellular physiology, including modifications of ion transporters, cytoskeletal reorganisation, cell-cycle arrest, metabolic adjustments, global translational changes, as well as profound effects on gene expression [reviewed in (Chen and Thorner, 2007, de Nadal and Posas, 2010, Hohmann *et al.*, 2007, Hohmann, 2009)]. The mechanisms of yeast osmo-adaption has become of great interest to various fields of biological studies for two main reasons: first, the key element responsible for osmotic stress response, the three-tiered cascade HOG MAPK pathway (refer to Figure 1.5), serves as a signal transduction module to provide general knowledge to the functions of all mitogen-activated protein kinase signalling pathways in diverse eukaryotic organisms; second, osmo-adaptation also plays an essential role in high eukaryotes in regards to cell growth and morphogenesis, and many mechanisms to respond to osmotic stress are conserved between yeast and humans [reviewed in (Saito and Posas, 2012, Hohmann, 2009, Duch *et al.*, 2012, Sheikh-Hamad and Gustin, 2004)].

1.3.1 Mechanisms of adaptation

Upon osmotic shock, the yeast elicits reprogrammation of cellular activities to mediate adaptation in HOG-dependent and HOG-independent manners. Immediately after osmotic shock, changes in

glycerol accumulation and re-establishment of ion homeostasis occur, accompanied by metabolic efflux changes to enhance glycerol production (Dihazi *et al.*, 2004, Albertyn *et al.*, 1994). Successful adaptation involves global reprogrammation of transcription and translation of the whole genome. Upon initial exposure to osmotic shock, global downregulation of transcription and translation occurs, which correlates with an increase in P-bodies containing mRNAs and factors of the decay machinery; yet, a small subset of mRNAs encoding stress-protective proteins rapidly associates with polysomes to be specifically translated, which is followed by the transcriptional upregulation of approximately 300 osmostress-responsive genes (Teixeira *et al.*, 2005, Romero-Santacreu *et al.*, 2009, Warringer *et al.*, 2010). Activated Hog1 controls the expression of majority of osmo-responsive genes by several mechanisms, including regulating transcription factors, associating with chromatin, driving transcription initiation and elongation, controlling mRNA processing and stability, and playing a role in translation (Saito and Posas, 2012, de Nadal *et al.*, 2011). However, not regulation mechanisms targeting gene expression during osmo-adaptation are HOG-dependent. Firstly, a subset of genes are upregulated in response to osmotic stress in a *hog1Δ* mutant (Romero-Santacreu *et al.*, 2009). Secondly, severe osmotic shock elicits a global mRNA stabilisation in a HOG-independent manner (Romero-Santacreu *et al.*, 2009). Finally, osmostress-induced transient inhibition of global translation is found to be HOG-independent, however, the pathway is required for the recovery of translation initiation during adaptation (Uesono and Toh, 2002, Garre *et al.*, 2012, Warringer *et al.*, 2010).

1.3.2 The HOG MAPK signalling cascade

In response to high external osmolarity, the HOG MAP kinase signalling pathway is activated to mediate a variety of cellular changes to allow adaptation. This well-studied MAP kinase module serves as a model for various signalling features, including cross-talk between pathways and the use of scaffolding proteins to insulate components of the different MAP kinase cascades (Yamamoto *et al.*, 2010, Harris *et al.*, 2001). At the core of this cascade lies Hog1 MAPK, which is conserved in diverse fungal species, with a human functional homolog, p38. In humans, p38 MAPK signalling is activated by stress stimuli, including cytokines, ultraviolet radiation, heat and osmotic shock, and its deregulation is implicated in a number of diseases such as chronic inflammatory and rheumatic diseases, cardiovascular dysfunction and various cancers [reviewed in (Cuenda and Rousseau, 2007, Cuadrado and Nebreda, 2010)]. It is interesting to note that the mammalian p38 is able to rescue osmosensitivity of the *hog1Δ* yeast mutant (Gustin *et al.*, 1998, Saito and Posas, 2012).

The HOG pathway is activated by two functionally redundant but mechanistically distinct SLN1 and SHO1 branches, and the signals generated from the two branches converge at MAPKK Pbs2, which then activates Hog1 by phosphorylation (Figure 1.5) (Saito and Posas, 2012, Hohmann, 2002, Chen *et al.*, 2001). Activated Hog1 phosphorylates specific targets both in the cytoplasm and, upon nuclear import, in the nucleus, which lead to downstream adaptation through the production of the osmolyte glycerol, re-organisation of ion transporters and water efflux, control of cell cycle progression, and regulation of gene expression (Saito and Posas, 2012).

1.3.2.1 The SLN1 branch

The SLN1 branch is activated by the osmosensor Sln1 which contains two transmembrane (TM) domains and a cytoplasmic histidine kinase domain. Under normal conditions, Sln1 is auto-phosphorylated and active, and in turn phosphorylates Ssk1 by transferring the phosphate group through Ypd1 (see Figure 1.5) (Hohmann, 2002, Zhi *et al.*, 2013). Sln1 and Ypd1 function as negative regulators of the HOG pathway as deletions of the genes *SLN1* and *YPD1* cause lethality due to over-activation of the pathway (Posas *et al.*, 1996). *In vivo*, Sln1 is found to respond to turgor pressure, and the decrease in turgor caused by high osmolarity inhibits histidine kinase activity, causing dephosphorylation of Sln1. Subsequently, unphosphorylated Ssk1 accumulates and activates the MAPKKKs Ssk2 and Ssk22 through its binding to the N-terminal regulatory domain of Ssk2 to mediate autophosphorylation. The MAPKKKs Ssk2/Ssk22 activates the signalling cascade through the specific phosphorylation of Pbs2, which consequently phosphorylates and thereby activates Hog1 (Hohmann, 2002, Zhi *et al.*, 2013, Saito and Posas, 2012).

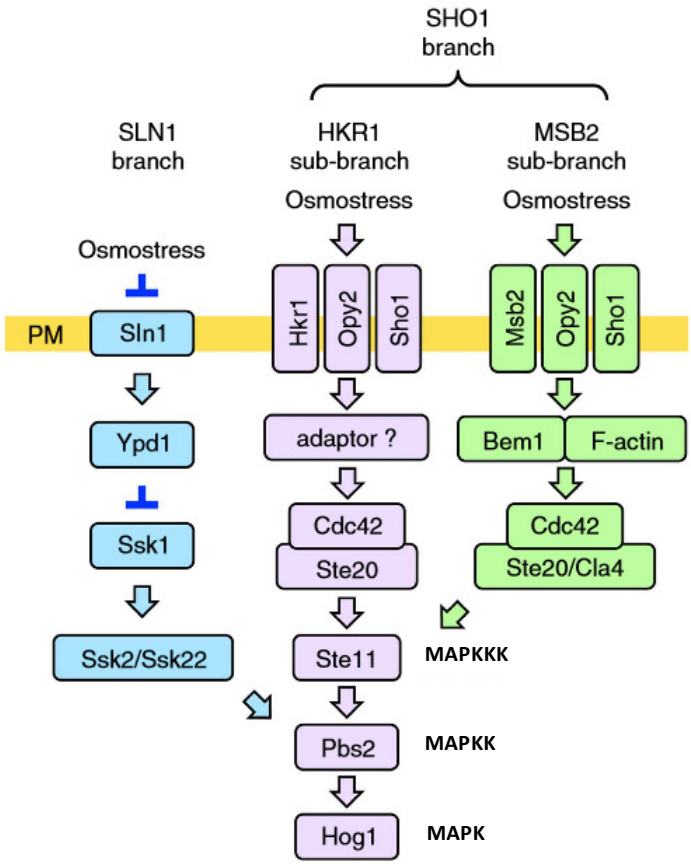


Figure 1.5 Schematic representation of the yeast HOG signalling pathway (Tatebayashi *et al.*, 2015). High external osmolarity activates the SLN1 and the SHO1 branches. Proteins involved in the SLN1 branch are coloured blue, proteins involved in the HKR1 sub-branch of the SHO1 branch are coloured lavender, and proteins involved in the MSB2 sub-branch of the SHO1 branch are coloured green. Proteins that share the same box represent proteins with redundant functions. Arrows represent activation and T-shaped bars represent inhibition. The horizontal yellow line represents the plasma membrane (PM).

1.3.2.2 The SHO1 branch

Unlike the SLN1 branch, the mode of activation of the SHO1 branch of the HOG pathway is not completely understood. Activation of this branch depends on three transmembrane osmosensors, Msb2, Hrk1 and Sho1, which were identified mainly based on their mutant phenotypes (Figure 1.6) (O'Rourke and Herskowitz, 2002, Takahashi and Pryciak, 2007, Maeda *et al.*, 1995, Tatebayashi *et al.*, 2007). Msb2 and Hkr1 share redundant functions, as it is necessary to disrupt both genes to completely inactivate the SHO1 branch, and both proteins belong to the conserved protein family of mucins, which are characterised as large transmembrane, highly glycosylated glycoproteins that

function in signal transduction (see Figure 1.6) (Singh and Hollingsworth, 2006, Cullen, 2011, Tatebayashi *et al.*, 2007). Their extracellular domains are highly Ser/Thr rich and contain numerous O-glycosylation sites that are glycosylated, which have been shown to have regulatory functions as their deletion converts Msb2 and Hkr1 to constitutively active forms (Takahashi and Pryciak, 2007, Cullen *et al.*, 2004). Addtionally, a conserved Hkr1-Msb2 homology (HMH) domain also forms part of their extracellular domain, and the deletion of this domain inactivates both proteins (Tatebayashi *et al.*, 2007). Two distinct modes of osmosensing have been proposed for the osmosensors Msb2 and Hkr1: one involves the cleavage of the regulatory glycosylated extracellular domains by the aspartyl protease Yps1 (Vadaie *et al.*, 2008); and the second involves stress-induced conformational change in the oligosaccharide structure of the extracellular domains (Tatebayashi *et al.*, 2007); however, the actual mechanism remains obscure.

The third osmosensor Sho1 is a relatively small protein of 367 aa and is composed of four TM segments with a C-terminal cytoplasmic SH3 domain and exists as an homo-oligomer through interactions of the TM domains (Figure 1.6) (Tatebayashi *et al.*, 2015). Osmotic stress is shown to induce structural modifications to the TM domains which changes their cross-linking efficiencies and this seem to be responsible for signal transduction across the plasma membrane (Tatebayashi *et al.*, 2015). Furthermore, Sho1 has been demonstrated to be a putative osmosensor through mutational studies, as in a mutant lacking Hkr1, Msb2 and Opy2 (combined with inactivation of SLN1 branch), Hog1 can be activated by osmotic stress when the expression of hyperactive Ste11, bypassing the need for Hkr1, Msb2 and Opy2 who mediate Ste11 activation by Ste20, is coupled with a membrane-tethered Ste50, bypassing the need for Opy2 (Tatebayashi *et al.*, 2015). The complexity of the functional relationships between Msb2, Hkr1 and Sho1 has been documented in various studies. It has been shown that both Msb2 and Hkr1 interact with Sho1 through their TM domains (Cullen *et al.*, 2004, Tatebayashi *et al.*, 2007). Deletion of Msb2 or Sho1 reduces cross-talk through Ste11, and a double mutation in both genes shows additional reduction in cross-talk, indicating that Msb2 and Sho1 have partially redundant roles in the activation of Ste11 (O'Rourke and Herskowitz, 2002). Recent studies conducted by the Saito laboratory have provided further insights into the inter-relationship between the three osmosensors. It was observed that signalling by the originally-identified redundant osmosensors Msb2 and Hkr1 is not equivalent and can be divided into two sub-branches. In the HKR1 sub-branch, the Hkr1 cytoplasmic domain is required to recruit Ste20 to the plasma membrane to activate Ste11. In contrast, the MSB2 sub-branch activates Ste11 through either Ste20 or Cla4, which are recruited by the scaffold protein Bem1, a protein that is involved in morphogenesis and establishment of cell polarity (Tanaka *et al.*, 2014, Chenevert *et al.*, 1992). Furthermore, Sho1 functions as an additional osmosensor in the HKR1 sub-branch (and possibly also

in the MSB2 sub-branch), since a Sho1 mutant defective in osmosensing is unable to activate the HKR1 sub-branch, and the Sho1-Hkr1 interaction is required for signalling through Sho1 (Figure 1.5) (Tatebayashi *et al.*, 2015).

Apart from its function as a putative osmosensor, Sho1 also plays an essential role as an adaptor to assemble Pbs2, Ste50 and Ste11 upon osmotic stress (Figure 1.5). The Sho1 cytoplasm SH3 domain binds very specifically to an N-terminal proline rich sequence (KPLPPLPV) of Pbs2 and serves to localise Pbs2 to the plasma membrane, and thus mediates the activation of Pbs2 by the MAPKKK Ste11 (Maeda *et al.*, 1995, Zarrinpar *et al.*, 2004). Interestingly, following osmostress stimulation, membrane localisation of Pbs2 decreases and Sho1-Pbs2 dissociates, and this dissociation is hindered in *ste20Δ* or *ste11Δ* mutants but not *hog1Δ*. These observations indicate a negative feedback by activated Pbs2 regulating the Sho1-Pbs2 interaction (Reiser *et al.*, 2000). Furthermore, the Ste11/Ste50 complex has also been shown to interact with Sho1, independent of the ability of the SH3 domain to bind proline-rich motifs, and this interaction is necessary for the efficient activation of Pbs2 by Ste11 (Zarrinpar *et al.*, 2004, Saito and Posas, 2012).

Remarkably, both Msb2 and Sho1 also function at the head of the FG pathway under nutrient-limiting conditions to activate the MAPKKK Ste11, the MAPKK Ste7 and the MAPK Kss1 (Roberts and Fink, 1994). It has been proposed that Msb2 maintains signal fidelity by differential glycosylation of its O-glycosylation domain dependent on the type of external signal detected (Yang *et al.*, 2009b, Lien *et al.*, 2013). Furthermore, Sho1 shows differential cellular localisation that is signal-dependent, as such that FG pathway activation localises Sho1 at highly polarised sites, and in contrast, Sho1 localises at punctate sites during osmotic stress (Pitoniak *et al.*, 2009). Most importantly, to prevent undesirable signal leakage during HOG activation, Sho1 and other osmostress-specific adaptors serve as docking proteins to bring Ste11 proximal to its substrate Pbs2 (Tatebayashi *et al.*, 2006, Lamson *et al.*, 2006).

Two important scaffold proteins, Ste50 and Opy2, are necessary for proper signalling through the SHO1 branch of the HOG pathway through the effective recruitment of Ste11 to the plasma membrane in order to phosphorylate its substrate Pbs2 (Figure 1.5). The Ste50 adaptor is composed of an N-terminal steril-α motif (SAM) domain and a C-terminal Ras association (RA) domain, and it has been shown that Ste50 binds to Ste11 through the SAM domains present in both proteins (Ramezani-Rad, 2003, Posas *et al.*, 1998b). This adaptor protein is essential for the activation of HOG pathway through the SHO1 branch, as mutations in the *STE50* gene in a strain that also harbours *ssk2Δssk22Δ* is unable to activate Hog1 (Posas *et al.*, 1998b). Furthermore, it was found that the Ste50 RA domain is essential for its localisation at the plasma membrane, and a Ste50 mutant lacking

the RA domain (Ste50-ΔRA) is unable to signal through the pathway (Wu *et al.*, 2006, Tatebayashi *et al.*, 2006). Membrane localisation of Ste50 is dependent on the adaptor protein Opy2, as forced membrane-targeting of the Ste50-ΔRA mutant can activate Hog1 by osmotic stress in the absence of Opy2, indicating that the Ste50-Opy2 interaction serves to membrane-localise Ste50 through its RA domain (Wu *et al.*, 2006, Tatebayashi *et al.*, 2007).

Opy2, another essential adaptor of the SHO1 branch, is a single-path TM protein containing a cytoplasmic domain comprised of four well-conserved regions (CR-A to CR-D) containing three independent Ste50 binding sites (Figure 1.6) (Yamamoto *et al.*, 2010). The essential function of Opy2 is to recruit the Ste50/Ste11 complex to the plasma membrane through Opy2-Ste50 interaction and serves as a membrane anchor for the MAPKKK Ste11 to mediate activation of Pbs2 (Yamamoto *et al.*, 2010, Wu *et al.*, 2006). Recently, it has been described that Opy2 also interact with Hkr1 and Sho1 through its CR and TM domains, respectively, and the formation of the Sho1-Opy2-Hkr1 complex serves to bring together Ste11, recruited by Sho1 and the binding of Ste50 to Opy2, with its kinase Ste20, recruited by Hkr1, for efficient Ste11 activation (Tatebayashi *et al.*, 2015).

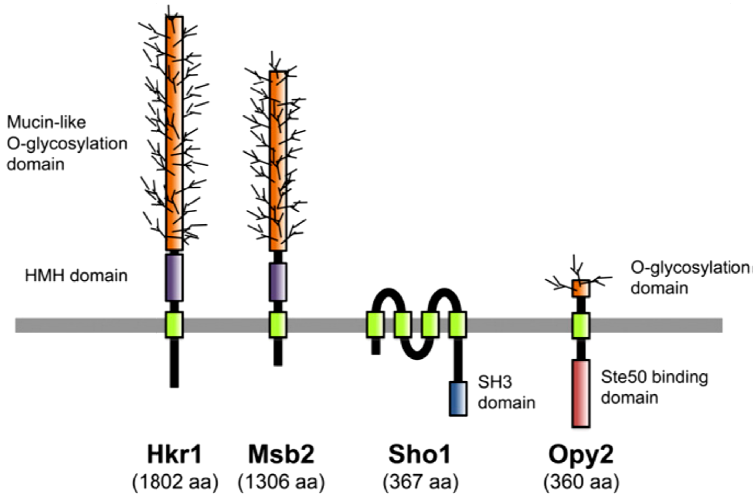


Figure 1.6 Schematic representations of four transmembrane proteins from the SHO1 branch (Saito and Posas, 2012). Hkr1, Msb2, Sho1 and Opy2 are represented schematically showing their transmembrane domains (green), extracellular O-glycosylation domains (orange), HMH domain (purple), Sho1 SH3 domain (blue), and Opy2 Ste50-binding domain (red). Grey line represents the plasma membrane, and size of protein is indicated as amino acid units.

The MAKKK Ste11 is a protein of 717 amino acids and is composed of three domains: a SAM domain and an auto-inhibitory domain, both located at the N-terminus, and a catalytic kinase domain located at the C-terminus (Hartwell, 1980). The activation of Ste11 is carried out by two major events: first, the binding of Ste50 to Ste11 via the Ste11 N-terminal SAM domain, which mediates the dissociation of the N-terminal inhibitory domain from the C-terminal kinase catalytic domain (Wu *et al.*, 1999); second, the phosphorylation of Ste11 by Ste20/Cla4 (Lamson *et al.*, 2006); however it was shown that neither of these two events is sufficient for full activation of the HOG pathway (Figure 1.5). Constitutively active Ste11 mutants, either by phospho-mimetic substitutions at the phosphorylation sites or by mutations in the auto-inhibitory domain, are only sufficient to activate the Hog1 MAPK in the absence of stress when overexpressed (Tatebayashi *et al.*, 2006, Lamson *et al.*, 2006). Furthermore, hyper-active Sho1 mutants in the SH3 domain are not able to induce pathway activation in the absence of stress without an additional hyper-active Ste11 mutation (Tatebayashi *et al.*, 2006). Thus, taken together, these results suggest that full activation of the HOG pathway requires a high concentration of activated Ste11 at the plasma membrane, mediated by scaffolds Ste50 and Opy2 (Posas *et al.*, 1998b, Wu *et al.*, 2006, Tatebayashi *et al.*, 2006).

The specific activation of Pbs2 by Ste11 without aberrant activation of other pathways is possibly mediated by three separate events: first, by the membrane recruitment of Pbs2 by Sho1 and the membrane recruitment of Ste11-Ste50 complex by Opy2 to membrane-localise both Ste11 and its substrate Pbs2 (Maeda *et al.*, 1995, Wu *et al.*, 1999, Yamamoto *et al.*, 2010); second, by the direct interaction between Ste11 and the cytoplasmic tail of Sho1, near the binding site of Pbs2 (Tatebayashi *et al.*, 2006); and finally, through the direct interaction between Ste11 and Pbs2 (Zuzuarregui *et al.*, 2012). Thus, multiple and dynamic interactions between the Opy2/Ste50/Ste11 complex and the Sho1/Pbs2 complex bring Ste11 in close contact with Pbs2 to enable efficient activation, however, the complexity of the HOG-associated membrane architecture, the relative contributions of these interactions, as well as their regulation by osmotic stress, has yet to be determined.

Activated Pbs2 catalyses the dual phosphorylation of the conserved Thr174 and Tyr176 residues in the MAPK Hog1, and this event occurs in the cytosol (Figure 1.5) (Brewster *et al.*, 1993, Hohmann, 2002). Phosphorylation causes a rapid concentration of Hog1 in the nucleus, while non-phosphorylated Hog1 has an even distribution between the cytoplasm and nucleus (Reiser *et al.*, 1999). Activated Hog1 phosphorylates a number of targets to elicit, among others, cell cycle arrest, activation of ion channels and gene expression changes (see Figure 1.7). In Chapter 3 of this thesis,

we provide insights to the interactions of signalling components the SHO1 branch of HOG pathway, namely Ste11, Sho1, Pbs2 and Msb2.

1.3.2.3 Negative feedback by Hog1 of upstream elements

The Hog1 MAPK is only transiently activated following osmotic stress, where Hog1 activation reaches a maximal level at around 5 min and is shown to gradually decrease to basal levels within 30 min (Maeda *et al.*, 1995). The downregulation of Hog1 activation requires its own kinase activity, as catalytically inactive Hog1 mutants show much prolonged persistence of the phosphorylated Hog1 (Wurgler-Murphy *et al.*, 1997). Several feedback mechanisms have been described to regulate the HOG pathway, including direct signal downregulation by glycerol accumulation (Brewster *et al.*, 1993, Albertyn *et al.*, 1994), and inactivation of signalling kinases by protein phosphatases through dephosphorylation [reviewed in Saito and Posas (2012)]. To attenuate Hog1 activity, protein tyrosine phosphatases Ptp2/Ptp3 and the type 2 protein phosphatases Ptc1-3 all contribute to its dephosphorylation, and a negative feedback loop is known to exist between Hog1 and Ptp2/Ptp3, where active Hog1 enhances Ptp2 activities and induces Ptp3 expression (Wurgler-Murphy *et al.*, 1997, Jacoby *et al.*, 1997, Saito and Tatebayashi, 2004). Furthermore, within the SHO1 branch, the inhibition of upstream elements of the HOG pathway by downstream components also seems to make major contributions to the attenuation of the system. To date, the direct phosphorylation of Sho1 and Ste50 by Hog1 has been described. Activated Hog1 was shown to phosphorylate Sho1 at a serine residue within the cytoplasmic linker region between the TM domains and the SH3 domain, however the functional role of this phosphorylation is still unknown (Hao *et al.*, 2007). The phosphorylation of Ste50 at multiple sites by Hog1 mediates its dissociation from the membrane anchor Opy2, thereby reducing Ste11 membrane localisation. The Opy2-Ste50/Ste11 interaction is essential for the activation of Hog1, hence, the dissociation of this complex serves to attenuate signal transduction (Yamamoto *et al.*, 2010). To further support the functional role of Ste50 phosphorylation, it is observed that a mutant expressing a phosphorylation-deficient version of Ste50 displays an increased duration of Hog1 activation (Yamamoto *et al.*, 2010). Overall, the downregulation of the HOG pathway through various negative feedback mechanisms plays an essential role in mediating effective cellular adaptation following osmotic stress.

In Chapter 3 of this thesis, we address the roles of Hog1 and Pbs2 as negative regulators of the SHO1 branch through several molecular mechanisms.

1.3.2.4 Regulation of signalling between HOG and other pathways

Several of the MAPK pathways in *S. cerevisiae* share common factors, including the HOG MAPKKK Ste11, also shared by the pheromone and FG pathways, and the HOG osmosensor Msb2, shared by the FG pathway, yet specific external signals only elicit a specific downstream response, preventing undesirable cross-talk between pathways (reviewed in Saito (2010)). Signal fidelity is maintained and regulated by several mechanisms: first, signal-specific docking interactions mediate direct binding between consecutive components in the pathway, for example, to prevent Ste11-stimulated activation of FG and pheromone pathways following osmotic stress, Sho1 acts as a scaffold to dock both Ste11 and the osmostress-responsive substrate Pbs2 to mediate specific phosphorylation of Pbs2 by Ste11 (Maeda *et al.*, 1995, Zarrinpar *et al.*, 2004); and second, cross-pathway inhibition mechanisms are employed, for instance, activated Hog1 readily inhibits FG responses (Pitoniak *et al.*, 2009). The intricate relationship between the MAPK pathways is highlighted in recent studies where Babazadeh *et al.* were able to generate HOG-independent osmo-adaptation by artificially rerouting signalling through the pheromone pathway (Babazadeh *et al.*, 2014). Moreover, in another study it was observed that stimulation with pheromone activates the HOG pathway, and that pheromone-induced HOG activation also facilitates the acute response to hyperosmotic stress (Baltanas *et al.*, 2013).

1.3.3 Transcriptional response upon osmotic stress

Changes in external osmolarity have profound effects on gene expression, and regulation of transcription is essential for long-term adaptation (de Nadal *et al.*, 2011, de Nadal and Posas, 2010). Within minutes of osmotic shock, global transcription rate drops by 50 % (Romero-Santacreu *et al.*, 2009). Despite this initial reduction in global transcription, expression of hundreds of genes are induced, and the most strongly activated genes increase their expression more than 100-fold in 20 minutes, indicating that yeast cells are evolved to prioritise transcription of a specific subset of genes to mediate osmostress cellular protection (see Figure 1.8A) (Capaldi *et al.*, 2008, O'Rourke and Herskowitz, 2004). It has, therefore, become of great interest to study the mechanisms and factors responsible for the transcriptional changes during osmotic stress response.

1.3.3.1 Hog1 regulates transcription during osmotic stress

In mammalian cells, the Hog1 homologue p38 controls the expression of more than 100 genes once activated by environmental stresses (Ono and Han, 2000). In *S. cerevisiae*, 5-7 % of genes become transiently induced following osmotic stress, and among them, between 60-80 % are fully or partially dependent on Hog1 activity (Figure 1.8). The involvement of Hog1 kinase in gene expression is further emphasised by the observation that genes considered highly induced are more dependent on Hog1 than genes that are only moderately induced (Posas *et al.*, 2000, Romero-Santacreu *et al.*, 2009). Moreover, a large number of these Hog1-dependent osmostress-responsive genes are implicated in carbohydrate metabolism, gene stress protection, protein biosynthesis and signal transduction (Figure 1.8 and Table 1.1) (Hohmann, 2002, de Nadal *et al.*, 2002).

Activated Hog1 controls gene expression at all levels of the transcription cycle by regulating transcription initiation factors, chromatin modification events, components of the general transcription machinery and transcription elongation (see Figure 1.7) (de Nadal *et al.*, 2002, Saito and Posas, 2012, De Nadal *et al.*, 2004). One of the well-characterised functions of the MAPK is its role in transcription initiation, in which it regulates several TFs through either direct phosphorylation, recruitment to chromatin or other mechanisms to control the expression of most osmostress-responsive genes. These TFs include transcription activators Hot1 (Gomar-Alba *et al.*, 2013, Alepuz *et al.*, 2003), Smp1 (de Nadal *et al.*, 2003), the likely redundant Msn2 and Msn4 (Capaldi *et al.*, 2008), and the transcription activator/repressor Sko1 (Proft and Struhl, 2002, Proft *et al.*, 2001). These factors act either independently or in combination with other factors to regulate highly specific promoters, and in some cases Hog1 not only regulates these TFs but also regulates its associated co-factors (Saito and Posas, 2012, Hohmann, 2002). The relationship between Hog1 and osmostress TFs seems to be reciprocal, as some TFs are able to recruit Hog1, and Hog1 associates to chromatin during osmotic stressthrough binding of TFs (Cook and O'Shea, 2012, Alepuz *et al.*, 2001). However, it has been suggested that Hog1-dependent phosphorylation of TFs is not an absolute requirement for transcription initiation, and activated Hog1 may be able to directly recruit the RNAPII machinery to osmo-responsive genes by tight association with the largest subunit Rpb1 of RNAPII, therefore, these results suggest that Hog1 itself may act as a TF through the binding of other TFs (Alepuz *et al.*, 2003, Alepuz *et al.*, 2001). Recently, Hog1 is shown to control the redistribution of RNAPII from housekeeping to osmostress-responsive genes upon osmotic shock, in which RNAPII, either in a complex with Hog1 or modified by Hog1, has reduced affinity to non-stress genes, allowing Hog1 to briefly hijack the transcription machinery and associated factors to carry Hog1-dependent transcription on Sko1- and Hot1-specific genes (Cook and O'Shea, 2012, Nadal-Ribelles *et al.*, 2012, Sole *et al.*, 2011). In addition to its association at gene promoters, Hog1 also associates

with the coding region and 3´ end of osmo-responsive genes, where it interacts with components of the RNAPII elongation complex (Alepuz *et al.*, 2003, Proft *et al.*, 2006, Pokholok *et al.*, 2006) and stimulates chromatin remodelling (Klopf *et al.*, 2009, Mas *et al.*, 2009) (Figure 1.7).

Overall, activated Hog1 is critical in the cellular response to hyperosmotic stress by playing a role in all steps of the transcription cycle to ensure high and timely expression of osmostress-responsive genes.

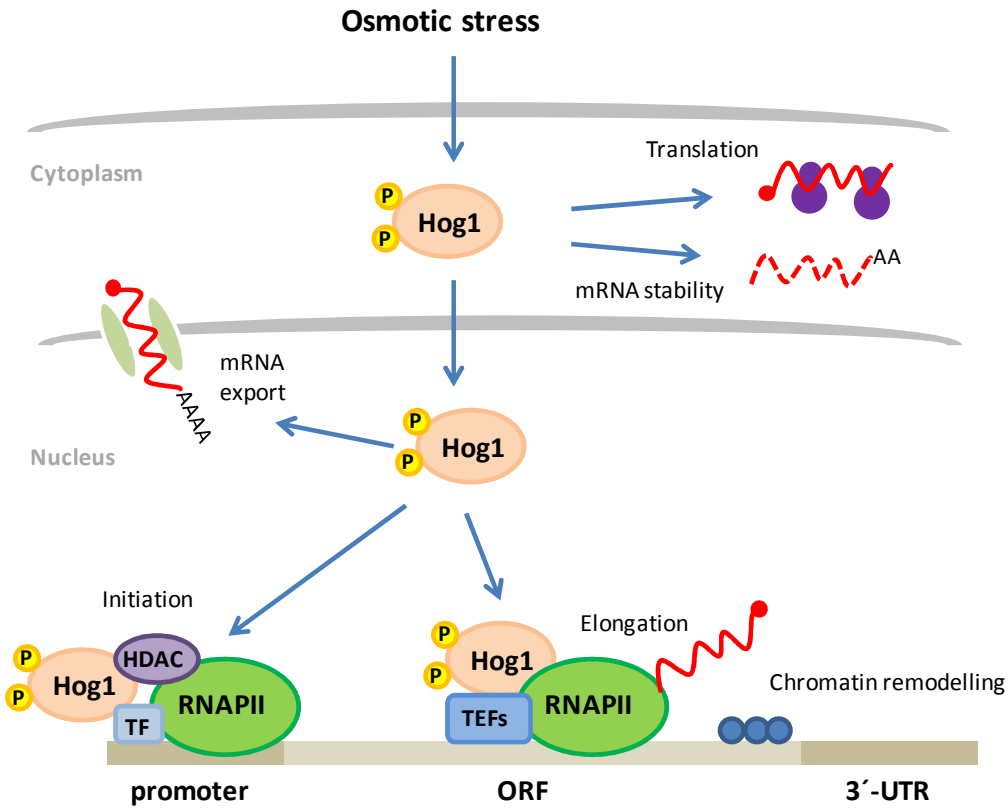


Figure 1.7 Role of activated Hog1 in gene expression following osmotic stress (modified from Saito *et al*). Upon osmotic stress stimulation, activated Hog1 regulates translation and mRNA degradation in the cytoplasm. In addition, phosphorylated Hog1 accumulates in the nucleus and is known to phosphorylate and activate transcription factors (TFs). Hog1-TFs are recruited to promoters of stress-responsive genes to induce the formation of pre-initiation complex (PIC). Furthermore, Hog1 also regulates transcription elongation and chromatin modifications, and has been proposed to regulate mRNA export. The compartments represented are the cytoplasm and the nucleus. Each Hog1-dependent process is labelled, and an osmo-responsive gene is schematically shown with promoter, ORF and 3´ UTR (Saito and Posas, 2012).

1.3.3.2 Transcriptional activators under control of Hog1

Hog1 regulates several unrelated TFs, and each of them is responsible for controlling the expression of a subset of osmostress-responsive genes, either directly or in combination with other factors (see Table 1.2) (Ni *et al.*, 2009, Capaldi *et al.*, 2008, de Nadal and Posas, 2010). Presently, Smp1, Msn2/Msn4, Sko1 and Hot1 have been identified to function in HOG-dependent transcription (Ni *et al.*, 2009, Capaldi *et al.*, 2008, de Nadal and Posas, 2010). The most important characteristics of each of these osmo-TFs are described below.

Smp1: a member of the MEF2C family of TFs, was first identified as a factor that induces a LacZ reporter attached to *STL1*, a gene that encodes a glycerol/proton symporter and is identified as the strongest induced gene during osmotic stress that is completely dependent on Hog1 activity (de Nadal *et al.*, 2003). The mode of activation of Smp1 is dependent on Hog1 kinase activity, where, upon osmotic stress, Hog1 phosphorylates Smp1 at multiple sites in its C terminal domain (de Nadal *et al.*, 2003). Activated Smp1 then transcriptionally activates a specific subset of genes (Table 1.2) (de Nadal *et al.*, 2003).

Msn2/Msn4: although the TFs Msn2 and Msn4 only share 32 % identify, their zinc finger DNA-binding domains are so similar that they have been predicted to bind the same stress response elements (STREs) on target promoters (Martinez-Pastor *et al.*, 1996). The STRE is characterised by the core sequence CCCCT in both orientations and it exists usually in two or more copies upstream of Msn2/4 target genes (Treger *et al.*, 1998). Msn2 and Msn4 seem to have redundant functions, however, Msn2 generally makes a much bigger contribution in the induction of STRE-dependent reporter genes (Schmitt and McEntee, 1996). The activities of Msn2/Msn4 are regulated by the PKA (**P**rotein **K**inase **A**), TOR (**T**arget **o**f **R**apamycin) and HOG pathways (Gorner *et al.*, 1998, Heinisch *et al.*, 1999, Rep *et al.*, 2000). In response to a number of stresses, including osmotic stress, the normally cytoplasmic Msn2/4 is translocated into the nucleus, where it binds to a subset of genes, including stress-protective genes *CTT1* and *HSP12*, to mediate transcription (Table 1.2) (Martinez-Pastor *et al.*, 1996). The mechanism by which Msn2/4 is regulated by Hog1 during osmotic stress is still unclear, however it has been described that mRNA induction of genes controlled by these factors is diminished in a *hog1Δ* mutant (Rep *et al.*, 2000). Furthermore, using bioinformatic approaches, Capaldi *et al.* has demonstrated that the majority of genes controlled by Msn2/4 are also dependent on Hog1. In addition, although osmostress-induced Msn2/4 nuclear import is dependent on Hog1, some Msn2 nuclear import is also observed in the absence of Hog1, indicating the involvement of other pathways (Capaldi *et al.*, 2008).

Sko1: is a member of the ATF/CREB family of AP1-related transcription factors (Nehlin *et al.*, 1992). Under normal conditions, Sko1 acts a transcription repressor of osmo-responsive genes via the general co-repressor complex Cyc8-Tup1 (Proft and Serrano, 1999). Interestingly, phosphorylation by activated Hog1 upon osmotic stress converts Sko1 from a repressor to an activator, and phosphorylated Sko1 recruits both Hog1 and the SAGA and SWI/SNF chromatin-modifying complexes (Proft and Struhl, 2002). Furthermore, Sko1 is found to co-localise with Hog1 and Hot1 at many osmostress promoters and the presence of these three factors recruits Hog1 to the ORFs of the same genes to mediate RNAPII transcription (Cook and O'Shea, 2012). A genome-wide study revealed that Sko1 binds ~40 target regions and amongst them include *GRE2* and *HXT5*, which encode a stress-defense protein and a sugar transporter, respectively (Table 1.2) (Proft *et al.*, 2005).

Hot1: is a protein of 719 amino acids that was originally identified in a two-hybrid search for proteins interacting with Hog1 (Rep *et al.*, 1999b). Hot1 targets a subset of genes which are amongst the most highly induced, including the well-studied *GPD1*, which encodes a NADH-dependent glycerol-3-phosphate dehydrogenase, and *STL1* (Table 1.2) (Capaldi *et al.*, 2008, Cook and O'Shea, 2012). The interaction between Hot1 and Hog1 is critical for the recruitment of the kinase to Hot1-dependent promoters, and furthermore, the binding of the Hot1/Hog1 complex, together with Sko1, to cis-promoter elements reallocates RNAPII from housekeeping genes to osmostress-responsive genes (Cook and O'Shea, 2012, Alepuz *et al.*, 2003, Alepuz *et al.*, 2001). A recent study conducted by Gomar-Alba *et al.* has characterised the interaction domain of Hot1 with Hog1, as well as identifying a specific short sequence that is responsible for Hot1 binding to DNA. Both sequences are required for osmo-tolerance as well as appropriate Hog1-mediated recruitment of RNAPII to target genes (Gomar-Alba *et al.*, 2013).

Under normal conditions, Hot1 is phosphorylated at basal levels, and upon osmotic stress, Hot1 becomes hyper-phosphorylated (Alepuz *et al.*, 2001). The phosphorylation of Hot1 was described by Alepuz *et al.* to be mediated by Hog1, however does not seem to be essential for gene expression (Alepuz *et al.*, 2003). Furthermore, residual phosphorylation is observed in a *hog1Δ* mutant, indicating that Hog1 is not the only kinase that phosphorylates Hot1 (Alepuz *et al.*, 2001). Recently, it has been described by Burns *et al.* that Hot1 is also phosphorylated by CK2 (**C**asein **K**inase II), however, CK2-dependent phosphorylation negatively regulates Hot1 activity and targets it to chromatin to modulate the expression of the Hot1-dependent gene *STL1* (Burns and Wente, 2014).

In Chapter 4 of this thesis, we describe the role of Cbc1 in the regulation of transcription during osmotic stress through its interactions with osmostress-TFs.

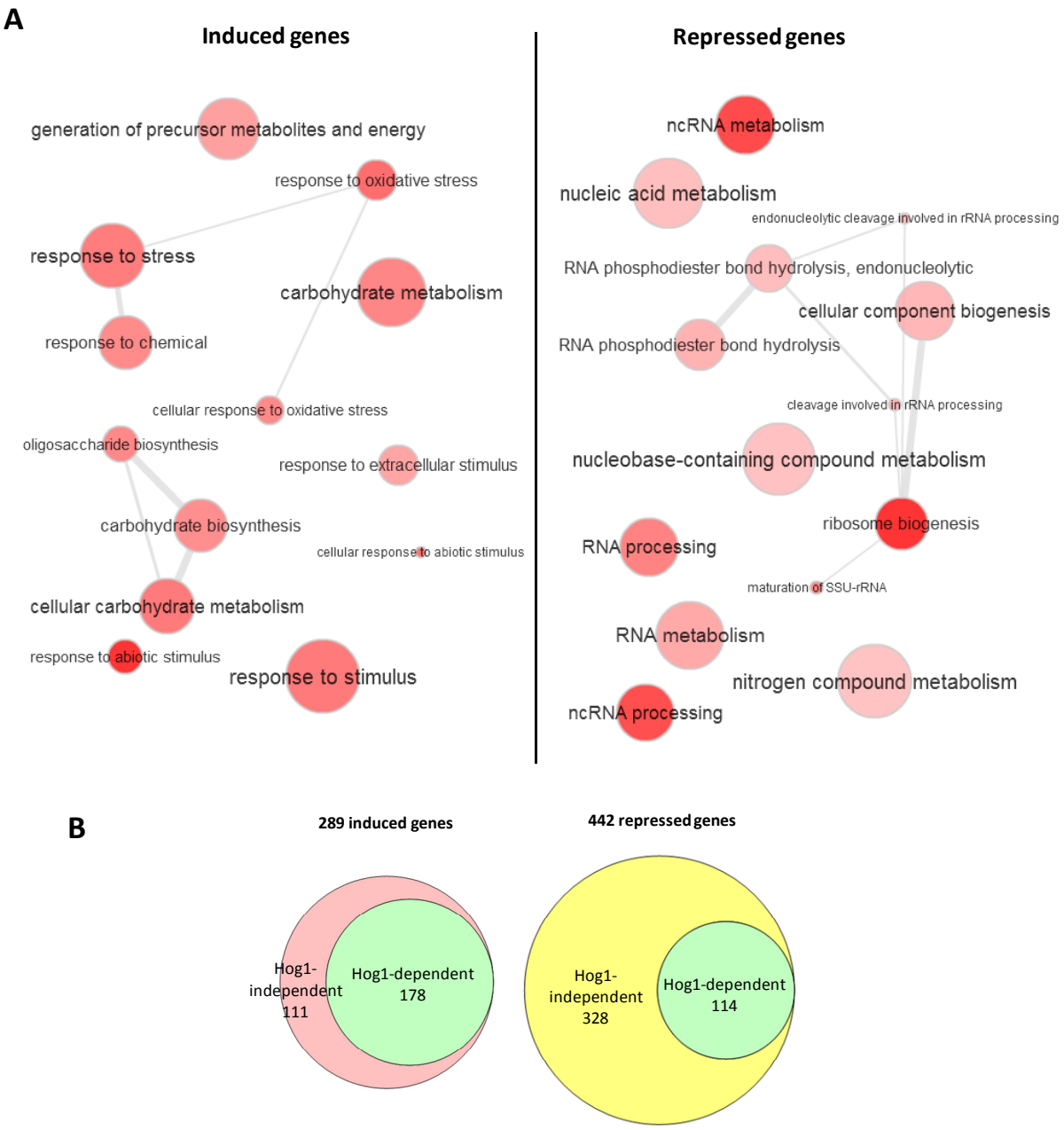


Figure 1.8 Functional categories and Hog1-dependency of genes induced or repressed by osmotic stress. All data are taken from Romero-Santacreu (2009). **(A)** Significantly represented function groups (p-value<e-05) of all osmostress-induced and -repressed genes, searched using the Gene Ontology (GO) Term Finder tool (*Saccharomyces* Genome Database), were graphed using the REVIGO tool (Supek *et al.*, 2011). Size of circles indicate GO term sizes, searched using UniProt (http://www.uniprot.org/), and grey lines indicate semantic similarities between functional groups which is measured using the simRel score (Schlicker *et al.*, 2006). Colour intensity of the circles indicates their p-value, in which dark and light red represent lower and high p-values, respectively. **(B)** Graphical representation of the number of osmostress-induced and repressed genes that are either Hog1-dependent or Hog1-independent. Venn diagrams were generated using the Venn Diagram Plotter (Littlefield, 2004).

Table 1.1 Over-represented functional categories of genes dependent on Hog1

Data taken from Romero-Santacreu (2009).

Category	Number of genes	GO functional categories	p-value
Hog1-dependent induced genes	178	-Response to abiotic stress	1e-05
		-Response to oxidative stress	7e-04
		-Carbohydrate biosynthesis process	7e-03
Hog1-dependent repressed genes	114	-Ribosome biogenesis	6e-12
		-RNA processing	9e-11

Table 1.2 Over-represented functional categories of genes dependent on specific transcription factors

Data taken from Romero-Santacreu (2009).

Category	Number of genes	GO functional categories	p-value
Hot1-dependent	73	-Response to abiotic stimulus	3e-06
		-Carbohydrate biosynthetic process	5e-06
		-Carbohydrate metabolic process	3e-05
Msn2-dependent	394	-Asparaginase activity	9e-03
Msn4-dependent	52	-Peroxidase activity	8e-03
Smp1-dependent	223	-Structural constituent of cell wall	1e-07
		-Helicase activity	1e-02
Sko1-dependent	33	No significant categories found	

1.3.4 Post-transcriptional regulation during osmotic stress

In addition to transcriptional changes, gene expression is also regulated at the post-transcriptional level, including global reprogrammation of translation and mRNA stability, which is critical for re-adaptation following hyperosmotic shock. The complicated process of post-transcriptional regulation ultimately achieves two main outcomes: the reallocation of cellular resources from cellular growth to stress adaptation; and the specific and efficient synthesis of proteins that function in osmostress cellular protection. Mechanisms employed to dictate translational regulation following osmotic

stress is of great importance, as defects in the reprogrammation of translation during the osmotic stress response in mammalian cells have been associated with such diseases as cancer, diabetes and inflammatory illnesses (Keene, 2007).

In response to osmotic stress, *S. cerevisiae* cells undergo an initial global inhibition of translation that is associated with a transient reduction in active ribosomes and a decrease of ribosome association to mRNAs, which then recovers during adaptation (Warringer *et al.*, 2010, Halbeisen and Gerber, 2009, Melamed *et al.*, 2008, Uesono and Toh, 2002). During mild osmotic shock, initial rapid global inhibition of translation is shown to be faster than the transcriptional response and seems to be independent of Hog1 activity, however, Hog1 does play a role in translation recovery as well as translation reprogrammation of many genes (Figure 1.7) (Garre *et al.*, 2012, Warringer *et al.*, 2010). Importantly, genome-wide studies show that, despite global translation inhibition following stress, the translation of a subset of genes is induced, and gene ontology studies describe these genes to function in general cellular protection from osmotic stress, including glycerol synthesis (*GPD1*) and sugar transporters (*STL1*) (Warringer *et al.*, 2010, Garre *et al.*, 2012, Melamed *et al.*, 2008). Conversely, a subset of genes is also shown to be translationally downregulated, and a large percentage of these genes encode for ribosomal proteins or are associated with other aspects of the translational machinery (Melamed *et al.*, 2008, Warringer *et al.*, 2010). The majority of gene expression changes are found to be homodirectional, where transcriptional and translational changes are complementary; however, many genes are found to display counterdirectional changes, where transcription and translation are inversely regulated (Warringer *et al.*, 2010). On the contrary, translational regulation under sever osmotic stress is mediated by pathways other than the HOG pathway (O'Rourke and Herskowitz, 2004, Melamed *et al.*, 2008).

mRNA stability, together with transcription, determines transcript steady-state levels, therefore, its regulation forms an important part of gene expression control (Perez-Ortin, 2007). Upon osmotic stress, a very rapid initial destabilisation of mRNA occurs independently of Hog1 that recovers after a short time (Romero-Santacreu *et al.*, 2009, Canadell *et al.*, 2015). In the early phase of adaptation, the mRNAs of osmostress-protective genes become transiently stabilised, while those encoding ribosomal proteins are destabilised. In contrast, it has been described that during recovery phase, there is a Hog1-dependent loss of stability of osmostress-protective mRNAs and a gain of stability of growth-relative mRNAs, including RP mRNAs (Figure 1.7) (Molin *et al.*, 2009, Romero-Santacreu *et al.*, 2009). Additionally, it has been observed that there is a decrease in RP pre-mRNA levels during osmotic stress, which is regulated by NMD (Garre *et al.*, 2013). Hence, this suggests that perhaps NMD plays a role in the regulation pre-mRNA stability of other genes during osmotic stress.

The response to osmotic shock corresponds to an increase in P-body formation (Romero-Santacreu *et al.*, 2009, Teixeira *et al.*, 2005), and it has been suggested that P-bodies harbour non-translating mRNPs to provide a way to transiently arrest translation of a subset of mRNA in order to re-establish these mRNAs to the translation cycle upon adaptation (Teixeira *et al.*, 2005, Romero-Santacreu *et al.*, 2009). Although Hog1 does not appear to be essential for P-body assembly, it has been observed that P-bodies assembly is delayed and prolonged in a *hog1Δ* mutant (Romero-Santacreu *et al.*, 2009).

On the other hand, severe osmotic stress produces a global non-specific mRNA stabilisation that is independent of Hog1, which correlates with an increase in P-body assembly observed in both wild type and *hog1Δ* mutant, suggesting that stabilised mRNAs are retained in P-bodies (Romero-Santacreu *et al.*, 2009). These results also correlate with delayed and prolonged global translation inhibition during severe osmotic stress that is Hog1-independent (Uesono and Toh, 2002). Taken together, it can be envisioned that severe osmotic stress induces a Hog1-independent mechanism that yields translation inhibition, mRNA stabilisation, and movement of stabilised mRNAs into P-bodies.

1.4 The response to pheromones

In response to mating pheromones, the yeast cell undergoes a series of physiological changes in preparation for mating. These include significant changes in gene expression, cell-cycle arrest, polarised growth towards the mating partner through the formation of the sexual project, the shmoo, and ultimately cell fusion of mating partners.

1.4.1 MAPK pathway of the pheromone response

The cellular response to pheromones is mediated by the pheromone MAPK signalling pathway, which is arguably the most throughly characterised MAPK multi-component signalling network in any eukaryote (see Figure 1.9). This pathway is activated through the binding of cell surface G-coupled receptors Ste2/Ste3 to pheromones a-/α-factor released by cells of the opposite mating type. Receptor occupancy activates a coupled G protein on the inner face of the plasma membrane, resulting in the dissociation of the βγ subunit complex (Gβγ), which activates the PAK kinase Ste20 that is anchored to the Rho GTPase Cdc42 at the plasma membrane, and interacts with the core

scaffold protein Ste5. After pheromone stimulus, Ste5 is rapidly translocated to the plasma membrane by Gβγ, where it initiates and amplifies mating signalling (Mahanty *et al.*, 1999, Pryciak and Huntress, 1998). Ste5 simultaneously binds Ste20 and all other components of the MAPK module through distinct domains, which results in the transduction of signal through a MAPK signalling cascade involving the common MAPKKK Ste11 shared by HOG and FG pathways, the MAPKK Ste7 and the MAPK Fus3/Kss1 (Figure 1.9) [reviewed by (Bardwell, 2005, Merlini *et al.*, 2013, Zhou *et al.*, 1993).

Fus3 and Kss1 are partially redundant kinases that regulate yeast mating processes, including induction of mating-specific genes, cell-cycle arrest and polarised morphogenesis [reviewed by Bardwell (2005)]. Activated Fus3 enters the nucleus and phosphorylates Dig1 and Dig2, which are negative regulators of the transcription factor Ste12, leading to the transcription of genes required for cell and nuclear fusion, including *FUS1* (Figure 1.9) (Oehlen *et al.*, 1996, Tedford *et al.*, 1997, Errede and Ammerer, 1989). Activated Fus3 also binds and phosphorylates Far1, which is a multifunctional regulator of the mating process (Merlini *et al.*, 2013, Gartner *et al.*, 1998). Far1 induces pheromone-dependent cell-cycle arrest by regulating the cyclin-dependent kinase Cdc28 (Gartner *et al.*, 1998, Peter *et al.*, 1993). A second, apparently independent, function of Far1 is to determine the site of cell polarisation. During mating, Far1 recruits Cdc24, the guanine nucleotide exchange factor (GEF) for Cdc42, and translocates it from the nucleus to the cell cortex, where the Far1-Cdc24 complex interacts with Gβγ at the site of receptor activation. The complex further recruits Cdc42 and the scaffold Bem1 away from the bud site in order to ensure orientated cellular polarisation towards a morphogenetic gradient (Figure 1.9) (Arkowitz, 1999, Gulli and Peter, 2001, Wiget *et al.*, 2004, Gulli *et al.*, 2000). However, the disruption of *FAR1* does not affect the shmooing ability of the cell, but impairs the localisation of polarity factors at the shmoo, thus leading to the formation of a mislocalised shmoo at the bud site (Nern and Arkowitz, 2000). In contrast, mutations in *FUS3* cause profound cell fusion defects (Elion *et al.*, 1990, Nelson *et al.*, 2004), indicating that polarised cell growth requires other factors regulated by Fus3, and one such factor is the formin Bni1 (Matheos *et al.*, 2004).

1.4.2 The polarisome

Bni1, a major contributor to polarised growth during the response to pheromones, belongs to the highly conserved family of formins, all characterised by the presence of the FH2 (**F**ormin **H**omology 2) domain, which functions in the nucleation of actin filaments and enables barbed end elongation

(Pruyne *et al.*, 2002, Faix and Grosse, 2006, Kovar, 2006). Formins are also characterised by the presence of proline-rich FH1 domain, which binds profilin, a protein that facilitates actin subunit delivery (Kovar, 2006, Evangelista *et al.*, 1997). Bni1 together with Bnr1 constitute the only two existing formins in *S. cerevisiae*, and while Bni1 has roles in both yeast budding and mating, Bnr1 only functions in budding (Goode and Eck, 2007, Imamura *et al.*, 1997, Qi and Elion, 2005). Following pheromone stimulation, the Bni1 protein has been identified to undergo two post-translational changes: first, Bni1 is phosphorylated by Fus3 (Matheos *et al.*, 2004); second, Cdc42 binds to Bni1 and relieves an auto-inhibitory loop to activate the FH2 domain to mediate actin polymerisation (Dong *et al.*, 2003). Both Fus3 and Cdc42 have been shown to be required for the localisation of Bni1 at the shmoo tip (Matheos *et al.*, 2004, Qi and Elion, 2005). In a positive feedback loop, Bni1 contributes to the polarised recruitment of the MAPK scaffold Ste5, the Cdc42-GEF Cdc24 and Fus3, which results in the amplification of signal and efficient Fus3 activation (Qi and Elion, 2005).

At the shmoo tip, Bni1 constitutes part of a protein complex, termed the polarisome, with Spa2, Bud6 and Pea2, and together, the polarisome mediates polarised actin assembly towards the pheromone gradient. Bni1 has been found to physically interact with Spa2, through its Spa2-binding domain, and Pea2 (Fujiwara *et al.*, 1998, Sheu *et al.*, 1998, Tcheperegine *et al.*, 2005), in which Spa2 and Pea2 have been described to act upstream of Bni1 to regulate the timing of shmoo formation (Bidlingmaier and Snyder, 2004). Finally, the polarisome localises the cell fusion protein Fus1, whose expression is induced by pheromone signalling, at the shmoo tip to initiate the process of cell fusion. Interestingly, components of the polarisome as well as Fus1 are required for the termination of projection growth and delocalisation of actin (Figure 1.9) (Bidlingmaier and Snyder, 2004). Moreover, to ensure signal specificity, Fus1 has been found to directly interact with Sho1 to downregulate HOG signalling during cellular response to pheromone in order to ensure efficient cell fusion (Nelson *et al.*, 2004).

In Chapter 5 of this thesis, we describe the role of the translation elongation factor eIF5A in the yeast pheromone response.

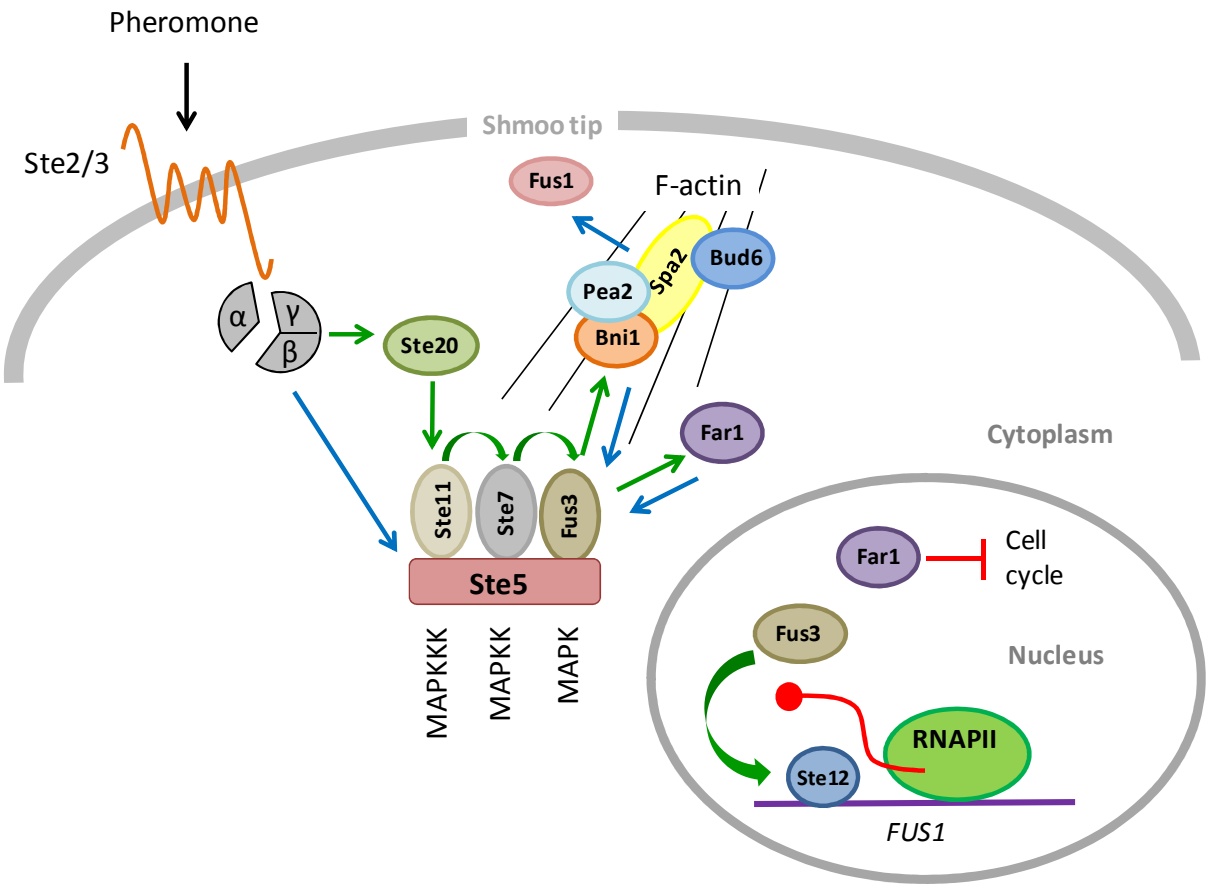


Figure 1.9 Schematic representation of the yeast pheromone response. Binding of a or α pheromone to G-coupled receptors Ste2 or Ste3, respectively, results in the dissociation of the βγ subunit, which initiates the signalling cascade involving consecutive phosphorylations of MAP kinases Ste11, Ste7 and Fus3. Activated Fus3 translocates to the nucleus and activates transcription of pheromone-responsive genes, such as *FUS1*, through the TF Ste12. Fus3 phosphorylates and activates Far1, which mediates cell cycle arrest in the nucleus. In the cytoplasm, Far1 in turn localises Fus3 at the shmoo site, and there Fus3 recruits the polarisome, consisting of Pea2, Spa2, Bud6 and Bni1. Formin Bni1 polymerises actin (straight black lines) towards the shmoo tip. The cell fusion protein Fus1 is recruited to the shmoo tip by the polarisome. Green arrows indicate sequential activation of the pathway components, blue arrows indicate recruitment of proteins to be localised at the shmoo tip, and red T signifies inhibition.

1.5 The RNA cap-binding complex CBC

As nascent pre-mRNA emerges from the RNA exit channel of RNAPII and reaches 22-25 nucleotides during transcription, a characteristic 7-methylguanosine (m^7GpppN) cap structure is attached co-transcriptionally to the 5´ end of the nascent mRNA by three sequential enzymatic steps, which are catalysed by Cet1, Ceg1 and Abd1 in yeast. This cap structure is recognised and bound by cap-

binding proteins (Mazza *et al.*, 2001, Mazza *et al.*, 2002, Worch *et al.*, 2005). To date, two cap-associated complexes have been described: the highly conserved CBC, consisting of a cap-binding subunit Cbp20 and an auxiliary protein Cbp80 (termed Cbc2 and Cbc1, respectively, in *S. cerevisiae*), binds the cap in the nucleus; and the eIF4F complex in the cytoplasm (refer to Figure 1.4) (Sonenberg and Hinnebusch, 2009, Lewis and Izaurralde, 1997, Topisirovic *et al.*, 2011). The m^7GpppN cap influences many aspects of mRNA life cycle: including splicing (Izaurralde *et al.*, 1994), 3´ end formation (Flaherty *et al.*, 1997), nuclear export (Gorlich *et al.*, 1996), stability (Furuichi *et al.*, 1977, Das *et al.*, 2000), translation (Shatkin, 1985), and, documented more recently, transcription (Lahudkar *et al.*, 2011) (see Figure 1.10).

CBC was first purified from nuclear extracts of HeLa cells based on its affinity for m^7GpppN, and it was demonstrated to be consisted of 20 kDa and 80 kDa peptides which were termed Cbp20 and Cbp80 respectively (Izaurralde *et al.*, 1994, Izaurralde *et al.*, 1995a). Cbp20 is found to be unstable as a monomer as its presence is undetectable in Cbc80-depleted extracts (Izaurralde *et al.*, 1995a), and in contrast, Cbp20 does not seem to be required for Cbp80 stability (Wong *et al.*, 2007). The two subunits of the CBC bind mRNA cap synergistically, as neither has sufficient affinity to the structure alone (Izaurralde *et al.*, 1994, Izaurralde *et al.*, 1995a). The cap-binding pocket resides in Cbp20, however, Cbp80 is required to increase its affinity to the cap structure in which their interaction causes a conformational change in Cbp20. The structure of Cbp80 consists of three helical domains connected by two linkers, where the N-terminal helical domain mediates its interaction with Cbp20 (Calero *et al.*, 2002, Mazza *et al.*, 2001). In addition, Cbp80 contains a nuclear localisation signal (NLS) in its N-terminus, and most likely, the two subunits are co-imported into the nucleus (Izaurralde *et al.*, 1995b).

The importance of CBC to cellular function has been extensively described in mammalian, yeast and plant cells. Although in *S. cerevisiae* the *CBC1* and *CBC2* genes are not essential for cell viability, they have been shown to be required for cell growth and proliferation (Fortes *et al.*, 1999). In *Arabidopsis thaliana*, disruption of the CBC genes is not lethal, but results in developmental delays, reduced stature and abscisic acid (ABA) hypersensitivity (Hugouvieux *et al.*, 2001). Finally, siRNA knockdown of CBC in mammalian cells results in the deregulation of ~400 genes as well as significant reduction in cell proliferation (Narita *et al.*, 2007). In all, CBC has been found to play an important role in cellular function from plants to mammals.

1.5.1 Functional roles of CBC

The CBC has been described to function in many aspects gene expression, including pre-mRNA splicing, RNA export, mRNA stability, translation, non-sense mediated decay (NMD) and various stages of transcription, and it can be assumed that many new roles are yet to be elucidated (Figure 1.10).

First, in the yeast nucleus CBC forms part of the splicing commitment complex, and its deletion causes a reduction in the recruitment of several factors of the splicing machinery to the nascent transcript, resulting in inhibition of co-transcriptional spliceosome assembly and reduced splicing (Figure 1.10) (Zhang and Rosbash, 1999, Gornemann *et al.*, 2005). In mammalian cells, a direct interaction between CBC and U4/U6.5U tri-snRNPs (small non-coding nuclear RNA-protein complex), which together form the active site of the spliceosome, has been observed (Pabis *et al.*, 2013). Furthermore, CBC has been described to facilitate alternative splicing by engaging with P-TEFb and Ser2-phosphorylated RNAPII (Lenasi *et al.*, 2011).

Second, it was found in *Xenopus* that CBC was required for the nuclear export of U small nuclear RNAs (U snRNAs) through its interaction with PHAX (**PH**osphorylated **A**daptor of RNA e**X**port) (Izaurralde *et al.*, 1995a, Hamm and Mattaj, 1990). In *S. cerevisiae*, CBC has been found to physically interact with SRP1 (importin-α) in the nucleus and once in the cytoplasm, importin-β dissociates CBC from RNA, suggesting a role for CBC in the nuclear export of RNA (Gorlich *et al.*, 1996). Furthermore, CBC also directly interacts with the RNA-binding protein Npl3, and since Npl3 has been described to carry poly(A) mRNA from nucleus to cytoplasm, therefore, this study reinforces the idea that CBC may play a role to facilitate mRNA nuclear export (Figure 1.10) (Shen *et al.*, 2000, Lei *et al.*, 2001).

Third, the cap structure protects RNA from degradation by 5′-3′ exonucleases in both the nucleus and the cytosol, in which capped mRNA is far more stable than its uncapped counterpart (Grudzien *et al.*, 2006). In part, the stability is mediated by CBC as it competes with decapping complexes for binding to the m^7GpppN cap (Grudzien *et al.*, 2006, Schwartz and Parker, 2000). Additionally, CBC has been found to inhibit the initial step in mRNA degradation by inhibiting the PARN deadenylase which catalyses poly(A)-tail removal (Balatsos *et al.*, 2006). Furthermore, Cbc1 has been described in yeast to drive a novel nuclear degradation pathway, the DRN (**D**ecay of **R**NA in the **N**ucleus) pathway, that acts on mRNAs partially retained in the nucleus (Das *et al.*, 2003).

Fourth, CBC drives the first round of translation, known as the pioneer round of translation, through its interaction with the translation machinery; however its mode of function is still unclear. It was first discovered, in a yeast synthetic lethality screen, that the deletion of Cbc1 results in lethality

when combined with an eIF4G protein deficient in eIF4E binding, which led to the observation that CBC physically interact with eIF4G *in vitro*, suggesting that CBC may play a role in translation initiation (Fortes *et al.*, 2000). However, a later study by the same laboratory suggested that the eIF4G-CBC interaction is dispensable in yeast and not required for mRNA translation (Baron-Benhamou *et al.*, 2003). In human cells, CTIF (**C**BC-dependent **T**ranslation **I**nitiation **F**actor), a factor required during the initiation of the pioneer round of translation, is found to interact with Cbp80 to mediate the recruitment of the 40S ribosomal subunit to CBC-bound mRNA (Kim *et al.*, 2009, Choe *et al.*, 2012). More recently, it has been demonstrated in yeast that Cbc1 displays a synthetic lethal relationship with eIF4E. In addition, cells lacking Cbc1 are hypersensitive to the translation inhibitor cycloheximide, indicating translational defects, and Cbc1 itself is observed to be associated to translating polysomes (Garre *et al.*, 2012). Taken together, these results indicate that Cbc1 indeed plays a role in mRNA translation (Figure 1.10).

Finally, both yeast Cbc1 and mammalian Cbp80 have been implicated in NMD. In mammalian cells, during the pioneer round of translation, Cbp80 ensures translation of correct mRNA by targeting aberrant mRNA that contains premature translation termination codons to NMD, in which Cbp80 directly interacts with Upf1, a major facilitator of NMD, and this interaction is required to mediate NMD (Figure 1.10) (Maquat *et al.*, 2010, Gao *et al.*, 2005, Ishigaki *et al.*, 2001). In *S. cerevisiae*, a deletion in *CBC1* causes a pronounced increase in steady-state levels of some long mRNAs, similar to what is observed for mutant lacking Upf1 (Das *et al.*, 2000). However, in contrast to mammalian cells, both CBC- and eIF4E-bound transcripts can be targeted to NMD (Culbertson and Neeno-Eckwall, 2005, Gao *et al.*, 2005)

Overall, CBC has been demonstrated to play a role in many aspects of gene expression, and finally, its role in transcription is starting to be unveiled.

1.5.2 Role of CBC in transcription

The complex role of CBC in transcription is just beginning to be elucidated, and recent studies suggest that CBC functions in various stages of the RNAPII transcription cycle, as well as influencing chromatin modifications (Figure 1.10).

First, Hossain *et al.* shows that CBC affects the modification state of histone H2B in *S. cerevisiae* by ensuring proper Sus1-mediated deubiquitination. This is of special importance as rounds of ubiquitination and deubiquitination of H2B occur during transcription elongation (Hossain *et al.*,

2009). Furthermore, this study demonstrates that a deletion in CBC has a global effect on gene expression, affecting ~20 % of all yeast genes (Hossain *et al.*, 2009). The role of CBC in histone modifications is further emphasised in a later study by the same group demonstrating that CBC is required for the establishment of proper histone marks during active transcription, where it has been observed that CBC mediates Set2-dependent K36 trimethylation of H3, as deletion of either Cbc1 or Cbc2 leads to a decrease in H3K36me3 throughout the coding regions of several genes (Hossain *et al.*, 2013). Second, two distinct studies describe genetic interactions between CBC and kinases that phosphorylate RNAPII CTD at Ser2, Bur1 and Cdk9, in which the deletion of CBC markedly reduces the co-transcriptional recruitment of both kinases, leading to abrogated Ser2 phosphorylation, a hallmark of transcription elongation (Hossain *et al.*, 2013, Lidschreiber *et al.*, 2013) (Figure 1.10). CBC is also proposed to recruit transcription elongation factors, including Spt6 (Lidschreiber *et al.*, 2013) and P-TEFb (Lenasi *et al.*, 2011), to promote productive transcription elongation. Finally, genetic studies by Lahudkar *et al.* show that Cbc1 physically interacts with the TF Mot1 to promote Mot1 recruitment to several yeast promoters, which in turn stimulates PIC formation and hence transcription initiation at these promoters (Figure 1.10) (Lahudkar *et al.*, 2011). However, CBC does not seem to regulate promoter clearance or PIC formation in the first round of transcription, as RNAPII accumulation at promoters is not observed in a *cbc1Δ* mutant, indicating that CBC stimulates subsequent rounds of transcription (Lahudkar *et al.*, 2011). Furthermore, CBC appears to regulate PIC formation following mRNA capping, as defects in mRNA capping events yield reduced recruitment of RNAPII and TBP (Lahudkar *et al.*, 2011).

Despite mounting evidence, other studies indicate that the active role of CBC in transcription is more complex. It has been described by Wong *et al.* that CBC does not play a critical role in promoting the rate or processivity of transcription elongation by RNAPII, as both RNAPII runoff and RNAPII occupancy experiments show no difference between wild type and a mutant with a deletion in *CBP80* gene (Wong *et al.*, 2007).

Overall, recent studies provide evidence for the emerging role of CBC in transcription, and this thesis further describes the mechanisms employed by CBC to promote transcription.

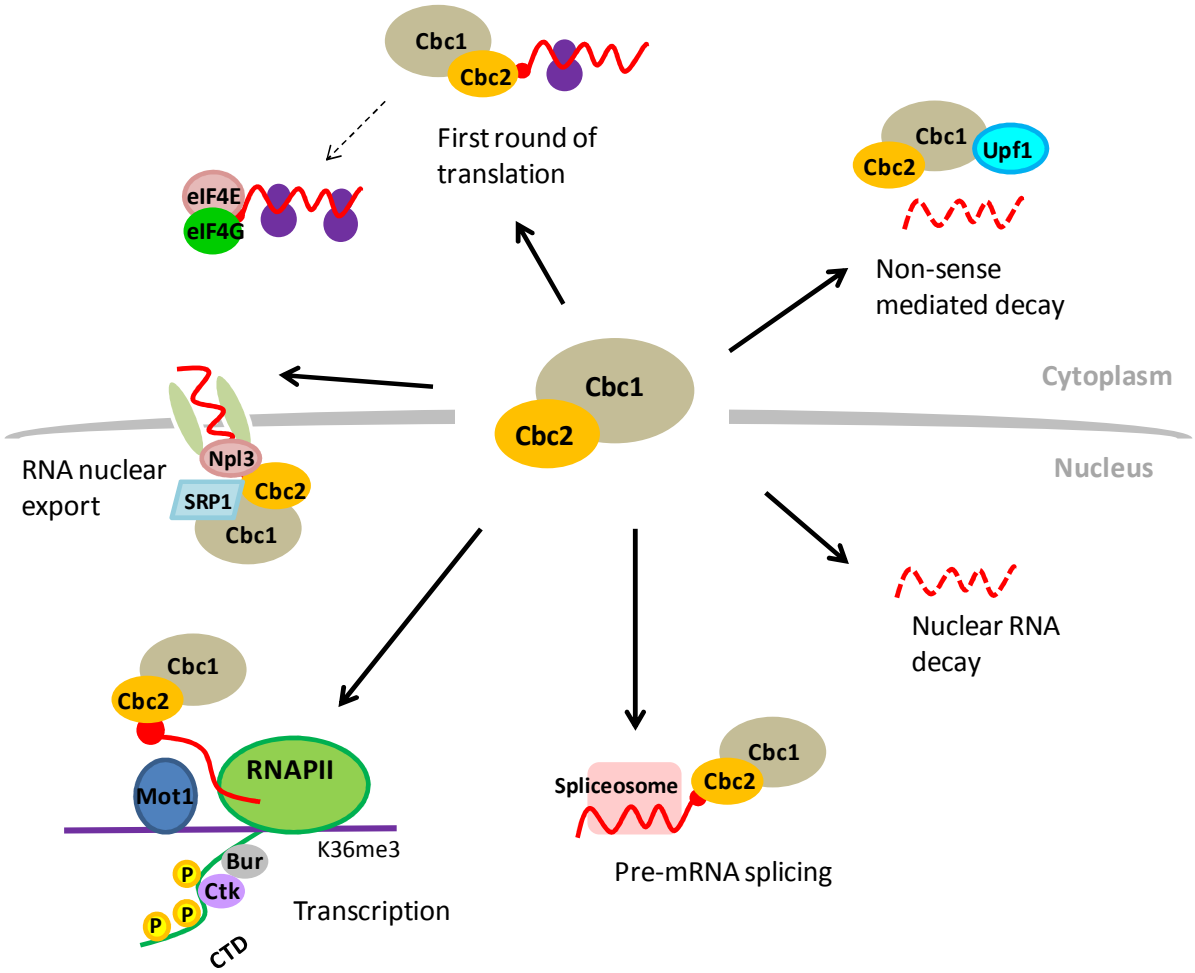


Figure 1.10 The cap-binding complex (CBC) have roles in various aspects of gene expression. In the nucleus, CBC, composed of Cbc1 and Cbc2 (Cbp80 and Cbp20, respectively, in mammalian cells) promotes transcription through the recruitment of Mot1 and/or RNAPII kinases Bur1 and Ctk9, as well as promoting Set2-dependent trimethylation of H3K36. CBC also participates in pre-mRNA splicing and the degradation of nuclear mRNA. CBC interacts with Srp1 and Npl3 and may have a role in RNA nuclear export. In the cytoplasm, CBC mediates the pioneer round of translation, from which aberrant mRNAs are sent to non-sense mediated decay (NMD). After replacement of CBC by eIF4F on correct mRNAs, CBC recycles back to the nucleus.

1.5.3 Role of CBC in hyperosmotic stress

A previous study conducted in our laboratory originally identified the *CBC1* gene to be important in osmotic stress response through a screening in search for deletion mutants that showed reduced tolerance to NaCl, and it was observed that *cbc1Δ* showed sensitivity and loss of viability under

osmotic stress. Cbc1 was further identified as a coordinator of the rapid translational response after hyperosmotic stress in *S. cerevisiae* (Garre *et al.*, 2012). In response to osmotic stress, global translation is rapidly inhibited to allow selective translation of osmostress-responsive mRNAs that encode stress-protective proteins (Yamasaki and Anderson, 2008, Warringer *et al.*, 2010). Using polysome fractioning studies, Cbc1 is shown to be important in the re-initiation of translation after osmotic stress, as a strain harbouring a deletion in the *CBC1* gene show a delayed recovery of polysomes after osmotic shock. The delay in translation recovery observed for *cbc1Δ* corresponds to a delay in P-body disassembly after adaptation, indicating that a role for Cbc1 in P-body disassembly, the return of mRNA to polysomes, and reinitiation of translation. Furthermore, Cbc1 is required for the rapid translation of specific osmostress-responsive mRNAs, as *CBC1* deletion reduces the percentage of these mRNA associated to polysomes during osmotic stress (Garre *et al.*, 2012). Interestingly, Garre *et al.* have observed that mRNA induction of osmostress-responsive genes, as well as recovery of RP mRNA, is delayed in *cbc1Δ* mutant in response to osmotic stress (Garre *et al.*, 2012).

Another recent study conducted in *Arabidopsis thaliana* shows that Cbp20 and Cbp80 function in a coordinated fashion to modulate the salt stress response (Kong *et al.*, 2014). Their findings show that *cbp20* and *cbp80* mutants exhibit increased sensitivity to salt stress, and that both proteins function synergistically to modulate pre-mRNA alternative splicing, as well as protein ubiquitination and sumoylation during osmotic stress (Kong *et al.*, 2014).

In all, yeast and *Arabidopsis* results indicate that CBC plays a key role in cellular response to osmotic stress by regulating several stages of mRNA life, and therefore gene expression, although all precise mechanisms involved are still unclear.

1.6 The role of translation factor eIF5A

The essential protein eIF5A (**e**ukaryotic Translation **I**nitialtion **F**actor **5A**) is evolutionarily conserved from archaea to mammals, with a distant ortholog, EF-P, in eubacteria (Park *et al.*, 2010). eIF5A is the only known protein to undergo a unique post-translational modification known as hypusination, where the amino acid residue hypusine, a derivative of spermidine, is enzymatically added to a conserved lysine residue of the protein. This modification has been shown to be essential for eIF5A activity (Schnier *et al.*, 1991). Recent studies have identified several cellular functions for eIF5A, including the formation of the first peptide bond (Benne and Hershey, 1978), translation initiation

and elongation (Benne and Hershey, 1978, Saini *et al.*, 2009), cell wall integrity (Galvao *et al.*, 2013), and mRNA degradation (Schrader *et al.*, 2006, Zuk and Jacobson, 1998b). Furthermore, hypusinated eIF5A has been implicated in the oncogenesis of several cancer types and has been identified as a target for therapeutic research (Cracchiolo *et al.*, 2004, Taylor *et al.*, 2007); however, further investigation is required to uncover the complexity and essentiality of eIF5A in cellular processes. This thesis will focus mainly on eIF5A as a translation elongation factor and its functional role in the yeast response to pheromones.

1.6.1 Isoforms of eIF5A

In *S. cerevisiae*, eIF5A is encoded by two highly homologous genes, *TIF51A (HYP2)* and *TIF51B (ANB1)*. The *TIF51A* gene is essential under normal aerobic conditions in which *TIF51B* expression is not observed, whereas cells under anaerobic conditions will only express *TIF51B* (Schnier *et al.*, 1991, Wohl *et al.*, 1993). The two genes are members of a duplicated gene cluster and encode proteins that share 90 % amino acid identity, as such that they can substitute each other, indicating identical functionality (Kang *et al.*, 1992). All eukaryotes contain a homolog of eIF5A, and while in some organisms, such as humans, eIF5A is encoded by two genes, in others, such as *Drosophila*, it is encoded by only one gene (Figure 1.11).

In humans, two isoforms of eIF5A exist, eIF5A-1 and eIF5A-2, and both harbour the hypusine modification (Clement *et al.*, 2006, Caraglia *et al.*, 2013). The first isoform, eIF5A-1, is constitutively expressed in all cells and is particularly abundant in proliferating cells. It is, in fact, overexpressed in human cancer cells, and its mRNA expression was found to be regulated by the human c-Myc oncogenic transcription factor (Caraglia *et al.*, 2013, Coller *et al.*, 2000). In contrast, the expression of eIF5A-2 is normally very low and tissue-specific, found mainly in testis and certain parts of the brain. Similarly, overexpression of the *EIF5A2* gene has been detected in several cancer cell lines, including ovarian and colorectal cancer (Clement *et al.*, 2006, Guan *et al.*, 2004), suggesting a role in cancer development. Furthermore, *in vitro* overexpression of eIF5A-2 has been observed to induce cellular transformation, as well as associated with a role in tumour metastasis, and clinically, its expression has been linked to poor prognosis (Guan *et al.*, 2004, Tang *et al.*, 2010, He *et al.*, 2011). There is 60-65 % identity between human and yeast eIF5A, where the N-terminal region is highly conserved amongst eukaryotes, and in contrast, the C-terminus is less conserved (Figure 1.11).The sequence of 12 amino acids surrounding the Lys residue that undergoes the hypusine modification (STSKTGKHGHAK) is invariable in all eukaryotes (Figure 1.11A), suggesting the importance of

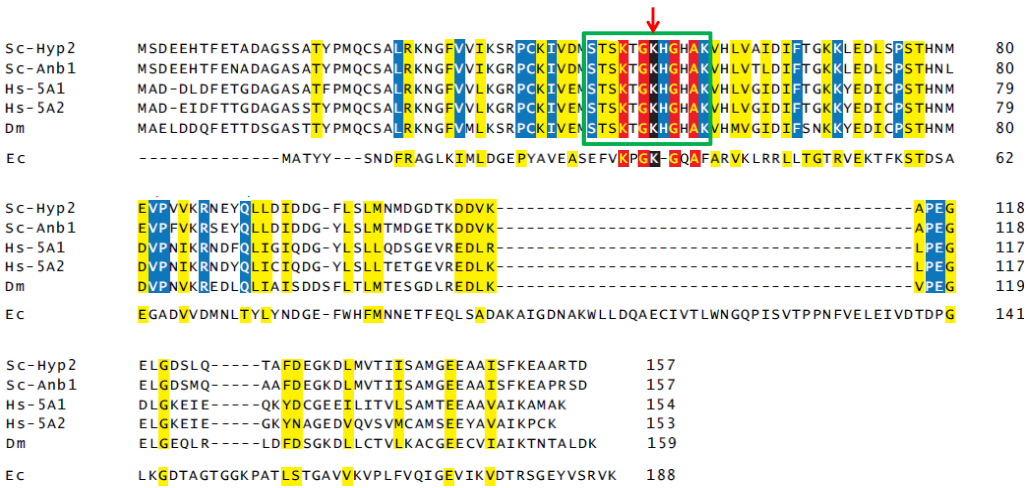
hypusination to fundamental cellular function throughout eukaryotic evolution (Joe and Park, 1994). In *Drosophila melanogaster*, eIF5A is encoded by only one gene, whereas in *C. elegans*, the eIF5A homolog is encoded by two genes, IFF-1 and IFF-2 (van Oers *et al.*, 1999, Hanazawa *et al.*, 2004).

Eukaryotic eIF5A has an ortholog in bacteria, elongation factor P (EF-P), and, although the two proteins share no more than 20 % amino acid sequence identity, they share significant structural similarities (Figure 1.11A and Figure 1.12A). While yeast eIF5A consists of a two-lobed structure, EF-P is composed of three domains, and the N-terminal domains of both proteins superimpose, while domains II and III of EF-P are structurally similar to one another as well as to the C-terminal domain of eIF5A (Figure 1.12A) (Dever *et al.*, 2014, Rossi *et al.*, 2014). A post-translational modification correlated with hypusination occurs on the conserved lysine or arginine residue of EF-P, termed lysinylation. Although the hypusine and lysyl-lysine residues are chemically distinct, they are very similar structurally and are expected to serve the same functional purpose (Figure 1.12) (Dever *et al.*, 2014, Rossi *et al.*, 2014).

1.6.2 From spermidine to hypusine

Hypusine [N^{ε}-(4-amino-2-hydroxybutyl)-L-lysine] is a spermidine-derived amino acid residue that is post-translationally added to the eIF5A protein in a process that involves two enzymatic steps. The first step involves the enzyme deoxyhypusine synthase (DHS), encoded by the yeast essential gene *DYS1,* transferring an aminobutyl moiety from the polyamine spermidine to a specific lysine group in the N-terminus of eIF5A. In the second step, the enzyme deoxyhypusine hydroxylase (DOHH), encoded in yeast by the *LIA1* gene, yields the mature and active hypusinated eIF5A protein through the addition of a hydroxyl group (see Figure 1.12B) (Park *et al.*, 1993, Abbruzzese *et al.*, 1989). It is remarkable that both enzymes, which act on just one protein, are evolutionary conserved (Park *et al.*, 2010). It is noteworthy that the enzyme DOHH is not essential in budding and fission yeasts (Park, 2006, Weir and Yaffe, 2004), however, in contrast the loss of DOHH is recessively lethal in *C. elegans* (Sugimoto, 2004), *Drosophila* (Patel *et al.*, 2009) and mouse (Sievert *et al.*, 2014), suggesting that the hydroxyl group on hypusine is more critical for eIF5A function in higher eukaryotes. Furthermore, recent studies showed that recombinant human eIF5A produced in bacteria that are either hypusinated or deoxyhypusinated results in equivalent abilities to stimulate translation *in vitro*, indicating that deoxyhypusinated eIF5A appears to be at least partly functional (Park *et al.*, 2011).

A



B

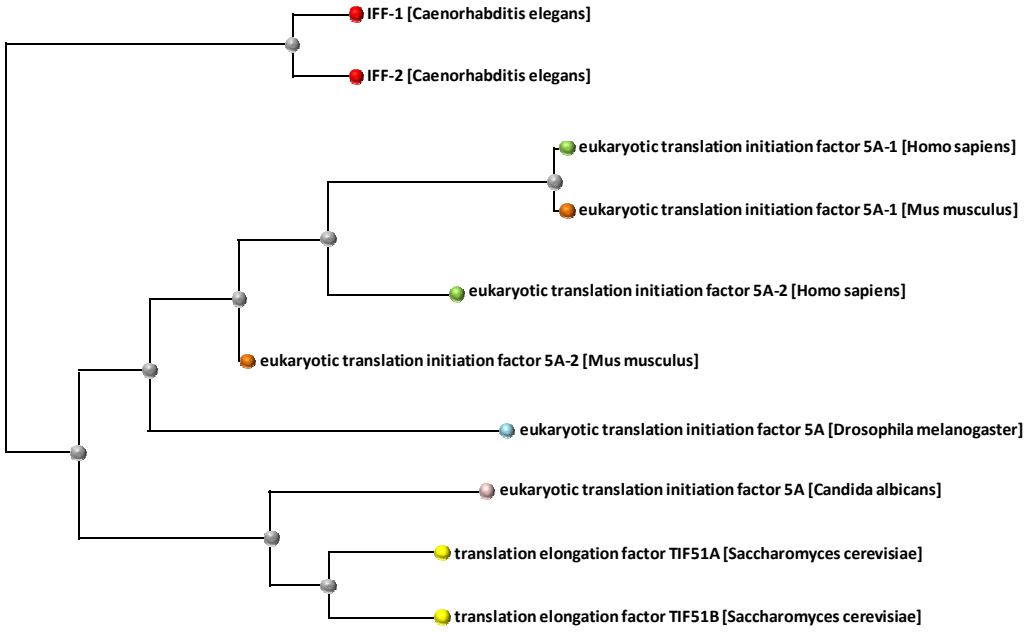


Figure 1.11 Evolutionary homologs of eIF5A. (A) Amino acid sequence alignment of genes encoding homologs TIF51A (Sc-Hyp2) and TIF51B (Sc-Anb1) in *S. cerevisiae*, eIF5A-1 (Hs-5A1) and eIF5A-2 (Hs-5A2) in *H. sapiens*, eIF5A in *D. melanogaster* (Dm) and EF-P in *E. coli*. Residues in red are identical in all eIF5A and EF-P, residues in blue are identical only in eukaryotic eIF5A, and residues in yellow are conserved between all eIF5A and EF-P (Dever *et al.*, 2014). The 12 amino acid residues surrounding the lysine residue that becomes hypusinated (in black and indicated by a red arrow) are identical in all eIF5A homologs and it is marked by a green box. **(B)** Phylogenic tree alignment of eIF5A homologs in *S. cerevisiae* (Tif51A and Tif51B; yellow circle), *Candida albicans* (pink circle), *H. sapiens* (eIF5A-1 and eIF5A-2; green circle), *M. musculus* (eIF5A-1 and eIF5A-2; orange circle), *D. melanogaster* (blue circle), and *C. elegans* (IFF-1 and IFF-2; red circle) utilising the BLAST Tree View tool (NCBI). The Fast Minimum Evolution algorithm (Desper and Gascuel, 2004) was applied, where the evolutionary distance (or dissimilarities) between two sequences is modeled as the expected fraction of amino acid substitutions per site given the fraction of mismatched amino acids in the aligned region.

The bacterial EF-P undergoes lysinylation catalysed by the enzymes YjeK, YjeA and YfcM. YjeK first converts α-lysine to β-lysine, which is then added to EF-P by YjeA to form β-lysyl-lysine. Finally, YfcM adds a hydroxyl group to complete the post-translational modification of EF-P [reviewed in (Figure 1.12B) (Dever *et al.*, 2014). Similar to the non-essential nature of yeast DOHH *LIA1*, loss of *yfcM* in *E. coli* does not affect growth or antibiotic sensitivity, raising the question to the function of the hydroxyl modification on EF-P (Bullwinkle *et al.*, 2013).

Polyamines (putrescine, spermidine and spermine) are clearly essential for life since inactivation of genes encoding early steps in the pathway of their biosynthesis is lethal; however, their exact mode of action is far from being resolved (Terui *et al.*, 2007, Pegg and Wang, 2009). In *S. cerevisiae*, the metabolic synthesis of spermidine requires the proteins encoded by genes *SPE1*, *2* and *3*. Under spermidine-limiting conditions, yeast *spe2Δ* mutants show morphological abnormalities and most of intracellular spermidine is used for the hypusine modification of eIF5A, suggesting that activation of eIF5A is the most important function of spermidine in yeast cells (Chattopadhyay *et al.*, 2008a). In mammalian cells, spermidine regulates translation at initiation and elongation steps by unknown mechanisms that are partially dependent on eIF5A. These data suggest that the role of polyamines in translation can account for their essentiality in cellular proliferation (Landau *et al.*, 2010). Spermidine is also indispensable for fertilisation efficiency in eukaryotes, from humans to yeast (Lefevre *et al.*, 2011). During the pheromone response of *S. cerevisiae*, spermidine is essential for the formation of the shmoo towards the pheromone gradient in order to mediate cell fusion (Bauer *et al.*, 2013). Spermidine is not only crucial for yeast mating but also essential for effective fertilisation in *C. elegans*, suggesting that its role in fusion of haploid gametes is conserved in higher eukaryotes (Bauer *et al.*, 2013).

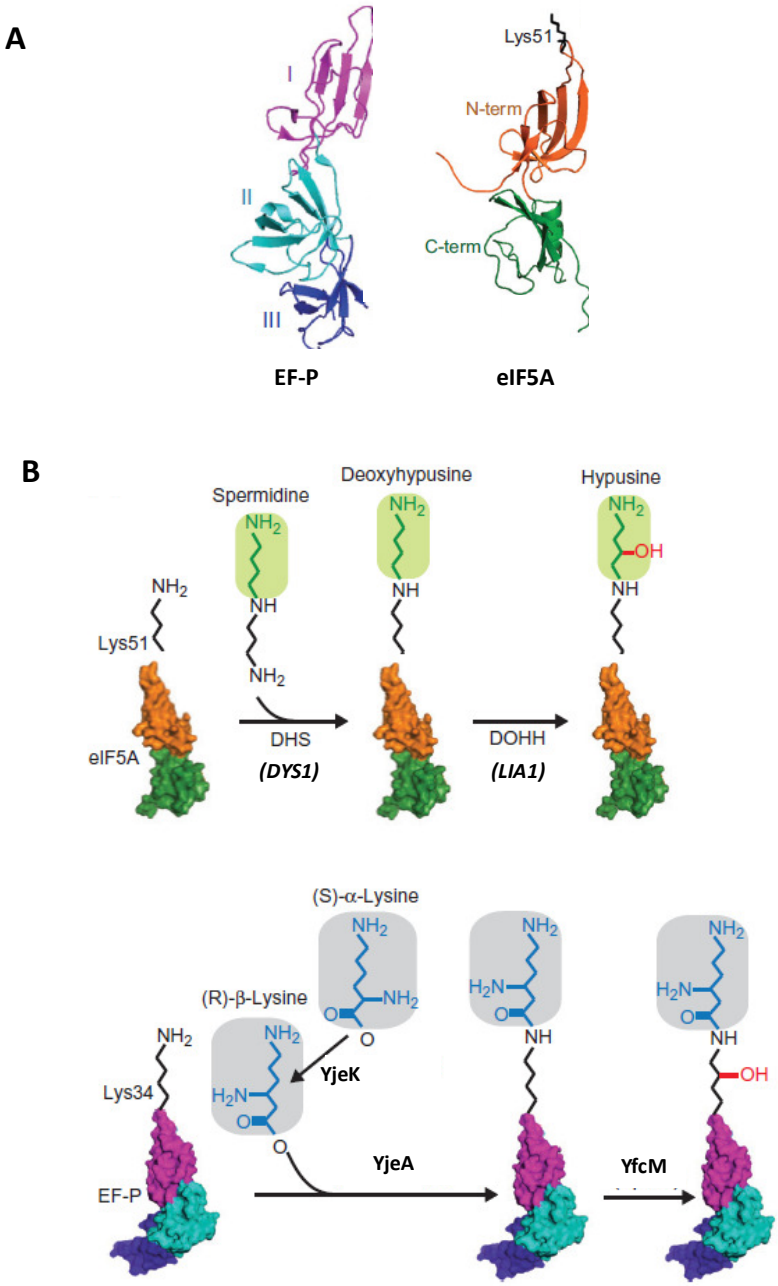


Figure 1.12 Bacterial EF-P and eukaryotic eIF5A post-translational modification pathways (Dever *et al.*, 2014). (A) Structure of bacterial EF-P (left) showing domain I (magenta), domain II (cyan) and domain III (blue). Structure of *S. cerevisiae* eIF5A showing N-terminal domain (orange) and C-terminal domain (green). **(B top panel)** In eukaryotes, n aminobutyl moiety is transferred from spermidine to the ε-amino group of the specific lysine residue (Lys50 and Lys51 in human and yeast eIF5A, respectively) by the enzyme deoxyhypusine synthase (DHS), encoded by the *DYS1* gene in yeast. The enzyme deoxyhypusine hydroxylase (DOHH), encoded by the *LIA1* gene in yeast,, adds a hydroxyl group to convert the deoxyhypusine to hypusine. **(B bottom panel)** In bacteria, the lysine aminomutase YjeK first converts α-lysine to β-lysine, followed by the addition of β-lysine to a specific lysine residue (Lys34 in E. coli EF-P). Finally, YfcM hydoxylates the lysine residue. Colour scheme used is the same as **(A).**

1.6.3 Molecular and physiological functions of eIF5A

eIF5A was originally characterised as a translation initiation factor for its ability to stimulate the transfer of methionine from Met-tRNAi in the 80S initiation complex to the aminoacyl-tRNA analog puromycin, an *in vitro* assay that mimics the formation of the first peptide bond (Kemper *et al.*, 1976). Similarly, the structurally-related bacterial ortholog, EF-P, was also identified by its ability to carry out translation *in vitro* using *E. coli* (Glick and Ganoza, 1975). To further establish eIF5A as a factor of translation, endogenous eIF5A was observed to bind with actively translating 80S ribosomes in a RNA- and hypusine-dependent manner (Jao and Chen, 2006). However, it was shown that depletion of eIF5A in yeast only causes a partial decrease (30 %) in protein synthesis rate, arguing against its role as a general translation initiation factor (Kang and Hershey, 1994). On the other hand, evidence for the essentiality of eIF5A to cellular proliferation is clear, as its depletion in yeast causes cell cycle arrest at G1 phase, and similarly in mammalian cells, inhibition of hypusination lead to G1/S cell cycle arrest (Hanauske-Abel *et al.*, 1994b, Kang and Hershey, 1994). Later reports, using polysome analyses, showed that eIF5A may in fact act as a translation elongation factor, in which inactivation of a yeast eIF5A temperature-sensitive mutant resulted in polysome accumulation in the absence of the translation elongation inhibitor cycloheximide, yielding polysome profiles similar to those of elongation factor mutants (Saini *et al.*, 2009). Furthermore, it was shown that depletion of eIF5A increases the transit time of ribosomes along mRNAs, and genetic analyses revealed a physical interaction between eIF5A and the known elongation factor eEF2 (Saini *et al.*, 2009, Dias *et al.*, 2012). Recently, it has been shown that active bacterial EF-P is essential for translation of proteins with three or more consecutive proline residues by alleviating ribosome stalling and stimulating the formation of peptide bonds between two proline residues (Ude *et al.*, 2013, Doerfel *et al.*, 2013). The relief of ribosome stalling events by eIF5A has also been recently described in *S. cerevisiae*. It was shown that eIF5A is required for the translation of polyPro-containing reporters with three or more consecutive proline residues both *in vivo* and *in vitro* (Gutierrez *et al.*, 2013). Although through recent research, the molecular function eIF5A is beginning to be understood, the physiological roles and molecular targets of eIF5A have yet to be elucidated.

Several functional studies give insights to the physiological role of eIF5A. First, it has been observed that depletion of eIF5A in yeast and inhibition of eIF5A hypusination in higher eukaryotes cause cell cycle arrest at G1 phase, which suggests a function in the control of cell cycle progression (Hanauske-Abel *et al.*, 1994a, Kang and Hershey, 1994). Furthermore, eIF5A has also been linked to nucleocytoplasmic transport and mRNA decay, including the nonsense-mediated decay (NMD) pathway, although is it unclear whether this is direct or indirect (Zuk and Jacobson, 1998a, Valentini *et al.*, 2002, Ruhl *et al.*, 1993, Schrader *et al.*, 2006).

In yeast, the overexpression of factors involved in proper actin organisation can suppress the temperature-sensitive phenotype of eIF5A mutants (Valentini *et al.*, 2002, Zanelli and Valentini, 2005). It was also documented that eIF5A temperature-sensitive mutants showed changes in actin dynamics at restrictive temperature (Chatterjee *et al.*, 2006). A functional association between eIF5A and cell wall integrity has been established from studies showing that growth defects caused by the inactivation of eIF5A and deoxyhypusine synthase (*DYS1*) are partially suppressed in the presence of the osmotic stabiliser sorbitol, and additionally, a *DYS1* mutant is sensitive to cell wall-perturbing compounds (Galvao *et al.*, 2013, Valentini *et al.*, 2002). Furthermore, the overexpression of several components of the PKC pathway also restores growth of eIF5A mutants (Valentini *et al.*, 2002). In mice, the knockout of eIF5A causes early embryonic lethality, indicating a role in mammalian development (Nishimura *et al.*, 2012). In *C. elegans*, mutation of eIF5A produces slow larval growth, structural abnormalities and sterile adults (Hanazawa *et al.*, 2004). In all, eIF5A knockout and depletion studies, together with the reported roles of spermidine, suggest that the translation factor may play a biological role in the control of cytoskeleton organisation and fertility, which will be addressed Chapter 5 of this thesis.

Chapter 2

Materials and Methods

2.1 Media and cultures for *Saccharomyces cerevisiae*

2.1.1 Media

S. cerevisiae cells were grown in YPD or synthetic complete (SC) media. YPD was prepared with 1 % yeast extract (Pronadisa #1702.00), 2 % bacteriological peptone (Pronadisa #1616.00) and 2 % glucose (Panreac #141341-1210).

SC medium was used for the selection of plasmids, in which the required amino acids, lacking the selection marker, were added to a SC-drop out base that lacked the five essential amino acids (adenine, uracil, histidine, leucine and tryptophan). SC-drop out medium was prepared with 0.67 % yeast nitrogen base (Difco #291940), 2 % glucose, 0.138 % SC-drop out mix (Formedium #DSCK322). Filter-sterile amino acids were added to SC-drop out medium as follows with the final concentration indicated: adenine (20 mg/L; Sigma #A8626), uracil (20 mg/L; Sigma #V0750), histidine (20 mg/L; Sigma #H8000), leucine (60 mg/L; Sigma #L8000) and tryptophan (20 mg/L; Sigma #T3300).

To induce expression of plasmids driven by the *GAL* promoter, transformed cells were grown to exponential phase in SC selective medium and then transferred to SCgal selective medium [0.67 % yeast nitrogen base (Difco #291940), 2 % galactose, 0.138 % SC-drop out mix (Formedium #DSCK322)] and incubated for 4 hours.

For geneticin (G418 sulphate) selective plates, a final concentration of 200 mg/L of geneticin (Roche #1464981) was added to YPD after autoclave. For KCl and NaCl salt plates, the salts (KCl VWR #726764-1000; NaCl Panreac #131659-1211) were added at the appropriate concentrations as powder to YPD media before autoclave. For calcium chloride plates (Chapter 4), $CaCl_2$ was added as power to YPD media at the appropriate concentrations in combination with 5 mM succinic acid and buffered to pH 5.5.

LB medium was prepared to grow *E. coli* and consisted of 1 % NaCl, 1 % tryptone (Pronadisa #1612.00), 0.5 % yeast extract. To select plasmid-transformed *E. coli*, Ampicilin (Sigma #A-9518) was added to LB to a final concentration of 50 µg/ml.

For all agar plates, 2 % agar (Pronadisa #1800-00) was added before autoclave. All media were autoclaved for 20 minutes at 121 ºC before use.

2.1.2 Growth conditions

Yeast cellular growth in liquid media was determined by optical density (OD) at 600 nm (OD_{600}). OD_{600} units refer to absorbance at OD_{600} multiplied by the volume of liquid culture in millilitres. All wild type strains and null mutants were grown at 30 ºC (or otherwise indicated). Thermal-sensitive mutants were maintained at the permissive temperature of 25 ºC, and at exponential phase, were transferred to the the restrictive temperature of 37 ºC to annul gene expression. Yeast strains were maintained on YPD agar plates and stored at 4 ºC. For experiments, yeast cells were grown in liquid media with shaking at 190 rpm until exponential phase (OD_{600} 0.5-0.6).

2.1.3 Treatments

For osmotic stress treatment, exponentially growing cells were either treated with KCl in powder to a final concentration of 0.6 M for experiments described in Chapter 4 or with liquid stock of 5 M NaCl to a final concentration of 0.4 M for experiments described in Chapter 3. Control (or t=0) samples were collected before salt treatment.

For pheromone stimulation experiments described in Chapter 5, exponentially growing cells were incubated with α-factor (Sigma #T6901, or kindly provided by Dr Gustav Ammerer, University of Vienna, Austria) at a final concentration of 10 μg/ml for 2 hours or otherwise indicated.

2.2 S. cerevisiae strains

S. cerevisiae strains used in this thesis are described in Table 2.1, and the construction of new yeast strains is described below.

2.2.1 Construction of new strains by gene disruption

To construct the yeast strain PA703 *ste11Δmsb2Δ*, the strain CF83 *msb2Δ* was transformed with the *STE11::ADE2* disruption plasmid (GA2126) following digestion with BamHI (New England Biolabs #R3136) for 30 mins at 37 ºC, and transformants were selected on SC-ade plates. Transformants were tested for the successful disruption of the *STE11* gene by its inability to mate with a wild type *MATα* strain (W303α). To achieve this, transformants were mixed with W303α cells at exponential

phase and plated on SC-drop out plates lacking the five essential amino acids, and those that did not yield colonies contained successful *STE11* gene disruption.

2.2.2 Construction of new strains by genomic tagging

To genomically tag the C-termini of genes of interest with HA, a PCR-based genomic tagging technique described by Knop *et al.* was employed with changes (Knop *et al.*, 1999). Briefly, the plasmid GA2256 HA6-HIS3 served as a template for subsequent PCR reactions to generate a cassette, and all PCR reactions were carried out as described in section 2.6.4. Specific primers containing 45 bases complementary to the gene of interest with an additional 18 bases complementary to the vector were designed as previously described (Knop *et al.*, 1999). For the HA genomic tagging of *BNI1* wild type (yeast strains PAY723 and PAY725) and C-terminal-truncated (yeast strains PAY727 and PAY729) versions used in Chapter 5, the primer pairs BNI1-1/BNI1-2 and BNI1-2/BNI1-5 were used respectively (see Table 2.3). For HA genomic tagging of *HOT1* (yeast strains PAY730 and PAY732) used in Chapter 4, the primer pair HOT1-1HA/HOT1-2HA were utilised (see Table 2.3). Size of PCR products were checked by agarose gel electrophoresis (section 2.6.3) and DNA was then ethanol precipitated (section 2.6.1). The resulting cassettes were then transformed in the corresponding yeast strains and transformants were selected on SC-his plates. Correct expression of genomic tags was confirmed by western blot analyses (section 2.6.6).

To generate the strains PAY739 and PAY742 containing a genomic 6HA tag of a *BNI1* mutant harboring the deletion of polyPro stretches (amino acids 1239-1307) (*BNI1ΔPro*) in Chapter 5, the C-terminus *BNI1-HA* sequence was amplified from genomic DNA of the strain PAY723 using primers BNI1-2 and BNI1-6. The use of the primer BNI1-6 resulted in the deletion of nucleotides 3715 to 3921 in *BNI1*, which encode for the polyPro stretches of the Bni1 protein. The resulting PCR product was transformed in *tif51A-1*^{ts} and wild type cells and transformants were selected in SC-his. Correct tagging of Bni1 proteins were confirmed by protein size using western blot analyses.

Table 2.1 *S. cerevisiae* strains used in this thesis.

Strain	Genotype	Relevant genotype	Source
BY4741	MATa *his3Δ leu2Δ met15Δ ura3Δ*	wild type	Euroscarf
W303	MATa *ade2-1 can1-100 his3-11,15 leu2-*	wild type	Dr. G. Ammerer,

	3,112 trp1-1 ura3-1		University of Vienna, Austria
W303α	MATα *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	wild type	Dr. G. Ammerer, University of Vienna, Austria
lia1Δ	MATa *his3Δ leu2Δ met15Δ ura3Δ lia1Δ::KanR*	*lia1Δ*	Euroscarf
spe2Δ	MATa *his3Δ leu2Δ met15Δ ura3Δ spe2Δ::KanR*	*spe2Δ*	Euroscarf
tif51BΔ	MATa *his3Δ leu2Δ met15Δ ura3Δ tif51BΔ::KanR*	*tif51BΔ*	Euroscarf
bnr1Δ	MATa *his3Δ leu2Δ met15Δ ura3Δ bnr1Δ::KanR*	*bnr1Δ*	Euroscarf
bni1Δ	MATa *his3Δ leu2Δ met15Δ ura3Δ bni1Δ::KanR*	*bni1Δ*	Euroscarf
tif51A-1	MATa *his3Δ leu2Δ met15Δ ura3Δ tif51A-1::KanMX*	*tif51A-1^{ts}*	(Li *et al.*, 2011)
tif51A-3	MATa *his3Δ leu2Δ met15Δ ura3Δ tif51A-3::KanMX*	*tif51A-3^{ts}*	(Li *et al.*, 2011)
PAY723	MATa *his3Δ leu2Δ met15Δ ura3Δ BNI1-6HA::HIS3*	BY4741 BNI1wt-HA	This study
PAY725	MATa *his3Δ leu2Δ met15Δ ura3Δ tif51A-1::KanMX BNI1-6HA::HIS3*	*tif51A-1^{ts}* BNI1wt-HA	This study
PAY727	MATa *his3Δ leu2Δ met15Δ ura3Δ bni1Δ1240-1954-6HA::HIS3*	BY4741 BNI1ΔCt-HA	This study
PAY729	MATa *his3Δ leu2Δ met15Δ ura3Δ tif51A-1::KanMX bni1Δ1240-1954-6HA::HIS3*	*tif51A-1^{ts}* BNI1ΔCt-HA	This study
PAY739	MATa *his3Δ leu2Δ met15Δ ura3Δ bni1Δ1239-1307-6HA::HIS3*	BY4741 BNI1ΔPro-HA	This study
PAY741	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 tif51A-1::KanMX bni1Δ1239-1307-6HA::HIS3*	*tif51A-1^{ts}* BNI1ΔPro-HA	This study
top2-1	MATa *ade2-1 can1-100 his3-11,15 leu2-*	*top2-1^{ts}*	(Brill and Sternglanz,

	3,112 trp1-1 ura3-1 top2-1^{ts}		1988)
xpo1-1	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 xpo1::LEU2 pKW457 (xpo1-1 in pRS313)*	*xpo1-1^{ts}*	(Stade *et al.*, 1997)
YAZ80	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ste11Δ::ADE2*	*ste11Δ*	Dr. G. Ammerer, University of Vienna, Austria
YAZ82	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ste11Δ::ADE2 sho1Δ::TRP1*	*ste11Δsho1Δ*	Dr. G. Ammerer, University of Vienna, Austria
YAZ87	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ste11Δ::ADE2 ste50Δ::KanMX*	*ste11Δste50Δ*	Dr. G. Ammerer, University of Vienna, Austria
YAZ120	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 sho1Δ::KanMX*	*sho1Δ*	Dr. G. Ammerer, University of Vienna, Austria
YID196	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssk2Δ::KanMX ssk22Δ::KanMX*	*ssk2Δssk22Δ*	Dr. G. Ammerer, University of Vienna, Austria
YAZ150	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssk2Δ::KanMX ssk22Δ::KanMX sho1Δ::TRP1*	*ssk2Δssk22Δsho1Δ*	Dr. G. Ammerer, University of Vienna, Austria
YAZ157	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ste11Δ::ADE2 opy2Δ::KanMX*	*ste11Δopy2Δ*	Dr. G. Ammerer, University of Vienna, Austria
YAZ195	MATα *his3Δ leu2Δ trp1Δ ura3Δ ssk2Δ::LEU2 ssk22Δ::LEU2 hkr1Δ::NatMX msb2Δ::KanMX*	*ssk2Δssk22Δhkr1Δ msb2Δ*	Dr. G. Ammerer, University of Vienna, Austria
YAZ196	MATα *his3Δ leu2Δ trp1Δ ura3Δ ssk2::LEU2 ssk22::LEU2 msb2Δ::KanMX*	*ssk2Δssk22Δmsb2Δ*	Dr. G. Ammerer, University of Vienna, Austria
YCF7	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ste50Δ::KanMX*	*ste50Δ*	Dr. S. Hohmann University of Gothenburg, Sweden

YCF163	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 opy2Δ::KanMX*	*opy2Δ*	Dr. G. Ammerer, University of Vienna, Austria
YCF173	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 opy2Δ::KanMX pbs2Δ::HIS3*	*opy2Δpbs2Δ*	Dr. G. Ammerer, University of Vienna, Austria
YCF189	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 msb2Δ::HIS3 hkr1Δ::KanMX*	*msb2Δhkr1Δ*	Dr. G. Ammerer, University of Vienna, Austria
YCF192	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssk1Δ::HIS3 ste11Δ::ADE2*	*ssk1Δste11Δ*	Dr. G. Ammerer, University of Vienna, Austria
YCF195	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssk1Δ::HIS3 sho1Δ::TRP1*	*ssk1Δsho1Δ*	Dr. G. Ammerer, University of Vienna, Austria
YCF209	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ste20Δ::TRP1*	*ste20Δ*	Dr. G. Ammerer, University of Vienna, Austria
YCF83	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 msb2Δ::HIS3*	*msb2Δ*	Dr. G. Ammerer, University of Vienna, Austria
KT060	MATα *his3Δ leu2Δ trp1Δ ura3Δ ssk2Δ::LEU2 ssk22Δ::LEU2 hkr1Δ::NatMX*	*ssk2Δssk22Δhkr1Δ*	(Tatebayashi *et al.*, 2007)
PAY185	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hog1Δ::KanMX*	*hog1Δ*	(De Nadal *et al.*, 2004)
PAY703	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ste11Δ::ADE2 msb2Δ::HIS3*	*ste11Δmsb2Δ*	This study
YVR10	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 pbs2Δ::HIS3*	*pbs2Δ*	(Reiser *et al.*, 1999)
YID140	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ste11Δ::ADE2 pbs2Δ::HIS3*	*ste11Δpbs2Δ*	Dr. G. Ammerer, University of Vienna, Austria

YID198	MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssk2Δ::KanMX ssk22Δ:KanMX ste50Δ::KanMX*	*ssk2Δssk22Δste50Δ*	Dr. S. Hohmann University of Gothenburg, Sweden
cbc1Δ	MATa *his3Δ leu2Δ met15Δ ura3Δ cbc1Δ::KanMX4*	*cbc1Δ*	Euroscarf
cbc2Δ	MATa *his3Δ leu2Δ met15Δ ura3Δ cbc2Δ::KanMX4*	*cbc2Δ*	Euroscarf
mot1	MATa *his3Δ leu2Δ met15Δ ura3Δ mot1-1033::KanMX*	*mot1^{ts}*	Euroscarf
PAY730	MATa *his3Δ leu2Δ met15Δ ura3Δ HOT1-6HA::HIS3*	BY4741 HOT1-HA	This study
PAY732	MATa *his3Δ leu2Δ met15Δ ura3Δ cbc1Δ::KanMX4 HOT1-6HA::HIS3*	*cbc1Δ* HOT1-HA	This study

2.3 Yeast plasmids

All plasmids used in this thesis are listed in Table 2.2.

2.3.1 Preparation of plasmids

To amplify plasmids, 1 µl of plasmids are transformed in 50 µl of DH5α™ Competent *E. coli* cells (Invitrogen #18265-017) following manufacturer´s instructions. Briefly, cells were mixed with plasmids and first incubated on ice for 30 minutes, then heat shock was performed at 42 ºC for 20 seconds in a water bath, followed by a further incubation on ice for 2 minutes. To cells, 300 µl of S.O.C. Medium (Invitrogen #1597798) was added, and samples were incubated at 37 ºC for 1 hour with shaking at 225 rpm. 20-200 µl of transformed cells was spread onto LB plates containing Ampicillin, which were incubated overnight at 37 ºC. Colonies were selected and incubated in 1.5 ml of LB liquid media containing Ampicillin at 37 ºC overnight with shaking at 190 rpm. Finally, for plasmid extraction, the GeneJET Plasmid Miniprep Kit (Thermo Scientific #K0502) was utilised according to manufacturer´s instructions.

Table 2.2 Plasmids used in this thesis

Plasmid	Description	Source
GA2256	*HA6-HIS3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PA283	pRS416 *SPA2-GFP, URA3*, 2µ	Dr. R. Arkowitz, Institute of Biology Valrose, France
GA1815	*FUS1-LacZ, URA3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PA159	YEp368 *STL1-LacZ, LEU2,* 2µ	Dr. E. de Nadal, University Pompeu Fabra, Barcelona, Spain (Alepuz *et al.*, 2003)
PB1028	*GAL-BNI1-4HA, URA3*, 2µ	Dr. D. Pellman, Dana-Farber Cancer Institute and Harvard Medical School, Boston, USA
PAZ56	YEp195 *STE11-H3-HA, URA3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PAZ85	YEp181 *STE11-HKMT, LEU2*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PAZ119	YCp33 *STE11-4H3-3HA, URA3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PAZ138	YCp111 *PBS2-9MYC-HKMT, LEU2*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PAZ139	YCp111 *PBS2-9MYC-HKMTinactive, LEU2*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PAZ140	YCp111 *SHO1-HKMTinactive, LEU2*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PAZ187	YCp111 3*MYC-HKMT, LEU2*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PAZ218	YCp111 *MSB2-3MYC-HKMT, LEU2*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PCF19	YCp111 *STE50-HKMTinactive, LEU2*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PCF54	YCp111 *SHO1-HKMT, LEU2*	Dr. G. Ammerer, University of Vienna,

		Vienna, Austria
PID225	YCp33 *SHO1-4H3-3HA, URA3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PID228	YEp195 *SHO1-4H3-3HA, URA3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PID238	YCp33 *SHO1ΔSH3-4H3-3HA, URA3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PID381	YEp195 *SHO1ΔSH3-4H3-3HA, URA3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PSS01012	YCp33 *SHO1W338A-4H3-3HA, URA3*	Dr. G. Ammerer, University of Vienna, Vienna, Austria
PA115	pRS313 *HOT1wt-3HA, HIS3*	(Alepuz *et al.*, 2003)
PA97	pRS316 *HOT1wt-3HA, URA3*	(Alepuz *et al.*, 2003)
PA149	YCplac33 *3HA-TBP1, URA3*	Dr. Kevin Struhl, Harvard Medical School, Boston, USA (Arkowitz and Lowe, 1997)
P15	YCplac111 *CBC1-3MYC-HKMT, LEU2*	Dr. Aurora Zuzuarregui, University of Valencia, Spain
CF1E5	YCplac22 *HOG1-4H3-HA, TRP1*	Dr. Aurora Zuzuarregui, University of Valencia, Spain
P12	YCplac111 *RCK2-3MYC-HKMT, LEU2*	Dr. Aurora Zuzuarregui, University of Valencia, Spain
GA2126	*STE11::ADE2* disruption plasmid, *ADE2*	Dr. G. Ammerer, University of Vienna, Vienna, Austria

2.4 Yeast transformations

Yeast transformation with yeast plasmids (listed in Table 2.3) was carried out following the One-Step transformation method as described by Chen *et al.* (Chen *et al.*, 1992). Briefly, freshly-grown yeast cells in stationary phase taken directly from agar plates were incubated with approximately 50-200 ng of plasmids in 150 µl of transformation solution [40 % PEG, 0.2 M LiAc, 0.1 M DTT] at 45 ºC for 45 mins. Transformants were plated onto SC selective plates.

Yeast transformation of PCR cassettes for genomic tagging or gene disruption were performed using a lithium acetate-based method described by Gietz *et al.* with modifications (Gietz and Woods, 2002). Briefly, 50 ml of exponentially growing cells (OD_{600} 0.8-1) were washed and suspended in 1 ml 0.1 M LiAc. For each cassette transformation, 50 µl of cells were used. To yeast cells, 5 µl of salmon sperm DNA (10 mg/ml; Sigma #D1626), denatured at 95 ºC for 5mins, was added to aid plasmid transformation. 50-200 ng of PCR cassettes were added with 300 µl of transformation solution [40 % PEG, 0.1 M LiAc] to yeast cells, and the samples were incubated at 42 ºC for 20 mins (15 mins for thermal-sensitive strains). Transformants were plated onto SC selective plates.

2.5 Growth and viability analyses

2.5.1 Analysis of cell growth by serial dilutions

To evaluate cellular growth (Chapter 4 and 5), cells were grown to exponential phase under experimental conditions. Cells were then diluted to OD_{600} 0.3 and 10-fold serial dilutions in the growth media were performed (1, 1^{-10}, 1^{-100} and 1^{-1000}). 3µl of each dilution was plated onto agar plates of interest and were then incubated at the indicated temperatures for at least 48 hours.

2.5.2 Cell viability assay

To test the viability of wild type, *tif51A-1*^{ts} and *tif51A-3*^{ts} under permissive and restrictive temperatures (Chapter 5), cell viability assays were performed as described by Li *et al.* (Li *et al.*, 2014b). Briefly, strains were transferred from 25 ºC to 37 ºC during exponential growth phase and controls were maintained at 25 ºC. Cell viability was measured at the indicated times using the Muse Count & Viability Assay Kit (Millipore #MCH100102) and the Muse™ Cell Analyzer (Millipore) according to manufacturer's instructions, in which the Muse Count & Viability Reagent differentially stains viable and non-viable cells based on their membrane permeability to two distinct DNA-binding dyes. Approximately 3000 counts were performed for each sample.

2.6 Molecular biology techniques

2.6.1 DNA extraction from yeast cultures

To extract yeast genomic DNA, 10 ml of cells were grown overnight, and were then washed and resuspended in 500 µl of 10prep solution [2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris pH=8.0, 1 mM EDTA] with 500 µl of phenol (Amresco #0945):chloroform:isoamyl alcohol 25:24:1. Cells were broken mechanically with glass beads using the Precellys® 24 homogeniser (Bertin Technologies) in two rounds of 20 seconds at 6500 rpm . Lysates were then washed with choloform:isoamyl alcohol 24:1 to eliminate organic residues. To separate aqueous and organic phases, samples were repeated centrifuged at 13000 rpm for 5 mins. The aqueous phase containing DNA was separated and DNA was precipitated by adding 0.1 volumes of 3 M NaAc and 2.5 volumes of 96 % ethanol and was at -80 ºC for 30 mins. The precipitate was isolated by centrifugation at 13000 rpm for 15 mins and washed with 500 µl of 70 % ethanol. To further pellet DNA, samples were centrifuged at 13000 rpm for 5mins and were then resuspended in 50 µl of TE [10 mM Tris, 1 mM EDTA, pH 8.0]. To eliminate RNA, samples were treated with 1 µl of RNAse (Roche #109169) by incubation at 37 ºC for 30 mins.

2.6.2 RNA extraction from yeast cultures

RNA extraction from yeast cells were carried out using a phenol-chloroform approach. Briefly, 10 OD_{600} units of exponentially growing cells were collected by centrifugation and were frozen in liquid nitrogen. Cells were resuspended in 500 µl of LETS buffer [0.1 M LiCl, 10 mM EDTA, 0.2 % SDS, 10 mM Tris-HCl, pH 7.4] and transferred to a tube containing glass beads and 500 µl of phenol (Amresco #0981):chloroform:isoamyl alcohol 125:24:1. Cells were broken mechanically using Precellys® 24 homogeniser (Bertin Technologies) in two rounds of 20 seconds at 6500 rpm and centrifuged to separate the aqueous from organic phase. The aqueous phase was then passed through 500 µl of phenol (Amresco #0981):chloroform:isoamyl alcohol 125:24:1, followed by 500 µl of chloroform:isoamyl alcohol 24:1 to discard residual organic matter and phenol. RNA was ethanol precipitated twice with 2.5 volumes of 96 % ethanol: first by LiCl (0.1 volumes of 5 M) and second by NaAc (0.1 volumes of 3 M), and were incubated at -20 ºC overnight. The RNA precipitate was isolated by centrifuging at 13000 rpm for 5 mins and washed with 500 µl of 70 % ethanol after each precipitation step. Pellet were air-dried and resuspended in 100 µl of water. Quantity and quality of

RNA was checked by NanoDrop 2000 (Thermo Scientific) and visualised by agarose gel electrophoresis.

2.6.3 Agarose gel electrophoresis

For fragments size determination and sorting of nucleic acids, horizontal agarose gel electrophoreses were carried out in TBE buffer [45 mM Tris-base, 45mM boric acid, 1 mM EDTA]. Agarose was dissolved in TBE buffer containing SafeView Nucleic Acid Stain (NBS Biologicals #NBS-SV1) at a concentration of 0.05 µl/ml of gel. To visualise nucleic acids, gels were exposed to a UV light source using the Molecular Imager Gel Doc^{TM} XR+ Imagining System (Bio-Rad).

2.6.4 Polymerase chain reaction (PCR)

Generally, PCR reactions were carried out using Mastercycler Personal (Eppendorf) and a program consisting of an initial denaturing step of 95 ºC for 4 mins, 30 cycles of amplification (95 ºC for 1 min, 56 ºC for 1 min, 72 ºC for 1min per kb of PCR product), and finally an extension of 7 mins at 72 ºC. For each 50 µl of reaction, 50-200 ng of DNA or plasmid, 5 µl of each 10 µM primer pair, 1 µl of 10 mM dNTPs (Fermentas #R0182), 3 µl of 50 mM $MgCl_2$, 0.5 µl of Taq DNA polymerase (5 u/µl; Biotools #10048) and 5 µl of the corresponding 10 X buffer (Biotools #10048) were added.

2.6.5 Quantitative real-time PCR (qPCR)

qPCR was utilised to quantify expression of mRNAs or to analyse DNA-protein interactions following chromatin immunoprecipitation. For mRNA expression, extracted RNA is treated with DNAse and then cDNA is synthesis by reverse transcription. For chromatin immunoprecipitation analyses (Chapter 4), DNA is purified as described in section 2.6.7 and directly used as template for qPCR.

For RNA isolation, unwanted DNA is eliminated by treating RNA samples with DNAse. Briefly, for each reaction, 2.5 µg of RNA was incubated for 15 mins at 25 ºC with 2.5 µl of RNAse-free DNAse (Roche #04716728001) and 2.5 µl of the corresponding 10 X incubation buffer (Roche #04716728001) added to a final volume of 25 µl. DNAse enzymatic reaction was terminated by adding 0.625 µl of 5 mM EDTA and incubating the samples for 10 mins at 65 ºC.

To synthesise cDNA from RNA template, a mix of 5 µl of DNAse-treated RNA, 1 µl of 10 µM Oligo d(T) (Thermo Scientific #SO132), 1.6 µl of 10 mM dNTPs pool (Fermentas #R0182) and 4.4 µl of water was incubated at 65 ºC for 10 mins. 4 µl of 10 X reverse transcriptase buffer (Thermo Scientific #EP0742) and 2 µl of 0.1 M DTT (Roche #70001022) were added to the mix and samples were incubated at 42 ºC for 2 mins. Finally, 1 µl of reverse transcriptase (Thermo Scientific #EP0742) and 1 µl of water were added, and enzymatic reactions were carried out at 42 ºC for 50 mins and reactions were then inhibited by incubation at 70 ºC for 15 mins.

All qPCR reactions were carried out using the CFX96 Real Time System C1000^{TM} Thermal Cycler (Bio-Rad) and analysed using the program Bio-Rad CFX Manager 2.1 (Bio-Rad). All primers used are listed in Table 2.3. For amplification, cDNA or DNA samples were diluted specifically for each primer pair. For each reaction of 10 µl, 5 µl of SYBR Premix Ex Taq^{TM} (Takara #RR420A) and 2.5 µl of sample were added, and volume and concentration of primers were added depending on the efficiency of primer pairs. To generate calibration curves, a pool of all undiluted cDNA samples (for mRNA analyses) or all undiluted INPUT samples (for ChIP analyses) were mixed and serial diluted (1/10, 1/50, 1/100, 1/500, 1/1000, 1/5000), and were then used as qPCR templates for each primer pair. For amplification of templates, an initial denaturing step was performed at 95 ºC for 10 secs, followed by 40 cycles of amplification consisting of annealing (95 ºC for 10 sec) and extension (55 ºC for 15 sec) where, after each cycle, plate was read to determine SYBR signal. For larger products, the extension time for each cycle at 55 ºC is increased to 20 sec. To generate melting curves of each primer pair, plates were incubated at 65 ºC with increment of 0.5 ºC per 0.5 sec until 95 ºC was reached.

For each reaction, a cycle threshold (Ct) value was determined as the number of cycles required for the fluorescent signal to exceed background levels. To determine specific mRNA amount of each sample, first, calibration curves for each primer pair were obtained by graphing the Ct of each dilution against the $\log_{10}$ of arbitrary units, which was calculated by multiplying an arbitrary number by the dilution factor. Second, utilising a line of best fit and linear interpolation, the relative template concentration was calculated from the calibration curve formula using the Ct value obtained for each sample. Finally, the relative template concentration was then normalised against the expression of an internal control and represented as fold change of wild type at time 0.

qPCR analyses of ChIP data is detailed in section 2.6.7.

Table 2.3 List of primers used in this thesis

Primer	Sequence1	Use
BNI1-1	AGATCCATAGGTGAGGCTAGCACAGGTAACAGGC TAAGTTTCAAATCCGGTTCTGCTGCTAG	*BNI1* full length genomic tagging
BNI1-2	GTTTGTTTTGGTATTACTGTTGTCATAATTTTTTGG TTTAATATTCCTCGAGGCCAGAAGAC	*BNI1* full length, C terminus truncated, and proline deletion genomic tagging
BNI1-3	ACATGTGGAAAACGGAAAGC	QPCR of *BNI1 ORF*
BNI1-4	AGATCTTCTGCGCCATCTGT	QPCR of *BNI1 ORF*
BNI1-5	GCAGAAGATCTCTCTACTCAATCATCTGTACTCTCC TCACAGCCGTCCGGTTCTGCTGCTAG	*BNI1* C-truncated genomic tagging
BNI1-6	GGCGCAGAAGATCTCTCTACTCAATCATCTGTACT CTCCTCACAGCTACCATCTGTATTATCT	*BNI1* proline deletion genomic tagging
M1	TCGTTCCAATTTACGCTGGTT	QPCR of *ACT1 ORF*
M2	CGGCCAAATCGATTCTCAA	QPCR of *ACT1 ORF*
HXK2-1	CAATTCCATTGGGTTTCACC	QPCR of *HXK2 ORF*
HXK2-2	GCAACATTGGAACAACATCG	QPCR of *HXK2 ORF*
HOT1-1HA	AAGAAAAGAACCATTGGCTGGTTGCAAG	*HOT1* genomic tagging
HOT1-2HA	TTTTCCCTTTTTCGTCGGTGCCAAGCTTG	*HOT1* genomic tagging
3-STL1-3c	GGGCAAATGGTTAGCAACTC	QPCR of *STL1* ORF
3-STL1-5b	CCGGAAGAAGTTTGGAGGAA	QPCR of *STL1* ORF
GRE3-1	CAAAACCATCCAGGCAGTAC	QPCR of *GRE3* ORF
GRE3-2	CTCTCTGAGTTGCCCATCTA	QPCR of *GRE3* ORF
3-GPD1-3	ATGGGAAGTCTTCGACAGAG	QPCR of *GPD1* ORF
3-GPD1-5	TTCTGCTGCCATCCAAAGAG	QPCR of *GPD1* ORF
M11	ACTTCCAGGTGGTAACAACGAA	QPCR of *RPL30* ORF
M12	AAATAGAGACAACACCGACTCTGAATAA	QPCR of *RPL30* ORF
GRE3-7	GGGGGCCTATCAAGTAAATT	QPCR of *GRE3* promoter -142F
5-GRE3-2	GAAGGATTGCACTGACAACT	QPCR of *GRE3* promoter -80R
STL1TATA-1	TTTTGTCCCCTTCAACGCTG	QPCR of *STL1* promoter -181F
STL1-2	TATGAGTGTGACTACTCCTG	QPCR of *STL1* promoter -113R
GPD1-9	TTGCTTCTCTCCCCTTCCTT	QPCR of *GPD1* promoter -298F
GPD1-10R	CAGCAGCAGCAGACATCTTT	QPCR of *GPD1* promoter +16R
FUS1-1F	CATGTGGACCCTTTCAAAAC	QPCR of non-coding region

		upstream of *FUS1*
FUS1-5	AGACAGCGCGAAAAGTGACA	QPCR of non-coding region upstream of *FUS1*
RPL30-4	TGTGGTGCACAGATGTAACG	QPCR of *RPL30* promoter -358F
RPL30-5	CAGCGTGGTTACGTGCTATC	QPCR of *RPL30* promoter -187R
RPL30-6	ACCACGCTGGTGTATTTTCA	QPCR of *RPL30* promoter -195F
RPL30-7	ACACTCGGTCTGTTTGTT	QPCR of *RPL30* promoter -42R
RPL33B-1	GGGGAAAGCAAATGAGAAAA	QPCR of *RPL33B* promoter -605F
RPL33B-2	GCCGGAAGAAGATGGTGTAA	QPCR of *RPL33B* promoter -307R
RPL33B-3	GCAGGTGTGACATGTGAAGG	QPCR of *RPL33B* promoter -192F
RPL33B-4	CAAAGAAACTGTTTTCGGACCT	QPCR of *RPL33B* promoter -46F

2.6.6 Western blot analysis

To quantify protein expression in yeast cells, protein was extracted from 20 ml of cells that were grown to OD_{600} ~ 0.5 at experimental conditions as previously described (Kushnirov, 2000). To prepare protein extracts, ~10 OD_{600} units of culture were harvested by centrifugation and frozen in liquid nitrogen. Upon defrosting, cell pellet was resuspended in 400 µl of 1 % glucose and treated with 0.25 M NaOH for 3 mins at room temperature to permeabilise cell membrane. Cells were pelleted by centrifugation and were resuspended in SDS lysis buffer [360 mM Tris pH 6.8, 18 % SDS, 54 % glycerol, 20 % β-mercaptoethanol, 0.12 % bromophenol blue]. Extracts were boiled for 5 mins and supernatant was collected and absorbance at OD_{280} was determined using Nanodrop as an indication of relative protein concentration.

Equal relative protein amounts, estimated using OD_{280} absorbance, were resolved on SDS-PAGE gel [7-10 % acrylamide mix (acrylamide: bis-acrylamide 30:0.8 %; National Diagnostics #EC-890), 0.75 M Tris pH 8.8, 0.2 % SDS, 0.001 % ammonium persulfate (VWR #33-603-267), 0.001 % TEMED] containing a pre-gel [4 % acrylamide mix (acrylamide: bis-acrylamide 30:0.8 %; National Diagnostics #EC-890), 0.25 M Tris pH 6.8, 0.2 % SDS, 0.001 % ammonium persulfate, 0.001 % TEMED]. Electrophoresis was carried out in electrophoresis buffer [25 mM Tris pH 8.3, 192 mM glycine, 0.02 % SDS] using Mini-PROTEAN® Tetra Cell (Bio-Rad #165-8004) at 100 V, room temperature, for 3-4 hours, depending on the size of proteins of interest.

Resolved proteins were then transferred onto nitrocellulose membrane (GE Healthcare #10401396) using Mini Trans-Blot® Cell (Bio-Rad #170-3930) in transfer buffer [25 mM Tris-Base, 192 mM glycine,

0.1 % SDS, 20 % methanol] with a magnetic mixer. Transfer was performed at 100 V for 12 mins, then 60 V for 30mins at room temperature for smaller proteins, and at 100 V for 1 h with ice for larger proteins.

Following protein transfer, membranes were visualised by incubation with Ponceau solution [0.1 % Ponceau S (Sigma-Aldrich #P3504), 1 % acetic acid]. Blocking was performed by incubation with PBS-Tween solution [1 X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), 0.1 % Tween-20 (Sigma-Aldrich #P1379)] containing 5 % skim milk for 1 hour at room temperature. Primary and secondary antibodies, listed in Table 2.4, were incubated in blocking solution at 4 ºC for 1 hour to overnight, depending on the antibody. Membranes were washed three times with PBS-Tween. Immunoblots were detected using ECL Prime kit (GE Healthcare #RPN2232) and the luminescent image analyzer ImageQuant LAS 4000 mini (GE Healthcare).

To quantify protein expressions, band intensities were analysed using the program ImageQuant TL (GE Healthcare) or the program ImageGauge 4.0 (Fuji Software). Protein expressions were normalised against loading controls of each samples and then expressed as fold change in respect to wild type time 0.

Table 2.4 Antibodies used in this thesis

Antibody (Source)	**Detected proteins**	**Working volume or concentration**
anti-HA (Dr. G. Ammerer, University of Vienna, Vienna, Austria)	HA-tagged proteins	1/10000 (WB)
High Affinity anti-HA (Roche #11867423001)	HA-tagged proteins	10µl/sample (ChIP)
anti-Hxk2 (Dr. F. Rández-Gil, IATA, Spain)	Hxk2	1/20000 (WB)
anti-eIF5A (S. R. Valentini, Säo Paulo State University, Brazil) (Valentini *et al.*, 2002)	eIF5A	1/2000 (WB)
anti-H3K9me3 (Dr. G. Ammerer, University of Vienna, Vienna, Austria)	H3K9 tri-methylation in M-track	1/2000 (WB)
anti-myc (Millipore #05419)	myc-tagged proteins	1/5000 (WB)
8WG16 (Covance #MMS-126R)	Rpb1 of RNAPII	1/10000 (WB) or

		10µl/sample (ChIP)
anti-RNA polymerase II (phospho S2; Abcam #ab5095)	Ser2 phosphorylated CTD of RNAPII	1/20000 (WB)
anti-Rap1 [y-300] (Santa Cruz #sc-20)	Rap1	1/2000 (WB) or 10µl/sample (ChIP)
anti-phospho-p38 (Cell Signaling #4811)	phosphorylated Hog1	1/2000 (WB)
anti-Pgk1 (Invitrogen #A6457)	Pgk1	1/10000 (WB)
anti-Cdc2 (Santa Cruz #sc-53)	Cdc28	1/2000 (WB)
anti-mouse HRP-conjugated (Promega #W4021)	mouse IgG	1/10000 (WB)
anti-rabbit HRP-conjugated (Promega #W4011)	rabbit IgG	1/10000 (WB)

2.6.7 Chromatin Immunoprecipitation (ChIP)

To determine enrichment of proteins bound to specific genes, ChIP experiments were performed as previously described with modifications (Alepuz *et al.*, 2001). Approximately 100 ml of OD_{600} 0.5 exponentially growing yeast culture was fixed with 1 % formaldehyde (Panreac #141328-1211) for 15 mins at room temperature. Formaldehyde was inactivated by treatment with 125 mM glycine for 5 mins at room temperature. Cells were then washed 4 times with cold TBS [50 mM Tris pH 7.6, 150 mM NaCl] and frozen at -20 ºC. Upon defrosting, cells were resuspended in 300 µl of lysis buffer [50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8.0, 1 % Triton X-100 (Sigma #T9284), 0.1 % sodium deoxycholate (Sigma #D5670), 1 mM PMSF (Sigma #P-7626), 1 mM benzamidine (Sigma #B6506), Protease Inhibitor Cocktail 1 tablet/25 ml (Roche #04693124001)] and mechanically broken by Precellys® 24 homogeniser (Bertin Technologies) in two rounds of 20 secs at 6500 rpm using glass beads. Following lysis, 150 µl of lysis buffer were added to each sample, and glass beads were eliminated by centrifugation. In order to generate short chromatin fragments (200-600 bp), lysates were sonicated in an ice bath using Bioruptor (Diagenode) for 2 rounds of 15 mins with cycles of 30 s on and 30 s off. Following sonication, 150 µl more of lysis buffer to added to the samples which were the centrifuged and 400 µl of supernatant were collected. For each sample, 10 µl of total extract were transferred to a new tube as INPUT which was maintained on ice until the reversal of cross-linking step.

Dynabeads were all provided by Life Technologies and antibodies used are listed in Table 2.4. Mouse antibodies were used in combination with Dynabeads Pan Mouse IgG (Life Technologies #11041), rabbit antibodies with Dynabeads Protein A (Life Technologies #10002D), and rat antibodies with Dynabeads Protein G (Life Technologies #10004D) (antibodies used are in Table 2.4). One day previous to cell extract, Dynabeads (50 µl/sample) were first washed with PBS/BSA [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05 % BSA], then incubated with the corresponding antibody overnight at 4 ºC with rotation (termed Dynabeads-Ab). A negative control of only Dynabeads without antibody was included. After antibody incubation, Dynabeads-Ab and negative control were washed 4 times with PBS/BSA and then resuspended in 30 µl of PBS/BSA per sample, which was added to the samples. Samples were incubated with Dynabeads-Ab at 4 ºC for 1.5 hours with rotation.

Following incubation, Dynabeads-Ab now bound with the corresponding proteins of interest were washed twice with lysis buffer, twice with lysis buffer containing 360 mM NaCl, twice with wash buffer [10 mM Tris pH 8.0, 250 mM NaCl, 0.5 % NP-40 (Fluka #74385), 0.4 % sodium deoxycholate, 1 mM EDTA pH 8.0], and then once with TE buffer. To elute bound samples, 50 µl of elution buffer [50 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1 % SDS] were added to Dynabeads and incubated at 65 ºC for 10 mins with rotation at 600 rpm. The elution process was repeated one more time and was termed IP sample. To both eluted IP samples and INPUT, elution buffer was added to a final volume of 150 µl, and to reverse cross-linking, all samples were incubated at 65 ºC with rotation at 600 rpm for 6-15 hours.

Once cross-linking was reversed, samples were treated with Proteinase K (Roche #03115887001) at 37 ºC for 1 h 30 mins with rotation at 600 rpm, in which to each sample 139.5 µl of TE, 3 µl of glycogen (20 mg/ml; Roche #901393) and 7.5 µl of Proteinase K (20 mg/ml) were added. DNA purification was performed using the GeneJET PCR Purification Kit (Fermentas #K0702) according to manufacturer's instructions. To determine enrichment of DNA regions bound to the proteins of interest, qPCR was performed as described in section 2.6.5 using specific primers listed in Table 2.3. Briefly, calibration curves were generated for each primer pair using a pool of all INPUT samples which were serial dilutes as described in 2.6.5. For each qPCR reaction of 10 µl, 5µl of SYBR Premix were added to 2.5 µl of either undiluted IP or 1/100 diluted INPUT samples, with specific primers whose concentration and volume depends on primer efficiency. qPCR protocols were performed and relative template concentrations were calculated as described in section 2.6.5.

To quantify the relative binding of proteins of interest to genes of interest, relative DNA enrichment was determined by, first, normalising the relative template concentrations against an internal

control, a region where the protein of interest was known to not bind. In our studies, a non-coding region upstream of the *FUS1* gene was utilised (primers FUS1-1F and FUS1-5). Second, IP enrichments were represented as a percentage of INPUT, and finally, all enrichments were represented as fold-change in respect to wild type time 0.

2.6.8 Beta-galactosidase assay

To investigate gene induction in response to pheromone (Chapter 5), the beta-galactosidase assay was employed as previously described with modifications (Garre *et al.*, 2012). The membrane protein *FUS1* expressed in a fusion plasmid containing the reporter gene *Lac*Z (PA288; see Table 2.2) and, as a positive control, osmotic stress-induced gene *STL1* coupled with *Lac*Z (PA159; Table 2.2) were utilised. Wild type strain, *bni1Δ*, and thermal sensitive strains *tif51A-1ts* and *tif51A-3ts* (Table 2.1) were transformed using the One-Step lithium acetate-mediated method and selected in appropriate synthetic media. Biological triplicates were grown at 25 ºC to the mid-log phase and transferred to the non-permissive temperature of 37 ºC for 4 hours and then incubated with 10 µg/ml of α-factor for 1 or 2 hours, and positive control cells were stressed with 0.6 M KCl for 45 mins. Upon cell collection, absorbance at OD_{600} was measured. To determine β-galactosidase activity, pellets were washed and resuspended with Buffer Z [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0]. For the permeabilisation of cell wall, samples were frozen in liquid nitrogen for 1 minute and immediately incubated at 37 ºC for 1 minute. To initiate enzymatic activity, samples were resuspended in Buffer Z containing 0.27 % of β-mercaptoethanol and 4 mg/ml ONPG (Sigma, #N1127) and were incubated at 30 ºC and the reaction time was recorded when samples turned yellow. Reaction was terminated by the addition of Na_2CO_3 with a final concentration of 0.27 M, and absorption of supernatant was measured at OD_{420}. To determine β-galactosidase activity, absorbance at OD_{420} was normalised against the time of reaction multiplied by the OD units of the culture used for the assay.

2.7 Microscopy

In Chapter 5, all microscopy were carried out at room temperature using the Axioskop 2 Florescence Microscope (Zeiss). Images were taken by the SPOT digital camera (Diagnostic Instruments) and viewed using the software Axio Vision Rel. 4.7 (Zeiss). For live-cell imaging, cells were concentrated in their respective experimental media and viewed using a Zeiss Plan-Neofluar objective lense with

63 X magnification and 1.0 numerical aperture in oil (Cargille). For fluorescent microscopy, the power unit AttoArc HBO 100W (Zeiss) was used to visualize the indicated fluorophores. Images were processed and sizes adjusted using the software Photoshop and final figures prepared with Power Point.

2.7.1 Analysis of shmooing efficiency

To study the formation of shmoos, null mutants were grown to the exponential phase at 30 ºC and temperature-sensitive mutants were transferred from 25 ºC to 37 ºC at the exponential phase and maintained at the restrictive temperature for 4 hours. Cultures were incubated with 10 µg/ml of α-factor for 2 hours at their respective experimental temperatures, and visualized using microscopy. Approximately 300 cells were manually counted and percentage of shmoo formation was determined based on phenotypic criteria as described by Sette *et al.* (Sette *et al.*, 2000).

2.7.2 Spa2 localisation

To specifically visualise the sub-localisation Spa2, an over-expression plasmid PA283, containing *SPA2-GFP* fusion (Table 2.2) and transformed in wild type and mutants. Transformants were grown in selective media under experimental conditions and treated with α-factor for 2 h. Spa2-GFP fluorescence was visualised by fluorescent microscopy (excitation/emission: 395/509 nm).

2.7.3 Phalloidine analyses of actin

For the visualisation of actin localisation, cultures were fixed with 4 % formaldehyde for 1 h at the respective experimental temperatures. Cells were collected by centrifugation and washed 3 times with PBS [140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4] containing 0.1 % BSA (PBS-BSA). Pellets were incubated with 9 % Alexa Fluor® 488 Phalloidin (Life Technologies #A12379) in PBS-BSA overnight at 4 ºC protected from light. To eliminate remaining phalloidin, cells were washed 3 times with PBS-BSA and twice with PBS, and resuspended in PBS to be visualised by fluorescent microscopy (excitation/emission: 346/442 nm).

2.8 M-track method

In Chapter 3, the M-track method, described by Zuzuarregui *et al.* (Zuzuarregui *et al.*, 2012), was utilised to evaluate protein-protein interactions of the SHO1 branch of the HOG pathway. Strains of interest (Table 2.1) were transformed with plasmids encoding fusion proteins of interest (Table 2.2) using the One-Step transformation method and selected in SC selective media. Transformants were grown in selective media to OD_{600} 0.6-0.8 and stressed, for 30 mins or otherwise indicated, with 0.4 M NaCl taken from a liquid stock of 5 M NaCl. Cells were collected by centrifugation and frozen in liquid nitrogen.

2.8.1 M-track western blot analyses

Total proteins were extracted and resolved on SDS-PAGE and transfer was performed as described in section 2.6.6. To visualise protein-protein interactions, membranes were blocked with blocking solution containing PBS-Tween and 1 % skim milk. To detect methylation signals, anti-H3K9me3 antibody was incubated in PBS-Tween containing 10 % yeast extract (see section 2.8.2) for 1-1.5 hours at 4 ºC. To detect myc- and HA-tagged fusion protein expressions, anti-myc and anti-HA antibodies were respectively used (Table 2.4). Western blots were visualised and analysed as described in section 2.6.6. To determine interaction intensities, methylation signal was normalised against HA expression for each sample, and this was then represented as a fold-change of the control strain without stress. To quantify Msb2 protein expression (Figures 3.8, 3.10-3.12), myc signals were normalised against myc signal of the control strain without stress. Figures shown in Chapter 3 are representative western blots, and the averages and standard deviations (SD) were calculated from 2 to 6 independent experiments.

2.8.2 Preparation of yeast extract for M-track analysis

To visualise histone methylation signal, anti-H3K9me3 was incubated with a blocking solution containing 10 % yeast extract (Chapter 3). For yeast extract preparation (stock of 100 %), 2 L of OD_{600} 1-1.5 of yeast cultures grown in YPD were collected by centrifugation at 6000 rpm for 15 mins at 4 ºC. Cells were then washed with water and cell pellets were frozen in liquid nitrogen. Cells were broken mechanically in liquid nitrogen using Mixer Mil 301 (Retsch) in 6 rounds of 3 mins. Each gram of yeast extract was resuspended in 10 ml of PBS-Tween and was mixed by rotation for 15 mins.

Dissolved extract was then centrifuged at 4000 rpm for 15 mins, and cell pellet was discarded. Complete Protease Inhibitor Cocktail (Roche #04693124001) was then added at a concentration of 1 tablet/25 ml.

2.9 Analysis of Genomic Run-On (GRO) data

In Chapter 4, genomic analyses were performed utilising the technique Genomic Run-On, first described by Garcia-Martinez *et al.* (2004), to determine the role of Cbc1 in transcription. All GRO experiments presented in this thesis were performed by Nikki De Clercq as previously described (Garcia-Martinez *et al.*, 2004) with changes detailed in Romero-Santacreu (2009) and analysed by Daniel A. Medina as described below. Briefly, yeast samples were collected at exponential phase in the absence (time 0) or presence of 0.6 M KCl for 8, 15, 30 and 45 minutes for both GRO experiment and mRNA amount determination, which were performed as previously described (Romero-Santacreu, 2009). Three independent experiments were performed for each time point.

The macroarray images were quantified using Array Vision software (Imaging Research Inc.). Values that were at least 1.2 times higher than the local background were taken as valid measurements. The TR and RA signal was corrected using a random primer genomic DNA label to correct the differences between macroarray chips. An average data set for each time point was created using Median Absolute Desviation normalization by ArrayStat software (Imaging Research Inc.).

mRNA amount (RA) and transcription rate (TR) were analysed as described in Medina (2015). Briefly, the RA adjusted by the concentration of total mRNA per cell as described (Garcia-Martinez *et al.*, 2004, Medina, 2015). Whole genomic wide data sets were processed with Microsoft Excel 2007 spreadsheet and the R-statistical language (R-Core-Team, 2014). The heatmap cluster was done using the gplots R-package, and the gene ontology (GO) enrichment in each cluster group was performed using the GOrilla online tool (Eden *et al.*, 2009). The redundant GO categories were filtered using the REVIGO online tool (Supek *et al.*, 2011). The list of targets genes regulated by transcription factors was obtained from Saccharomyces Genome Data Base (Cherry *et al.*, 2012) for Sko1, Msn2 and Msn4 factors, and from Yeastract (Teixeira *et al.*, 2014) for Hot1 and Smp1 factors, and for each group, the upregulated genes were then chosen from GRO data obtained from Romero-Santacreu (2009), where the TR show 2.5-fold or more increase during 0.6 M KCl treatment compared to time 0 in wild type. The list of target genes dependent and independent of Hog1 were

taken from Romero-Santacreu (2009). The list of RP genes were obtained from Saccharomyces Genome Data Base (Cherry *et al.*, 2012).

Chapter 3

Msb2 is a Ste11 membrane concentrator of the HOG pathway

3.1 Background

3.1.1 HOG pathway

In response to high external osmolarity, the yeast HOG MAPK signalling pathway is rapidly activated to mediate immediate changes to various cellular processes in order to allow adaptation. At the core of this cascade lies Hog1 MAPK, which is conserved between eukaryotes, with a functional homolog, p38, in humans (Yamamoto *et al.*, 2010, Harris *et al.*, 2001). Upon osmotic stress, Hog1 is activated by two independent and functionally distinct upstream branches that converge at the MAPKK Pbs2: the SLN1 branch propagated by the redundant MAPKKK Ssk2/Ssk22, and the SHO1 branch by the MAPKKK Ste11 (detailed in section 1.3.2 and refer to Figure 1.5) (Maeda *et al.*, 1995, Posas and Saito, 1997).

Activation of the SHO1 branch of the HOG pathway requires the osmosensors Msb2 and Hkr1 which seem to act redundantly but via slightly different mechanisms, as well as the osmosensor Sho1 (O'Rourke and Herskowitz, 2002, Takahashi and Pryciak, 2007, Tatebayashi *et al.*, 2007, Tanaka *et al.*, 2014). The mode in how these osmosensors function together to effectively propagate signalling is still unclear and is beginning to be elucidated. First, O´Rourke *et al.* showed that Msb2 and Sho1 may have partially redundant functions in the activation of Ste11, as a single deletion of Msb2 or Sho1 reduced cross-talk through Ste11, and a double mutation in both genes shows an additive reduction in cross-talk (O'Rourke and Herskowitz, 2002). More recently, it has been elucidated that perhaps the signalling generated from SHO1 branch can be divided into two sub-branches: one involving osmosensor Msb2 and the other involving Hkr1. In the HKR1 sub-branch, Hkr1 activates Ste11 by recruiting Ste20 to the plasma membrane, and in contrast Msb2 activates Ste11 through either Ste20 or Cla4, which are recruited by the scaffold Bem1 (Tatebayashi *et al.*, 2015, Tanaka *et al.*, 2014). Several evidence suggest that efficient signal propagation requires a high membrane concentration of Ste11, which is constitutively bound to Ste50 (Ramezani-Rad, 2003), which allows the Ste11-Ste50 complex to be recruited to the plasma membrane through the Opy2-Ste50 interaction (Wu *et al.*, 2006, Tatebayashi *et al.*, 2007). Once at the plasma membrane, Opy2 interacts with the osmosensors to bring together Ste11 and its kinases Ste20/Cla4 in order to mediate efficient phosphorylation and activation of Ste11 (Tatebayashi *et al.*, 2015).

Ste11, the central MAPKKK of the SHO1 branch, is also shared by two other distinct pathways, the pheromone and filamentous growth (FG) pathways. Activation of Ste11 in response to specific external stimuli induces, in each case, a specific and appropriate adaptive response (de Nadal *et al.*,

2002, Hohmann *et al.*, 2007, Yamamoto *et al.*, 2010, Lefevre *et al.*, 2011, Saito and Posas, 2012). During osmotic stress, Ste11 only activates Pbs2 and this signal fidelity is possibly achieved by three separate events: first, by the membrane recruitment of Pbs2 by Sho1, through the Sho1-SH3 domain, and the membrane recruitment of Ste11-Ste50 complex by Opy2 to membrane-localise both Ste11 and its substrate Pbs2 (Maeda *et al.*, 1995, Wu *et al.*, 1999, Yamamoto *et al.*, 2010); second, by the direct interaction between Ste11 and the cytoplasmic tail of Sho1, near the binding site of Pbs2 (Tatebayashi *et al.*, 2006); and finally, through the direct interaction between Ste11 and Pbs2 (Zuzuarregui *et al.*, 2012). Thus, multiple and dynamic interactions between the Opy2/Ste50/Ste11 complex and the Sho1/Pbs2 complex bring Ste11 in close contact with Pbs2 to enable efficient activation.

Negative feedback mediated by downstream components to regulated upstream elements of the HOG pathways has been previously described (Yamamoto *et al.*, 2010). First, as osmo-adaptation progresses, Pbs2 dissociates from Sho1, however, this dissociation is hindered in a strain lacking Ste11, indicating that phosphorylated Pbs2 mediates its own dissociation from Sho1 in a negative feedback loop (Reiser *et al.*, 2000). Second, to attenuate signalling following adaptation, Hog1 is known to phosphorylate Ste50 which mediates its dissociation from Opy2, thereby reducing Ste11/Ste50 membrane localisation (Yamamoto *et al.*, 2010). Furthermore, Hog1 has also been described to phosphorylate Sho1, however, the functional role of this phosphorylation is still unknown (Hao *et al.*, 2007).

In this chapter, we aim to characterise, using the novel M-track method (detailed below) (Zuzuarregui *et al.*, 2012), the protein interaction profile of the MAPKKK Ste11 with other components of the SHO1 branch during hyperosmotic stress, as well as identify possible feedback mechanisms employed by downstream components to regulate upstream signalling. In summary, first, we show that majority of Ste11 interactions can be detected at basal levels in the absence of stress and augments with osmotic stress treatment. Second, the Ste11-Sho1 interaction is independent of the Sho1-SH3 domain, but requires Ste50 and osmosensors Msb2 and Hkr1. Third, we have characterised a novel dynamic interaction between Ste11 and osmosensor Msb2, and this interaction requires Ste50 but not Sho1. Finally, we observed multiple negative feedback systems mediated by downstream factors Pbs2 and Hog1 to regulate protein expressions of upstream components as well as Ste11 interactions.

3.1.2 M-track method

A novel method to detect short-lived and stable protein-protein interactions has been described by Zuzuarregui *et al.* (2012). This *in vivo* assay utilises plasmids containing a bait protein fused to the catalytic domain of a mouse histone lysine methylthransferase (HKMT) and a prey protein fused to its substrate, epitope repeats of lysine 9 of histone H3 (H3K9). Methylation of H3 occurs when the bait and prey proteins come in proximity, and the signal is detected by highly specific antibodies. Furthermore, both bait and prey plasmids are tagged with either HA or myc to track plasmid expressions by western blotting (detailed in Figure 3.1) (Zuzuarregui *et al.*, 2012).

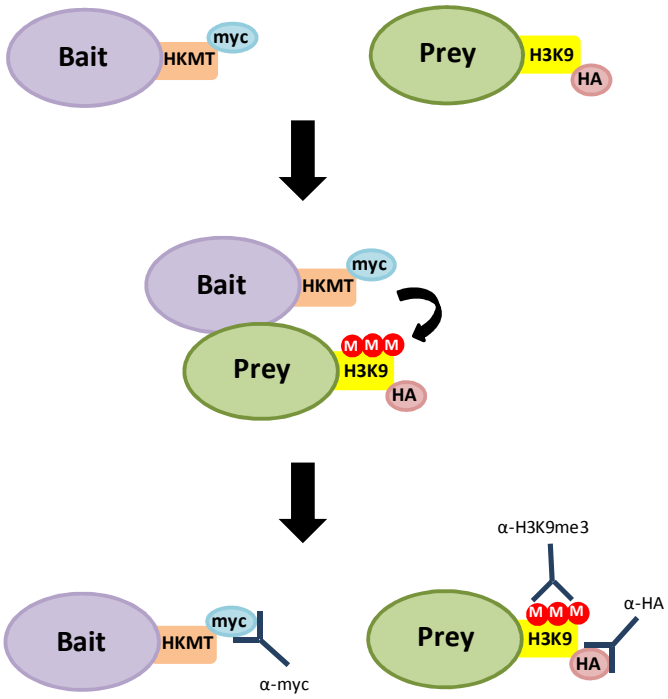


Figure 3.1 Schematic representation of the M-track method. Yeast cells are simultaneously transformed with two plasmids, one encoding a bait protein of interest fused to HKMT catalytic subunit with a myc tag, and the other encoding a prey protein fused to H3K9 epitope with an HA tag. Upon interaction, either transient or stable, H3K9 becomes methylated, which is then detected by a specific antibody. Antibodies against the myc and HA tags are hybridised to visualise bait and prey fusion protein expressions, respectively.

M-track method presents several advantages over currently-known protein-protein detection assays: first, transient interactions are readily detected, as yeasts do not have any counterparts with respect to substrate specificity; second, the catalytic epitope of HKMT is a mutant version that

possesses more than 20-fold the enzymatic activity of its wild type counterpart, indicating that the enzymatic reaction occurs at higher speed than protein interactions, providing high sensitivity to the assay; finally, the affinity between HKMT enzyme and the epitope of its substrate H3K9 is substantially lower than the affinity between any two interacting proteins, which ensures minimal false positives (Zuzuarregui *et al.*, 2012).

3.2 Results

3.2.1 Interaction between Ste11 and Pbs2 requires Ste50, Opy2, Msb2/Hkr1 and Sho1

Short-lived protein interactions are difficult to trace biochemically. In this thesis, we followed the interactions of several components of the SHO1 branch of the HOG pathway (Figure 1.5) utilising the previously described M-track method (Figure 3.1) (Zuzuarregui *et al.*, 2012).

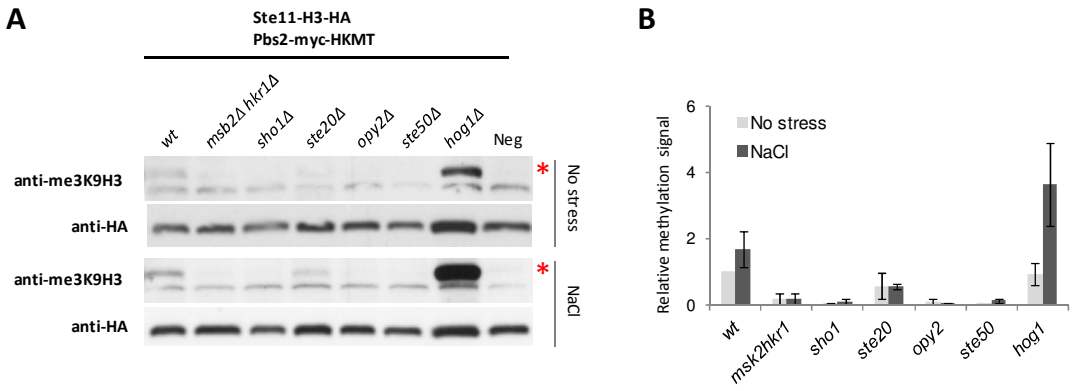


Figure 3.2 Stress-regulated Ste11-Pbs2 interaction requires osmosensors and scaffold proteins and is negatively regulated by Hog1. (A) Interaction of *STE11-H3-HA* (PAZ119) and *PBS2-HKMT* (PAZ138) was detected with anti-me3K9H3 antibody in the indicated mutants (YCF189, YAZ120, YCF209, YCF163, YCF7 and PAY185) and wild type (wt) W303 strain in the absence (no stress) or presence of Na-induced osmotic stress (0.4 M NaCl) for 30 mins. Asterisks indicate the specific methylation band. Negative control corresponds to the wild type W303 transformed with the plasmids *STE11-H3-HA* (PAZ119) and Pbs2 fused to a catalytically inactive methylase (PAZ139). Immunoblots were also developed with anti-HA antibody to detect *STE11-H3-HA* plasmid expression. **(B)** Quantification of the methylation signal normalised against HA signal and represented as fold-change of wild type in the absence of stress. The average and standard deviations (SD) of 2 to 6 independent experiments for each strain are shown. This figure shows one representative experiment.

Using this assay, the short-lived Ste11-Pbs2 interaction was recently studied and shown to increase during osmotic stress (Zuzuarregui *et al.*, 2012). Here, we further investigated the extent to which HOG signalling components were found to be necessary for obtaining this kinase-substrate interaction (Figure 3.2). As expected, the Ste11-Pbs2 signal was completely dependent on Sho1, the scaffold protein responsible for the recruitment of Pbs2. Furthermore, as shown in Figure 3.2, the Ste11-adaptor protein Ste50, the transmembrane anchoring protein Opy2, and the osmosensors Msb2 and Hkr1 were all necessary for this interaction. An interaction signal, albeit at low level, was detected in a strain carrying a deletion in the PAK kinase Ste20. Surprisingly, we observed a higher level of methylation in *hog1Δ* under non-stress conditions, which increased significantly after NaCl treatment. The higher Ste11-Pbs2 interaction signal in the absence of Hog1 was partially due to an increase in the Ste11 protein level, suggesting a negative feedback on Ste11 protein stability by Hog1. Nevertheless, the increase in Ste11 protein concentration alone (1.9 and 1.3 fold in *hog1Δ* in respect to wild type in non-stress and stress, respectively) does not justify the increase in the methylation signal (2.5 and 2.8 fold in *hog1Δ* in respect to wild type in non-stress and stress, respectively), and therefore, our results suggest that Hog1 activity might increase the dissociation rate of the Ste11-Pbs2 complex either directly or indirectly via other signalling components.

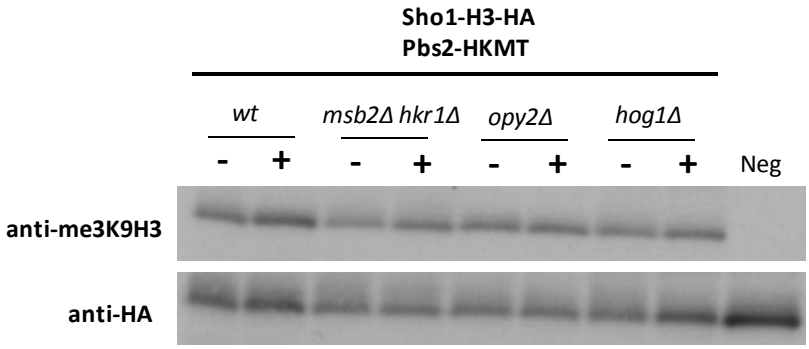


Figure 3.3 Interaction of Sho1 with Pbs2 is constitutive and independent of Opy2, Hog1 and Msb2/Hkr1. Interaction of *SHO1-H3-HA* (PID225) and *PBS2-HKMT* (PAZ138) was detected with anti-methylase antibodies (anti-meK9H3) in the indicated mutants (YCF189, YCF163 and PAY185) and wild type (K699) in the absence (-) or presence (+) of Na-induced osmotic stress. Negative control corresponds to a wild type strain expressing the plasmids *SHO1-H3-HA* (PID225) and a Pbs2 fused to a catalytically inactive methylase (PAZ139). Immunoblots were also developed with anti-HA antibody to detect *SHO1-H3-HA* expression.

3.2.2 Sho1-SH3 domain is not required for the Sho1-Ste11 interaction but influences its dissociation

The functional role of Sho1 in osmotic stress signalling is both complex and multi-disciplinary. As an adaptor, Sho1 is essential for the recruitment of both Pbs2 and Ste11 to the plasma membrane in order to mediate their interaction (Maeda *et al.*, 1995, Zarrinpar *et al.*, 2004). In this study, we observed that Sho1-Pbs2 interaction was constitutive and independent of Hog1, Opy2 and the Msb2/Hkr1 osmosensors (Figure 3.3), and this interaction has been previously described to be completely dependent on the Sho1-SH3 domain (Posas and Saito, 1997, Zarrinpar *et al.*, 2003). On the other hand, the interaction between Sho1 and Ste11 has been described to occur independently of the Sho1-SH3 domain (Zarrinpar *et al.*, 2004, Tatebayashi *et al.*, 2006). We therefore re-investigated the role of the SH3 domain of Sho1 in Sho1-Ste11 interaction utilising the M-track assay. First, we detected an increase in Ste11 interaction with full-length Sho1 during osmotic stress (Figure 3.4A and B). Second, in concordance with previous data (Zarrinpar *et al.*, 2004, Tatebayashi *et al.*, 2006), Ste11 was found to interact with a truncated Sho1 lacking the SH3 domain (Sho1SH3Δ), and this interaction was stronger than the interaction with full-length Sho1 (Figure 3.4C and D). Furthermore, Ste11 interaction showed a similar increase (Figure 3.4E) with a Sho1 version that was unable to bind Pbs2 as a result of a mutation in the SH3 domain (Sho1-W338A) (Tatebayashi *et al.*, 2006). Given that the assay was performed in a background in which it was still possible to induce Pbs2 activity via the SLN1 branch, these results collectively suggest that the physical presence of Pbs2 in the Ste11-Sho1-Pbs2 complex, and not its kinase activity, negatively regulates Ste11-Sho1 interaction.

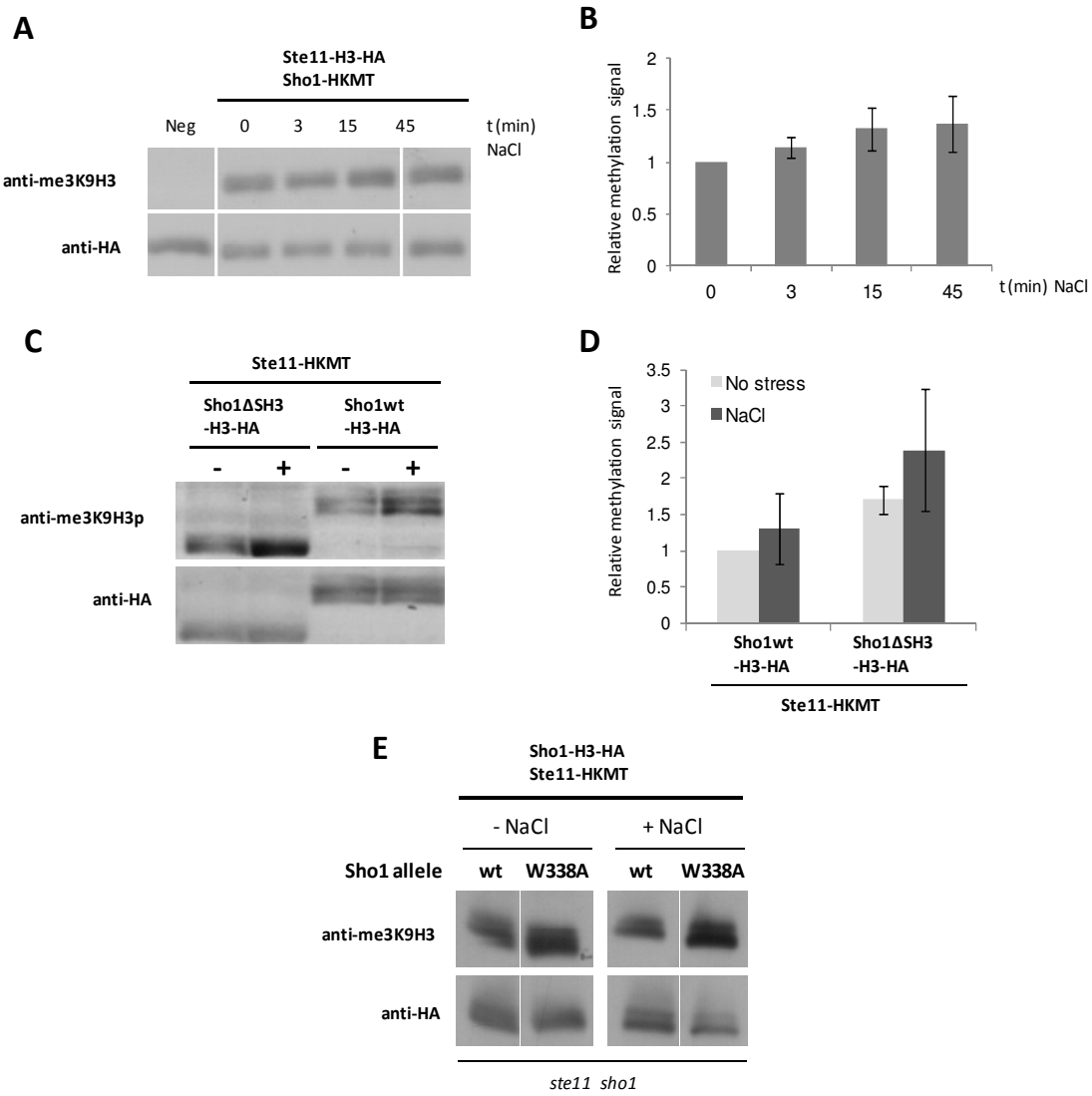


Figure 3.4 Ste11 interaction with Sho1 is regulated by stress and through the SH3 domain of Sho1. (A) *ste11Δ sho1Δ* mutant strain (YAZ82) containing the centromeric plasmids *STE11-H3-HA* (PAZ119) and *SHO1-HKMT* (PCF54) was incubated with 0.4 M NaCl at the indicated times. Negative control corresponds to a *ste11Δ* mutant strain (YAZ80) containing *STE11-H3-HA* (PAZ119) and an empty plasmid. All rows were cut from same western. Immunoblot was developed with the indicated antibody. **(C)** M-track analysis of the interaction between *STE11-HKMT* (PAZ85) and two *SHO1-H3-HA* versions: with (Sho1wt, PID228) and without the SH3 domain (Sho1ΔSH3, PID381) in *ste11Δsho1Δ* double mutant strain (YAZ82), in non-stress (-) or Na-induced osmotic stress (+) conditions. Fusion proteins were expressed from multicopy plasmids. Immunoblot was developed with the polyclonal anti-me3K9H3 and anti-HA antibodies. **(B)** and **(D)** Quantifications of the methylation signals in **(A)** and **(C)** respectively, normalised as in Fig. 3.2. The average and SD of 3 **(B)** and 2 **(D)** independent experiments are shown. The Western blots shown in (A) and (C) are representative experiments. **(E)** M-track analysis of the interaction between *STE11-HKMT* (PAZ85) and two *SHO1-H3-HA* versions: Sho1wt (PID225) and Sho1W338A (PSS041012) in *ste11Δsho1Δ* double mutant strain (YAZ82) before (-) or after (+) treatment with 0.4 M NaCl. Immunoblots were developed with the indicated antibodies. Figure show one representative experiment. All rows are from same western.

3.2.3 Interaction of Ste11 with Sho1 requires the scaffold Ste50 and osmosensors Msb2/Hkr1 but not the membrane protein Opy2

It has been previously described that the direct interaction of Ste11 with Sho1 is only observed in the presence of the Ste11-adaptor protein Ste50 (Zarrinpar *et al.*, 2004) or in the absence of Ste50 but in combination with Sho1 hyperactive mutants (Tatebayashi *et al.*, 2006). In this thesis, we confirmed that Ste11 was unable to bind Sho1 in the absence of Ste50 (Figure 3.5A and B), suggesting that this interaction requires the formation of the Ste11-Ste50 complex, which in itself was independent of the presence of Sho1 (Figure 3.6). Furthermore, as seen in Figure 3.6A and B, the Ste11-Sho1 interaction was somewhat partially dependent on the presence of Opy2, and the interaction signal was detected at wild type levels in a *ste20Δ* mutant, possibly due to the presence of the activity of redundant kinase Cla4 (Lamson *et al.*, 2002). It could be observed that downstream factors, Pbs2 and Hog1, were not required for this interaction; however, their deletion resulted in the upregulation of the Ste11 protein under osmotic stress (an increase of 1.3 fold in both mutants in respect to wild type; Figure 3.5), which confirmed the presence of a Hog1-mediated negative feedback, also seen in Figure 3.2, at the level of Ste11 protein. Moreover, the upregulation of Ste11 protein expression was also observed in the absence of stress (1.3 and 1.6 fold in *pbs2Δ* and *hog1Δ* respectively compared to wild type), suggesting that basal activation of Hog1 under non-stress conditions might be sufficient to mediate negative feedback (Figure 3.5A and B). Since an inhibition of negative feedback was observed in a *pbs2Δ* mutant where the Hog1 protein was present but inactive, we could conclude that not only the physical presence of Hog1, but its activity regulates Ste11 protein expression. Additionally, coupled deletion of both osmo-sensors, Msb2 and Hkr1, abolished the Ste11-Sho1 interaction under non-stress conditions, and only a very weak signal could be detected upon stress (Figure 3.5A and B).

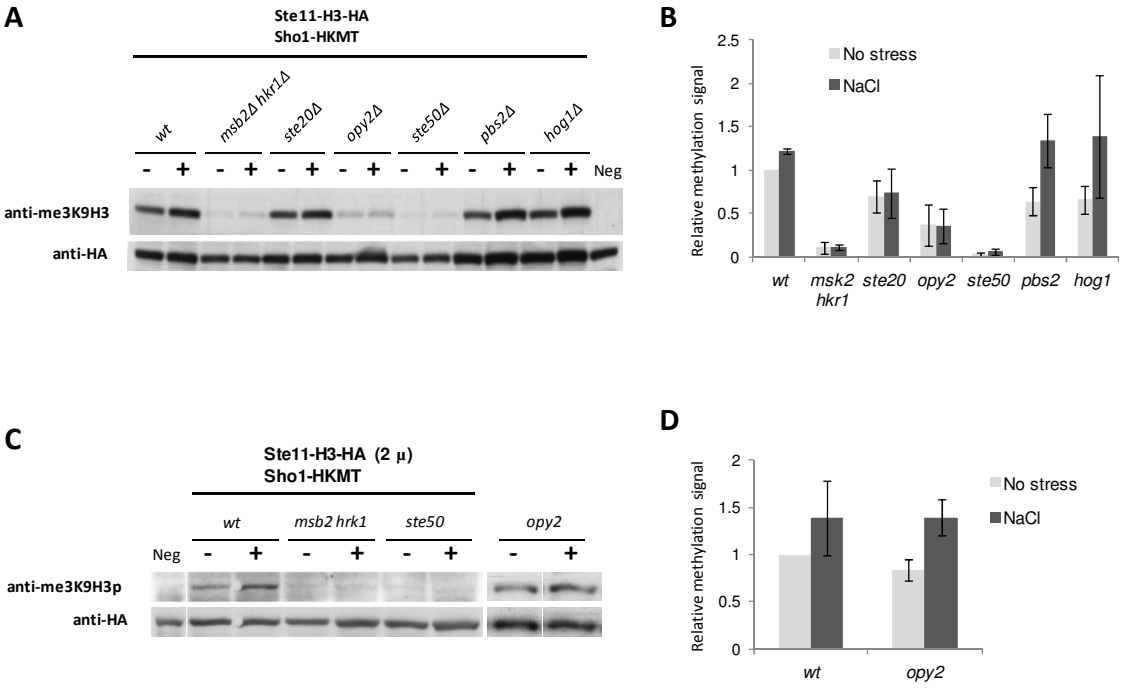


Figure 3.5 Ste11-Sho1 interaction requires osmosensors Msb2/Hkr1 and scaffold Ste50 but do not require Opy2. (A) Interaction of *STE11-H3-HA* (PAZ119) with *SHO1-HKMT* (PCF54) in the indicated mutants (YCF189, YCF209, YCF163, YCF7, YVR10 and PAY185) and wild type (K699) strain, with (+) or without (-) osmotic stress. Immunoblots were developed with the indicated antibodies. **(C)** Interaction of overexpressed *STE11-H3-HA* (multicopy plasmid PAZ56) with *SHO1-HKMT* (PCF54) in wild type (K699), *msb2Δhkr1Δ* (YCF189), *ste50Δ* (YCF7) and *opy2Δ* (YCF163) strains, under stress (+) and non-stress (-) conditions. Negative control used in **(A)** and **(C)** corresponds to a wild type strain transformed with the plasmids *STE11-H3-HA* (PAZ119) and Sho1 fused to a catalytically inactive methylase (PAZ140). **(B)** and **(D)** show the quantifications of the methylation signals in **(A)** and **(C)** respectively, normalised as in Figure 3.2. The average and SD of 3 or 4 independent experiments for each strain in **(B)** and 3 independent experiments in **(D)** are shown. The Western blots shown in **(A)** and **(C)** are representative experiments.

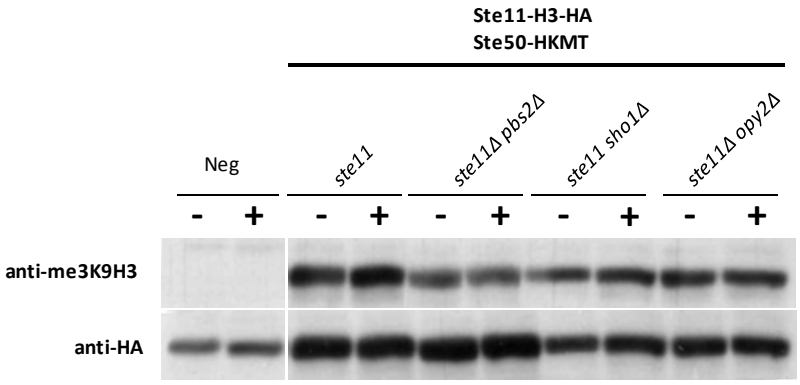


Figure 3.6 The Ste11-Ste50 interaction is constitutive, independent of Pbs2, Sho1 and Opy2. Interaction of *STE11-H3-HA* (PAZ119) and *STE50-HKMT* (PCF23) was detected with anti-methylase antibodies (anti-meK9H3) in the indicated strains (YAZ80, YID140, YAZ82 and YAZ157) in the absence (-) or presence (+) of Na-induced osmotic stress. Negative control corresponds to a wild type strain (K699) expressing the plasmids with *STE11-H3-HA* (PAZ119) and non-fused methylase (HKMT, PCF19). Immunoblots were also developed with anti-HA antibody to detect *STE11-H3-HA* expression. All rows are from the same western.

We then investigated whether the loss of Ste11-Sho1 interaction in osmosensors, *ste50Δ* and *opy2Δ* mutants could be restored by the overexpression of Ste11 protein through the use of a multicopy plasmid. In Figure 3.5C and D, we observed that the overexpression of Ste11 was able to restore interaction with Sho1 in *opy2Δ*, indicating that Opy2, although a known membrane anchor of the Ste11-Ste50 complex (Wu *et al.*, 2006), was not essential for Ste11-Sho1 interaction. This result suggests that Sho1 and Opy2 may have accumulative functions in the recruitment of the Ste11-Ste50 complex. Contrastingly, overexpression of Ste11 was unable to rescue interaction in mutants that lacked osmosensors Msb2/Hkr1 and Ste50. Altogether, these results indicate that the Ste11-Sho1 interaction is absolutely dependent on the osmosensors Msb2/Hkr1 and Ste50, but not on the membrane anchor Opy2.

3.2.4 Msb2 interacts dynamically with Ste11, and this interaction, as well as Msb2 protein, is negatively regulated by Hog1

In order to propagate signal via the SHO1 branch, Ste11 interacts, either directly or through Ste50, with several components of the pathway, including Ste20/Cla4, Opy2, Sho1 and Pbs2 (Saito and Posas, 2012). Moreover, we have shown in this thesis that the osmosensors Msb2 and Hkr1 are

required for the Ste11 interaction with Sho1, indicating a possibility that Ste11 may interact with components more upstream in the pathway. We therefore investigated the possible interaction between Ste11 and Msb2 using M-track. As shown in Figure 3.7A and B, Ste11 interacted with Msb2 in both the presence and absence of stress, with a slight increase in interaction during osmotic stress. Furthermore, in both *hog1Δ* and *pbs2Δ* mutants, we observed an increase in Ste11-Msb2 interaction normalised against Msb2 protein expression (2.0 ± 0.6 and 2.4 ± 0.7 fold in *hog1Δ* and *pbs2Δ*, respectively, compared to 1.4 ± 0.3 fold observed in wild type), suggesting that Pbs2 and/or Hog1 mediated the dissociation of the Ste11-Msb2 complex in a feedback regulation (Figure 3.7C-H). An approximately two fold increase in Msb2 protein expression was also observed in *pbs2Δ* and *hog1Δ* mutants in both stress and non-stress conditions (Figure 3.7C, E, F, H), and since activated Hog1 also has a functional role to mediate changes in transcription during osmotic stress response (see section 1.3.3.1), we therefore utilised RT-qPCR to determine whether Hog1 regulated *MSB2* mRNA levels. Interestingly, although *MSB2* mRNA expression was found to be upregulated in these mutants under stress conditions, no up-regulation was observed under normal conditions (Figure 3.8B). Additionally, to evaluate HOG signal transduction of these mutants under osmotic stress, we analysed the mRNA induction of the Hog1-dependent osmostress gene *STL1*, and it could be observed in Figure 3.8A that no *STL1* induction was detected in the strains *pbs2Δ*, *hog1Δ* and *ssk2Δssk22Δsho1Δ*, where HOG signalling was impeded. Taken together, these results suggested that during osmotic stress, active Hog1 mediated negative feedbacks on Msb2 protein and mRNA expression as well as on Ste11-Msb2 complex dissociation. However, under non-stress conditions, Hog1 only regulateed Msb2 protein levels, suggesting that basal Hog1 activity may be sufficient to this mediate feedback. Furthermore, not only the physical presence of Hog1 but its activity is necessary to regulate Msb2, as negative feedback is not observed in a strain where Hog1 was present but inactive (*pbs2Δ*; Figure 3.7).

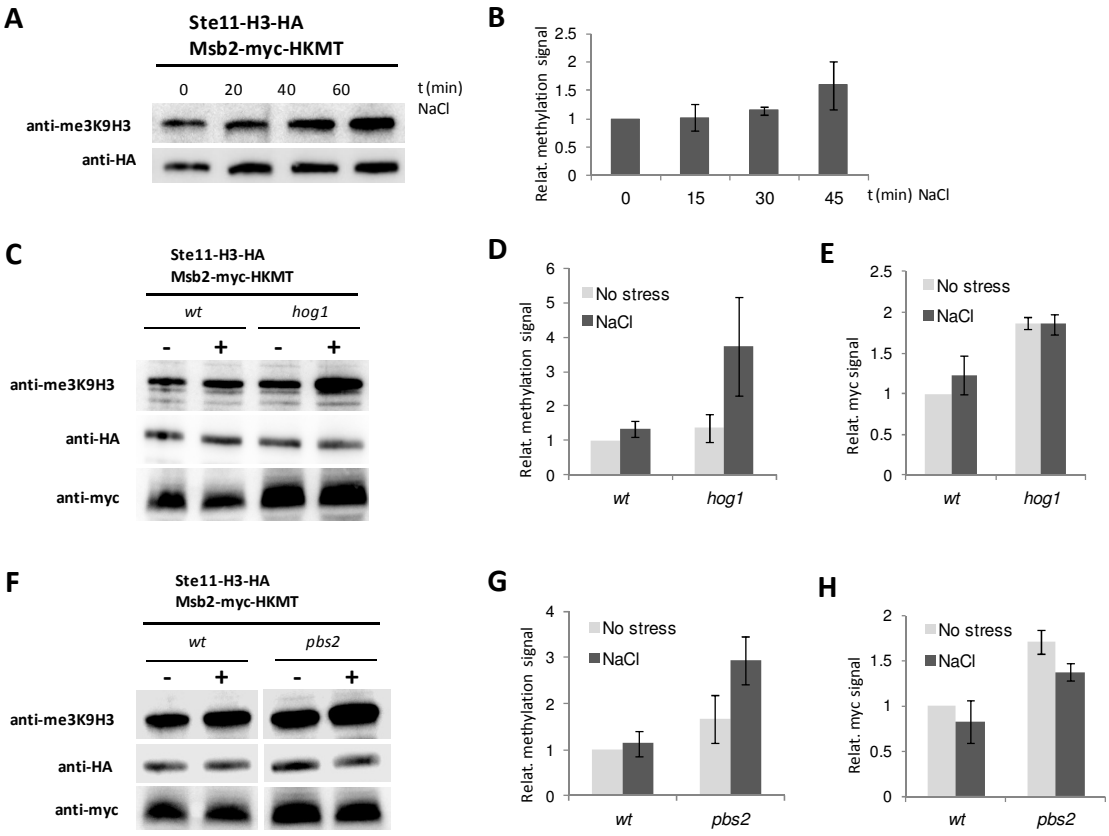


Figure 3.7 Msb2 interacts with Ste11, and Msb2-protein level and relative Ste11-Msb2 interaction are negatively regulated by Hog1. (A) *STE11-H3-HA* (PAZ119) interaction with *MSB2-MYC-HKMT* (PAZ218) in *ste11Δmsb2Δ* (PAY703) strain at the indicated times of osmotic stress. Immunoblots with the indicated antibodies are shown. **(C)** Ste11-Msb2 M-track interaction in wild type (K699) and *hog1Δ* mutant (PAY185) strains under stress (+) and non-stress (-) conditions. Immunoblots with the indicated antibodies are shown. **(F)** Ste11-Msb2 M-track interaction in wild type (*ste11Δ*, YAZ80, complemented with the Ste11 plasmid) and *pbs2Δ* mutant (*ste11Δpbs2Δ*, YID140, complemented with the Ste11 plasmid) strains under stress (+) and non-stress (-) conditions. Bands from each row are from the same Western blot. Immunoblots were developed with the indicated antibodies. **(B)**, **(D)** and **(G)** Quantifications of the methylation signals in **(A)**, **(C)** and **(F)** respectively, normalised as in Fig. 3.2. The average and SD of 3 **(B)**, 2 **(D)** and 3 **(G)** independent experiments are shown. **(E)** and **(H)** Quantifications of the myc signals in **(C)** and **(F)** respectively, compared to wild type signal in the absence of stress. The average and SD of 2 **(E)** and 3 **(H)** independent experiments are shown. The Western blots shown in **(A)**, **(C)** and **(F)** are representative experiments.

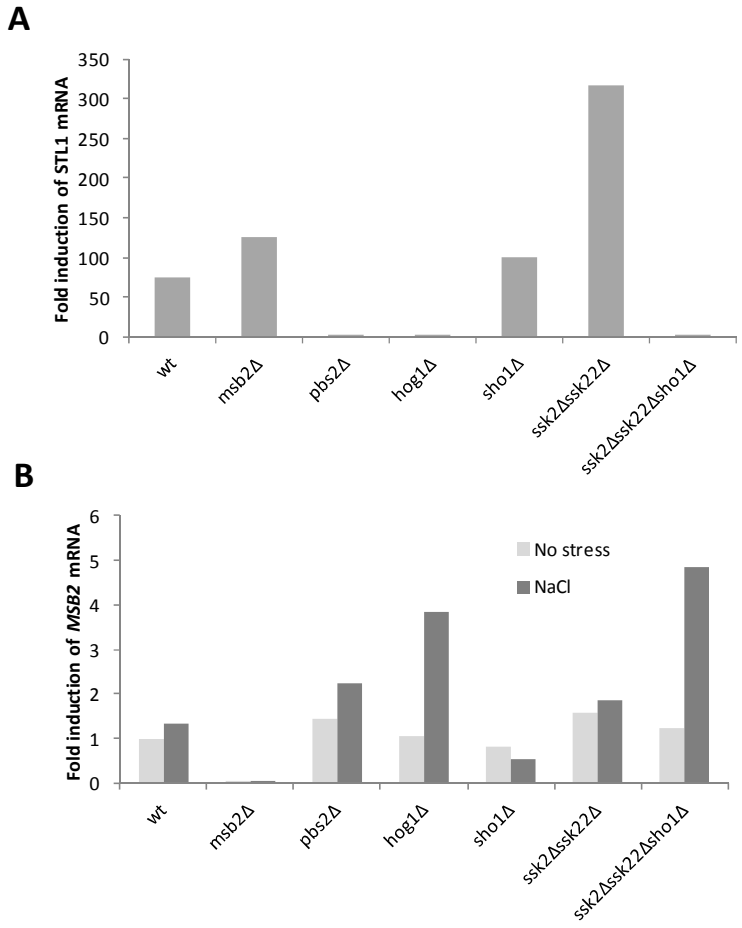


Figure 3.8 HOG-mediated negative feedback to regulate *MSB2* mRNA levels. RT-qPCR analyses of *STL1* **(A)** and *MSB2* **(B)** mRNA levels before and after 30 mins of 0.4 M NaCl osmotic stress (only after stress is shown for *STL1*) in wild type and deletion mutants: *msb2Δ* (CF83), *pbs2Δ* (YVR10), *hog1Δ* (PAY185), *sho1Δ* (YAZ120), *ssk2Δssk22Δ* (YID196) and *ssk2Δssk22Δsho1Δ* (YAZ150). mRNA levels were normalised against the mRNA expression of the endogenous control *ACT1*, and represented as fold-change of normalised mRNA levels in wild type under non-stress conditions. Data shown are the average of two experiments.

3.2.5 Sho1 is not essential for Ste11 interaction with Msb2

It has been previously described that membrane recruitment of Ste11 is mediated through either direct or indirect interactions with the scaffolds Ste50, Opy2 and Sho1 (Saito and Posas, 2012). Hence, it was of interest to determine whether these factors were necessary for the binding of Ste11 to Msb2 at the plasma membrane. Interaction signal was significantly reduced in *sho1Δ* and almost

not observed in *opy2Δ* and *ste50Δ*, initially indicating that the scaffold proteins Opy2 and Ste50, and to a lesser extent Sho1, were required for the Ste11-Msb2 interaction (Figure 3.9).

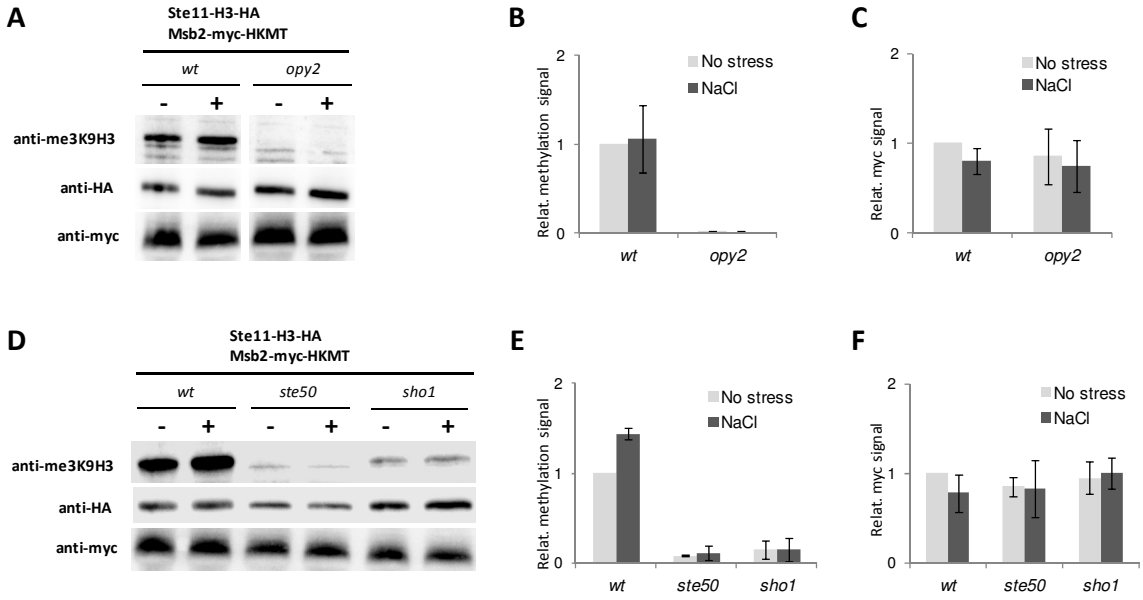


Figure 3.9 Ste11-Msb2 interaction requires Ste50 and Opy2, and to a lesser extend Sho1. *STE11-H3-HA* (PAZ119) interaction with *MSB2-MYC-HKMT* (PAZ218) in the absence of Opy2 (YCF163) **(A)** and in the absence of Ste50 (YAZ87) or Sho1 (YAZ82) **(D)** under stress (+) and non-stress (-) conditions. Immunoblots with the indicated antibodies shown in **(A)** and **(D)** were quantified as described in Fig 3.7, with methylation signals represented in **(B)** and **(E)** respectively, and myc signals in **(C)** and **(F)** respectively. All quantifications show the average and SD of 3 independent experiments. The Western blots shown in **(A)** and **(D)** are representative experiments. All rows were from same Western blot.

To check the essentiality of Opy2, Ste50 and Sho1 in Ste11-Msb2 interaction, we then overexpressed Msb2 by utilising mutants that were unable to propagate signal via the SLN branch through either a double deletion of Ssk2 and Ssk22 osmosensors or through the direct deletion of Pbs2 kinase, coupled with a deletion in the genes that encoded these three scaffold proteins. These mutants were unable to propagate HOG signalling, as *STL1* could not be induced in the *ssk2Δssk22Δsho1Δ* triple mutant (Figure 3.8A), and therefore upregulated Msb2 protein expression (Figure 3.10) due to the absence of HOG negative feedback. As shown in Figure 3.10, the overexpression of Msb2 was unable to restore the Ste11-Msb2 interaction in *ste50* (*ssk2Δssk22Δste50Δ*) and *opy2* (*opy2Δpbs2Δ)* deletion mutants to wild type levels (Figure 3.10A-F). However, the relative increase in Ste11-Msb2

interaction observed in *opy2Δpbs2Δ* with respect to *opy2Δ* was much higher than the increase observed in *ssk2Δssk22Δste50Δ* with respect to *ste50Δ* (19-fold versus 1.6-fold), suggesting that, even though both factors were essential for a physiological Ste11-Msb2 iteraction, Ste50 was required to an higher extent than Opy2 (Figure 3.10A-F). On the other hand, Sho1, shown to interact with both Msb2 and Ste11 (Cullen *et al.*, 2004, Zarrinpar *et al.*, 2004), was observed to be disposable for the Ste11-Msb2 interaction, as the overexpression of Msb2 protein in the triple mutant *ssk2Δssk22Δsho1Δ* was able to fully restore Ste11 binding to Msb2 (Figure 3.10G-I).

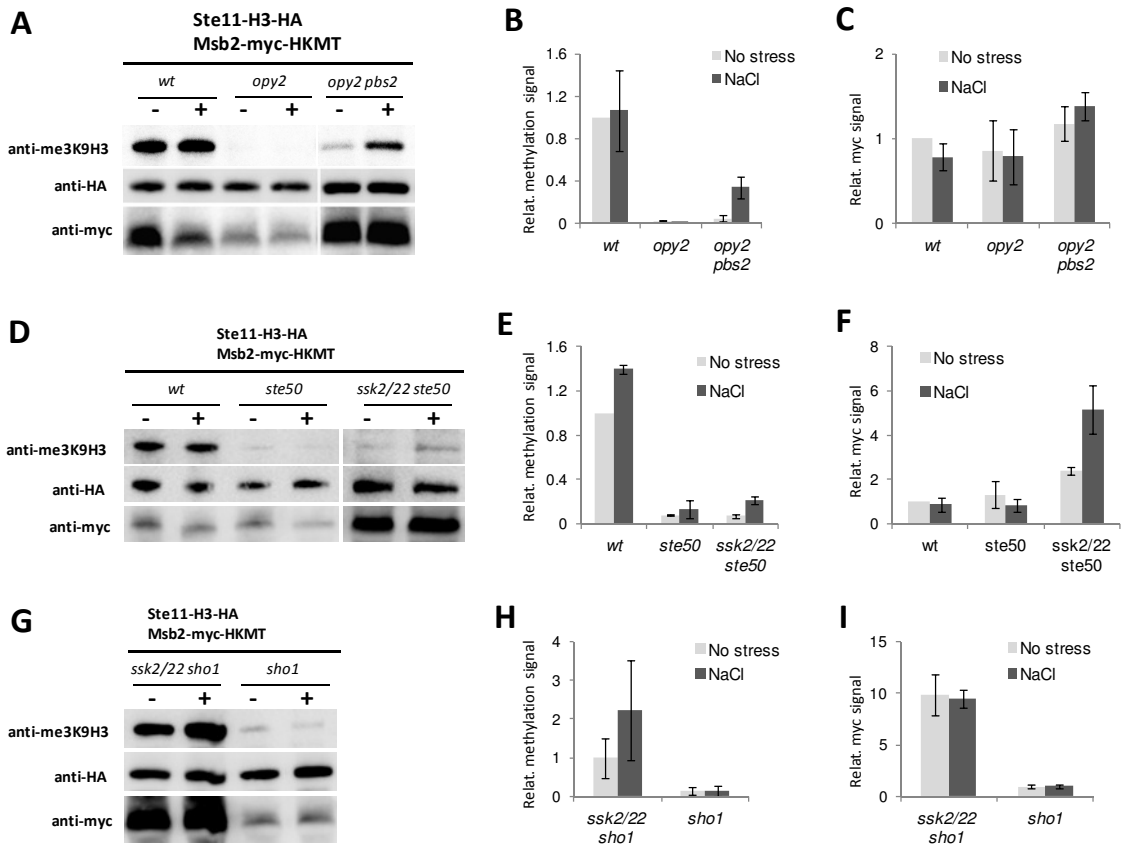


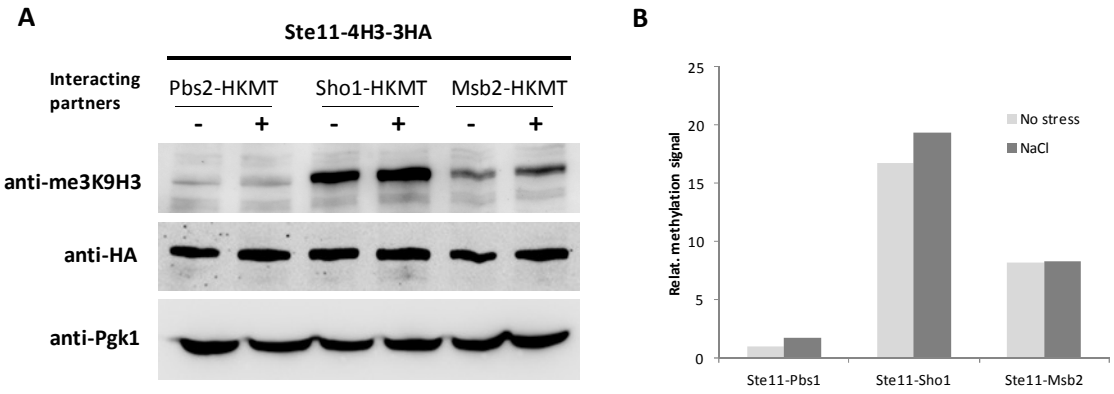
Figure 3.10 Increase in Msb2 protein completely rescues Ste11-Msb2 interaction in *sho1Δ*, but not in *ste50Δ* and *opy2Δ* mutants. (A) *STE11-H3-HA* (PAZ119) interaction with *MSB2-MYC-HKMT* (PAZ218) in wild type (K699), *opy2Δ* (YCF163) and *opy2Δpbs2Δ* (YCF173) strains under stress (+) and non-stress (-) conditions. **(D)** Ste11-Msb2 M-track interaction in wild type (K699), *ste50Δ* (YCF7) and *ssk2/22Δste50Δ* (YID198) strains under stress (+) and non-stress (-) conditions. **(G)** Ste11-Msb2 M-track interaction in *sho1Δ* (YAZ120) and *ssk2/22Δsho1Δ* mutant (YAZ150) strains under stress (+) and non-stress (-) conditions. Immunoblots with the indicated antibodies shown in **(A)**, **(D)** and **(G)** were quantified as described in Fig 3.7 for methylation signals represented in **(B)**, **(E)** and **(H)**, respectively, and myc signals represented in **(C)**, **(F)**

and **(I)**, respectively. Quantifications show the average and SD of 3 **(B and C)** and 2 **(E, F, H and I)** independent experiments. The Western blots shown in **(A)**, **(D)** and **(G)** are representative experiments. Parts of each row are from the same western.

Taken together, these results suggest that the scaffold protein Ste50 is essential for the interaction between the osmosensor Msb2 and the MAPKKK Ste11, and the transmembrane anchor protein Opy2 may be required to a lower extent. Importantly, the transmembrane Sho1 is not essential for Ste11-Msb2 interaction when Msb2 is over-expressed, but is required for an efficient Msb2-Ste11 binding *in vivo* (Figure 3.10).

3.2.6 Ste11 binds to Sho1 with high affinity

In our studies, we described several Ste11 interactions under different conditions, and one observation was evident: different Ste11 interaction partners yielded different strengths in methylation signals. It was therefore of interest to evaluate the possible differences in binding affinities of Ste11 to its substrate Pbs2, and to osmosensors Sho1 and Msb2. As shown in Figure 3.11, the Ste11-Pbs2 interaction resulted in the weakest methylation signal, which, in concordance with previous studies (Zuzuarregui *et al.*, 2012, Zarrinpar *et al.*, 2004), may be due to its extremely transient binding. Ste11 binding to Sho1, on the other hand, resulted in the highest methylation signal; in fact, their interaction gave rise to a signal that was 17 times higher than the Ste11-Pbs2 signal under non-stress conditions, indicating a robust and stable interaction between Ste11 and the co-osmosensor/scaffold Sho1. Finally, and most interestingly, the novel interaction between Ste11 and osmosensor Msb2 also yielded robust methylation signals (8 times higher than the Ste11-Pbs2 signal) both under stress and non-stress conditions, suggesting that this interaction was a stable and direct one.



3.11 Ste11 binds to Sho1 with higher affinity than to Msb2 and Pbs2. (A) *STE11-H3-HA* (PAZ119) interaction with *PBS2-9MYC-HKMT* (PAZ138), *SHO1-HKMT* (PCF54) and *MSB2-MYC-HKMT* (PAZ218) in W303 wild type under stress (+) and non-stress (-) conditions. Immunoblots were incubated with the indicated antibodies. **(B)** Relative methylation signals were normalised against both HA and the endogenous loading control Pgk1, and represented as fold-change of wild type without stress. Data shown are of one experiment.

3.3 Discussion

The osmotic environment of the budding yeast in the wild oscillates as fruits ripe and glucose concentration increases. Survival under hyperosmolarity depends on activation of the HOG pathway, for which the outcome is to accumulate compatible osmolites, such as glycerol, to increase internal osmolarity, control water efflux and adjust cell cycle progression [reviewed in (Saito and Posas, 2012, Saito and Tatebayashi, 2004, Hohmann *et al.*, 2007)]. The central element that regulates this response is the kinase Hog1, which is phosphorylated at conserved threonine and tyrosine residue by the MAPKK Pbs2 upon osmotic stress. However, for this event to occur, a number of upstream signalling components interact to mediate signal transduction via several kinases through two distinct branches, SLN1 and SHO1 (Saito and Posas, 2012, Posas *et al.*, 1998a). This thesis focuses on the interactions between the MAPKKK Ste11 and the sensory system, scaffold proteins and its substrate of the SHO1 branch to reveal how these interactions are inter-dependent in their roles to first propagate and then shutdown signalling in response to hyperosmotic stress.

3.3.1 Ste11 interacts with Msb2 and other signalling factors before and during osmotic stress

In this thesis, we have studied the interactions of the MAPKKK of the HOG pathway Ste11 with its substrate Pbs2, the osmosensor/scaffold Sho1, the Ste11-scaffold protein Ste50 and the membrane osmosensor Msb2 using a novel biochemically-based *in vivo* protein proximity assay (Zuzuarregui *et al.*, 2012). In concordance with previous studies (Posas *et al.*, 1998b, Tatebayashi *et al.*, 2006), Ste11 is observed to form stable interactions with Ste50 and Sho1 in the absence of osmotic stress, and the Ste11-Sho1 signal increases with osmotic stress (Figures 3.4-3.6). Furthermore, in agreement with a previous study by Zuzuarregui *et al.* (2012), we show that the short-lived Ste11-Pbs2 is weakly detected under normal non-stress conditions, and the signal increases substantially with osmotic stress (Figure 3.2). Most notably, we demonstrate that Ste11 comes into close contact with the osmosensor Msb2 under both normal and stress conditions (Figure 3.7). Taken together with the observation that Sho1-Pbs2 also forms a complex under non-stress conditions (Zuzuarregui *et al.* (2012) and Figure 3.3), our results suggest that the signalling components of the SHO1 branch bind or are in close contact with each other before osmotic stress in order to respond and propagate signal rapidly upon stress stimulation. However, it should be noted that the M-track assay is a proximity assay, and therefore it is not possible to distinguish direct from spatially-close indirect interactions. Moreover, it needs to be taken into account that the stoichiometry and overall structure of the signalling components is still unclear, and to add to its complexity, it has been established that at least Sho1 is able to assemble into multimeric structures (Tatebayashi *et al.*, 2015).

3.3.2 Inter-dependency between signalling factors to efficiently recruit Ste11 to the membrane

Investigating which additional factors are required for these interactions, we have made several interesting observations that might shed light on the staging and the dynamics of the SHO1 branch signalling system. First, all Ste11 interactions studied are fully dependent on the presence of the Ste11-adaptor protein Ste50 (Figures 3.2, 3.5, and 3.9), and here, we cannot rule out that all Ste11 interactions may be mediated indirectly via Ste50. Second, we have characterised a novel Ste50-dependent Ste11 interaction with the osmosensor Msb2 (Figure 3.9), and we believe that based on our genetic analyses, together with other evidence in literature, it can be envisioned that the interaction between Ste11 (in a complex with Ste50) and Msb2 may be direct. Although it appears that there is some inter-dependency between Sho1 and Msb2 in how effective they produce an interaction signal with Ste11, as overexpression of Ste11 does not restore Ste11-Sho1 interaction in

the absence of Msb2 and Hkr1 (Figure 3.5C and D), yet, in contrast, overexpression of Msb2 restores Ste11-Msb2 interaction in a *sho1Δ* mutant (Figure 3.10G and H). Thus, Msb2 (with Hkr1) is essential for Ste11-Sho1 interaction, but Sho1 is not absolutely required for the binding of Ste11 to Msb2. It is also of interest to note that Ste11 appears to bind to Sho1 with higher affinity than to Msb2 (Figure 3.11), which may suggest that Ste11 is recruited by two distinct modes: first, the Msb2 may function as an independent recruitment system for Ste11 to the plasma membrane, not absolutely requiring Sho1; second, the weaker binding of Ste11 to Msb2 may be required for the high affinity binding of Ste11 to Sho1 in order to form a stable complex encompassing all three factors (Ste11-Msb2-Sho1 complex). Apart from Sho1, the transmembrane protein Opy2 also seems to be auxiliary for Ste11-Msb2 interaction, as, similar to Sho1, Ste11-Msb2 signals can be partially restored in *opy2Δ* when Msb2 is overexpressed (Figure 3.10A and B).

Emerging evidence shows that Hkr1 and Msb2 are not so redundant after all, as Tanaka *et al.* describes several functional differences between the two so-called ¨redundant osmo-sensors¨ (Tanaka *et al.*, 2014). First, Hkr1 activates Ste11 uniquely via Ste20, however, Msb2 can activate Ste11 via either Ste20 or Cla4. Second, actin cytoskeletion is indispensable for signalling via Msb2 but not via Hkr1. Finally, Bem1, a SH3 domain-containing scaffold protein functioning in polarised growth pathways, is necessary for Msb2 activation, but not Hkr1 activation, of the HOG pathway by recruiting Ste20/Cla4 (Tanaka *et al.*, 2014). Altogether, these results suggest that the mechanisms of signal transduction of the two osmosensors are distinct, and although our results show that Msb2 functions as an independent membrane concentrator of Ste11, we cannot extend this observation to Hkr1 without further investigation. It may also be entirely possible that Msb2 binding to Ste11 is mediated through Bem1, and in effect, the activation of the MAPKKK may be propagated from the Msb2-Bem1-Ste20/Cla4 cascade. Nonetheless, the role of Hkr1 in Ste11 activation is of interest for further study, as a very recent study by Tatebayashi *et al.* (2015) suggests that Hkr1 forms a complex with Opy2 and Sho1, and the Sho1-Opy2-Hkr1 complex acts as an independent signalling sub-branch of the SHO1 branch. This may raise the possibility that Ste11 is recruited to the membrane by two distinct complexes: the Sho1-Opy2-Hkr1 complex and the Sho1-Opy2-Msb2 complex, with the emphasis that Msb2-mediated recruitment of Ste11 is only partially dependent on Sho1 and Opy2 and not at all dependent on Hkr1 (data not shown).

In agreement with many previous studies, Sho1 seems to provide the central hub for connecting the sensory system and the activation system of Ste11 (via Opy2 and Ste50) with its target the MAPKK Pbs2. While the comparatively weak Ste11-Pbs2 methylation signal decreases in the absence of Opy2, Sho1 and Msb2/Hrk1 (Figure 3.2), the Sho1-Pbs2 signal is completely independent of the

membrane proteins Msb2/Hkr1 and Opy2 (Figure 3.3). Accordingly, it appears that the interaction between Pbs2 and Ste11 might not contribute to the membrane recruitment of Ste11. This observation is further strengthened by our finding in which the absence of Pbs2-Sho1 interaction increases the Ste11 occupancy at Sho1 (Figures 3.4 and 3.5). We therefore suggest that the Msb2/Hkr1 sensory system as well as Opy2 might function as primary concentration machineries for the transfer of Ste11 to Sho1.

3.3.3 Hog1-dependent negative feedback regulates Msb2 and Ste11

A second important observation of this work is the apparent increase of the protein interaction signals when a signalling component is deleted. Principally, there are two plausible explanations for such a result; one, a defect in a negative feedback system, or two, the accumulation of intermediate signalling complexes, the dissociation of which depends on the function or structural presence of the deleted downstream component.

Several negative feedback systems have been proposed as essential for the transience and proper dynamics of the stress response mediated by the HOG pathway. Here we showe that at least two of them are executed over the protein level of upstream signalling factors: Ste11 (Figure 3.2 and 3.5) and Msb2 (Figure 3.7). For both proteins we have found that this regulation also occurs, but to a lesser extent, under non-stress conditions. Since the increase in Msb2 levels is not only observed in *pbs2Δ* and *hog1Δ* mutants but also in *ssk2Δssk22Δsho1Δ* mutant, we suggest that general Hog1 activity, and not only the presence of the Hog1 protein, is required for the negative feedback. It also confirms that basal Hog1 activity generated by the SLN1 branch (Macia *et al.*, 2009) is sufficient for this negative regulation. Furthermore, Msb2 is not only regulated at the protein level but also at mRNA level, as a Hog1 deletion upregulates *MSB2* expression by almost 4 times during osmotic stress (Figure 3.8). However, the contribution of mRNA regulation to protein expression is still unclear and requires further investigation, as Msb2 protein levels are regulated in the presence and absence of osmotic stress, whereas *MSB2* mRNA expression is only dependent on Hog1 during osmotic stress.

Other types of negative feedback mechanisms envisioned and proposed at protein binding levels have been detected by our work. Our data suggest that Ste11-Pbs2 and Ste11-Msb2 interactions are indeed highly influenced by Hog1 (Figures 3.2 and 3.7). According to previous studies, the effects could be caused by direct Hog1-dependent phosphorylation events connected to at least two factors: Ste50 and Sho1 (Yamamoto *et al.*, 2010, Hao *et al.*, 2008, Hao *et al.*, 2007). For Ste50, it has

been shown that substitutions of the phosphorylation sites not only increases the Opy2-Ste50/Ste11 interaction but also prolongs Hog1 activation (Yamamoto *et al.*, 2010). Since all Ste11 interactions are dependent on the Ste50/Ste11 complex, the phosphorylation state of this important scaffolding element alone could explain the changes in the protein interaction profile.

Our results also hint at the role of downstream components in the dissociation of signalling intermediates. One obvious finding to support this observation is the increase in the interaction signal between Ste11 and Sho1 when Pbs2 is unable to bind due to the absence of the SH3 domain in Sho1 (Figure 3.4). It is possible that the dissociation of Ste11 after activation of Pbs2 might be a prerequisite to keep the signalling system in a productive state, and to further emphasise this observation is that *hog1Δ* cells show an extraordinary increase in the Pbs2-Ste11 interaction (Figure 3.2).

In conclusion, we have characterised several *in vivo* interactions of the MAPKKK Ste11 with various components of the HOG pathway in order to achieve effective transduction of signal in response to hyperosmotic stress. First, the interaction between Ste11 and its substrate Pbs2 requires all upper components of the pathway. Second, the Ste11-Sho1 interaction does not require the Sho1-SH3 domain, and requires Ste50 and Msb2/Hkr1. Third, we have characterised a novel dynamic interaction between Ste11 and Msb2, where Msb2 acts as a membrane-concentrator of Ste11 requiring Ste50 but not Sho1. Finally, we have observed multiple negative feedbacks regulating protein levels and Ste11 interactions mediated by downstream components of the pathway, Pbs2 and Hog1. Our studies have provided further resolution to what is considered to be a complex and dynamic signalling system, as well as founding the basis for the use of novel biochemical tools to study *in vivo* protein interactions.

Chapter 4

The mRNA cap-binding protein Cbc1 regulates high and timely gene expression

4.1 Background

Cells respond to external osmotic perturbations with changes in gene expression that are coordinated in both magnitude and time. Transcriptional regulation encompasses one of the most important processes to influence gene expression outcomes, and therefore it is of interest to identify underlying mechanisms employed by specific factors to globally regulate transcription under normal growth conditions and in response to environmental changes, such as osmotic stress. In this chapter, our results, together with previous studies, show strong evidence that Cbc1, amongst its numerous functions in mRNA fate, is implicated in global transcription (detailed in section 1.5).

Cbc1, the auxiliary protein that forms part of the cap-binding complex (CBC) with the cap-binding subunit Cbc2, has been implicated in a number of post-transcriptional processes and may be considered a global regulator of mRNA fate. First, in the nucleus, CBC regulates pre-mRNA splicing by forming part of the splicing commitment complex (Zhang and Rosbash, 1999, Gornemann *et al.*, 2005, Pabis *et al.*, 2013), and regulates RNA nuclear export (Gorlich *et al.*, 1996) as well as nuclear degradation of transcripts retained in the nucleus (Das *et al.*, 2003). Second, the cap structure, in combination with CBC, protects RNA from 5´-3´ degradation in both the nucleus and the cytosol (Grudzien *et al.*, 2006, Schwartz and Parker, 2000). Third, in the cytoplasm, CBC drives the pioneer round of mRNA translation through its interaction with the translation machinery (Fortes *et al.*, 2000, Gao *et al.*, 2005). Finally, and more recently, a role for CBC in transcription has been elucidated, where CBC is found to genetically interact with transcription elongation factors, such as RNAPII CTD-Ser2 kinases Bur1 and Cdk9 (Hossain *et al.*, 2013, Lidschreiber *et al.*, 2013, Lenasi *et al.*, 2011). Furthermore, it has been demonstrated that CBC stimulates the formation of PIC at several yeast promoters through its interaction with Mot1 to enhance transcription initiation (Lahudkar *et al.*, 2011). Hence, mounting evidence suggests a possible role for CBC in the regulation of transcription at both initiation and elongation stages.

During exponential growth, transcription of RP genes is very costly and occupies approximately 10 % of all RNAPII activity, and is driven by the specific TF Rap1 (Lascaris *et al.*, 1999, Pelechano *et al.*, 2009). Upon increases in external osmolarity, expression of RP genes is rapidly downregulated combined with a 50 % decrease in global transcription rate, and the transcription machinery is reallocated to transcribe specific osmostress-responsive genes (Cook and O'Shea, 2012, Romero-Santacreu, 2009, Capaldi *et al.*, 2008, O'Rourke and Herskowitz, 2004). The transcriptional upregulation of the majority of these osmostress-responsive genes is dependent on the MAPK Hog1 and its recruitment of specific transcription activators, including Hot1, to a subset of gene

promoters. Once bound to the promoters, the TFs, together with Hog1, mediate PIC formation and recruit RNAPII to drive transcription (Posas *et al.*, 2000, de Nadal *et al.*, 2002, Alepuz *et al.*, 2001, Saito and Posas, 2012).

The interest for Cbc1 in the context of osmotic stress arose when the *CBC1* gene was identified to be required for osmotic stress response through a screening conducted in our laboratory in search for deletion mutants that showed reduced tolerance to NaCl (Romero-Santacreu, 2009). Cbc1 was further described as an important coordinator for the global re-initiation of translation after osmotic stress with additional roles in the translation of specific osmostress-responsive mRNAs (Garre *et al.*, 2012). Furthermore, Garre and co-workers have observed that induction of some osmostress-response mRNAs, as well as the recovery of ribosomal protein (RP) mRNAs, is delayed in *cbc1Δ* mutant following osmotic stress (Garre *et al.*, 2012), suggesting that Cbc1 may play a role in the transcription kinetics of induced genes. A genomic study was previously carried out in our laboratory, which is described in Section 4.2 ¨Previous results¨, and later, more molecular work conducted in this thesis is detailed in Section 4.3 ¨Results¨.

4.2 Previous results

4.2.1 Deletion of Cbc1 deregulates the global transcriptional response to osmotic stress

In a previous study conducted in our laboratory, we observed that the *cbc1Δ* mutant was sensitive to osmotic stress and Cbc1 was required for the rapid association of osmo-mRNAs to polysomes to be actively translated during osmotic stress (Garre *et al.*, 2012). Furthermore, we observed that a deletion in *CBC1* caused a delay in the mRNA induction kinetics of three osmostress-induced genes (Garre *et al.*, 2012). To address whether the role of Cbc1 in osmo-mRNA induction was general or gene-specific and whether these observed defects were transcription-dependent, we conducted a genomic study utilising the technique Genomic Run-On (GRO), previously described Garcia-Martinez *et al.* (2004) and detailed in Romero-Santacreu (2009). Here, we evaluated the changes in global transcription rate (TR) and global mRNA amount (RA) during mild osmotic stress stimulation with 0.6 M KCl for 8, 15, 30 and 45 minutes. TR for 5868 ORFs was determined as a measurement of the density of elongating RNAPII along each gene, and on the other hand, RA of each gene was determined by conventional radioactive cDNA-labelling methods. In concordance with previous data (Romero-Santacreu *et al.*, 2009), both global TR and RA, analysed as the median of all genes, rapidly decreased in the wild type strain upon initial osmotic shock, which recovered during adaptation

phase, with global TR recovering earlier than RA (Figure 4.1A). In contrast, a larger decrease in both TR and RA was observed in *cbc1Δ* compared to wild type, followed by a slower and lower recovery, indicating that Cbc1 is required for the reprogrammation of global TR and RA following osmotic stress (Figure 4.1A).

We then analysed the TR and RA profiles of individual genes and found that majority of genes displayed altered profiles in *cbc1Δ* compared to wild type (Figure 4.1B). The genes analysed were then divided into 11 clusters based on similarities in their TR profiles, and utilising Gene Ontology (GO) tool, a number of functional categories were found to be significantly enriched (Figure 4.1B). Majority of clusters showed lower TR and RA in *cbc1Δ* compared to wild type during osmotic stress, with clusters 5, encoding genes that function in reproductive and cellular developmental processes, and cluster 10, with no significant enrichment of functional categories, showing the most important differences (Figure 4.1B). Further analyses show that genes that correspond to stress responses fall under cluster 1, including the upregulated osmo-responsive genes *STL1* and *GPD1*, encoding a glycerol/proton symporter and a NADH-dependent glycerol-3-phosphate dehydrogenase, respectively. The TR and RA kinetics of cluster 1, showing the fastest and highest induction in wild type during osmotic stress, were delayed in *cbc1Δ*. Genes that encode proteins that function glycogen synthesis, including *GRE3*, fell under cluster 4 and was also affected in *cbc1Δ* in a similar manner to cluster 1. Genes involved in RNA processing and translation, such as those encoding ribosomal proteins (RP) for example *RPL30* and *RPL33B*, fall under cluster 6, encompassed the most significantly enriched functional groups (p-value of e-35.6 and e-30.6, respectively). The TR and RA of this cluster initially became repressed upon osmotic stress and then recovered to non-stress levels in wild type, however, it could be observed that in *cbc1Δ*, after the initial repression, the TR and RA of these genes do not recover to non-stress levels. Interestingly, genes encoding many osmostress-responsive transcription factors, including *HOG1*, *MSN2*, and *HOT1*, also show affected TR and RA kinetics in *cbc1Δ* during osmotic stress (Figure 4.1B). In general, all clusters show reduced and/or delayed TR and RA profiles in the absence of Cbc1, indicating a global role for Cbc1 in the reprogrammation of transcription during osmotic stress.

4.2.2 Function of Cbc1 in osmostress-induced transcription encompasses both Hog1-dependent and -independent genes

The majority of transcriptional regulation under osmostic stress is controlled by the MAP kinase Hog1 (de Nadal *et al.*, 2002, de Nadal and Posas, 2011), thus we aimed to determine the inter-

dependency of Cbc1 and Hog1 by analysing the effect of *CBC1* deletion on the transcription of Hog1-dependent and Hog1-independent genes. The genes that fall under these two groups were selected based on criteria of our previous data (Romero-Santacreu *et al.*, 2009), in which the RA of genes that were considered to be induced (289 genes) or repressed (442 genes) showed at least a 2.5-fold change during osmotic stress stimulation, and were then characterised as Hog1-dependent if they showed at least a 2-fold difference between wild type and *hog1Δ* (see Figure 1.8B). In this study, we utilised the GRO data to compare the median TR and RA of wild type and *cbc1Δ* for each of these groups and found that both Hog1-dependent and Hog1-independent clusters showed similar TR kinetics, where wild type showed rapid increase in TR at 8 mins following osmotic stress which decreased during recovery, whereas in contrast *cbc1Δ* displayed a delay in TR induction with maximum TR levels never reaching wild type levels (Figure 4.2A upper panel). For repressed genes, in wild type we observed a rapid decrease in TR following 0.6 M KCl treatment that begun to recover after 30 minutes which fully recovered after 45 minutes; yet, in contrast, no TR recovery was observed in the strain harbouring a *CBC1* deletion (Figure 4.2A lower panel). Again, the difference in TR kinetics observed in *cbc1Δ* extended to both Hog1-dependent and –independent genes. Taken together, we could conclude that Cbc1 has roles in TR induction of induced genes and TR recovery of repressed genes during osmotic stress, in which at least part of its function is independent of Hog1.

To date, five transcription factors (TFs) have been described to be involved in Hog1-dependent upregulation of genes during osmotic stress (Hohmann, 2002, de Nadal and Posas, 2011). To determine how the *CBC1* deletion affectes specific transcriptional regulons, we compared the TR and RA median of *cbc1Δ* and wild type of genes that fell under five clusters based on their transcriptional regulation by Hot1 (73 genes), Msn2 (394 genes), Msn4 (52 genes), Sko1 (33 genes) and Smp1 (223 genes), in which the list of genes dependent on these TFs were extrapolated from the YEASTRACT database (Teixeira *et al.*, 2014) or Saccharomyces Genome Database (Cherry *et al.*, 2012). In Figure 4.2B, it could be observed that for all five transcriptional regulon subsets, a deletion in *CBC1* resulted in delayed and reduced TR kinetics during osmotic stress response. In particular, an initial delay in TR induction was observed in *cbc1Δ* mutant for Hot1-, Msn2-, Msn4- and Sko1-dependent genes, which was followed by reduced induction coupled with a delayed downregulation of TR in *cbc1Δ* compared to wild type. For Smp1-dependent genes, while wild type displayed an initial decrease in TR profile which later recovered, *cbc1Δ* showed a larger initial TR decrease with reduced and delayed recovery (Figure 4.2B). These results indicate that Cbc1 plays an important role in the upregulation of osmostress-responsive gene expression, and its function encompasses all five Hog1-dependent transcriptional regulon subsets.

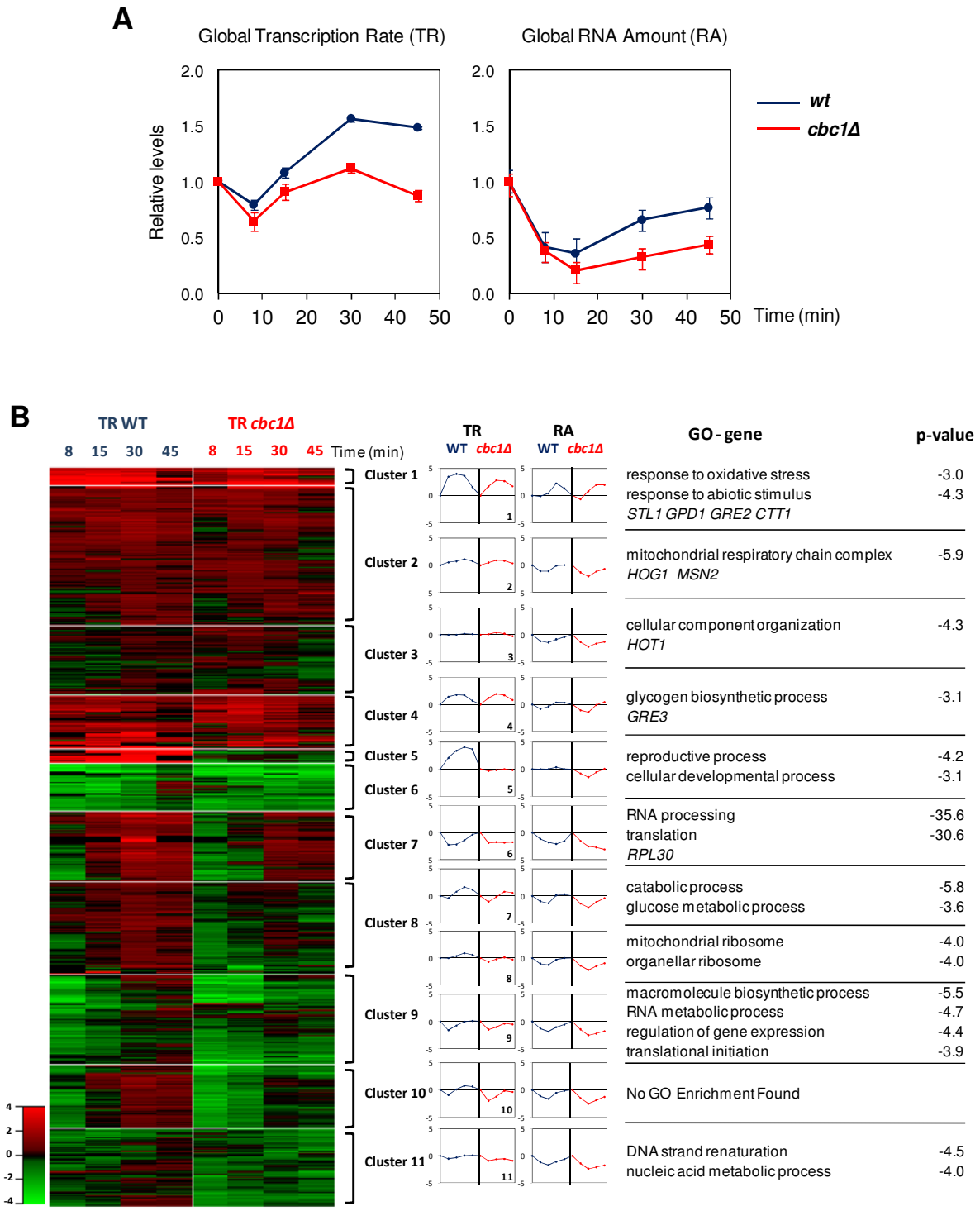


Figure 4.1 Effect of *CBC1* deletion on changes in TR and RA during the response to osmotic stress. Genomic data were obtained from the median of three independent genomic run-on (GRO) experiments normalised as described in Section 2.9, where wild type and *cbc1Δ* were treated with 0.6 M KCl for 8, 15, 30 and 45 mins or left untreated. **(A)** Graphical representations of the medians of transcription rate (TR) and mRNA amount (RA) for 5868 genes for wild type (blue line) and *cbc1Δ* (red) treated with 0.6 M KCl or untreated. Stressed samples were normalised, in each case, against the non-stressed sample of each

experimental group. Error bars represent standard error of all analysed genes. **(B)** Heat map (left panel) demonstrating the classification of all analysed genes based on TR, where each lane corresponds to one gene and each column corresponds to a sample treated with 0.6 M KCl for the indicated time for wild type and *cbc1Δ*. Stressed samples were normalised, in each case, against the non-stressed sample of each experimental group. Green represents relative TR repression and red represents relative TR induction. The middle panel shows graphical representations of the medians of TR and RA of the genes of each cluster for wild type and *cbc1Δ*. Right panel shows significantly enriched GO functional categories of each cluster with the p-value indicated. Representative genes studied in this thesis are written in italics.

According to results by Romero-Santacreu Romero-Santacreu (2009), osmostress-induced genes can be classified into two distinct clusters: genes that are very rapidly induced and rapidly induced. Very rapidly induced cluster contains 49 genes, including *STL1* and *GPD1*, and reaches maximum TR at 15 mins following osmotic stress stimulation, whereas rapidly induced cluster contains 129 genes, including *GRE3*, and reaches maximum TR later at approximately 30 mins (Figure 4.2C). A marked difference could be observed in TR profiles of both clusters for *cbc1Δ* compared to wild type, where in the cluster of very rapidly-induced genes, the TR profile of *cbc1Δ* was observed to be delayed in its induction reaching a higher maximum, and in rapidly induced genes, TR induction in *cbc1Δ* was delayed and never reaching wild type levels (Figure 4.2C).

Overall, the analyses of TR and RA profiles, utilising the method GRO, indicate that Cbc1 plays a role in the initial TR induction of induced genes and TR recovery of repressed genes during osmostic stress. Furthermore, the function of Cbc1 is observed to be more general, affecting most genes, and regulates transcription independently of Hog1, the TR kinetics of the genes, and the specific TF that drives transcription.

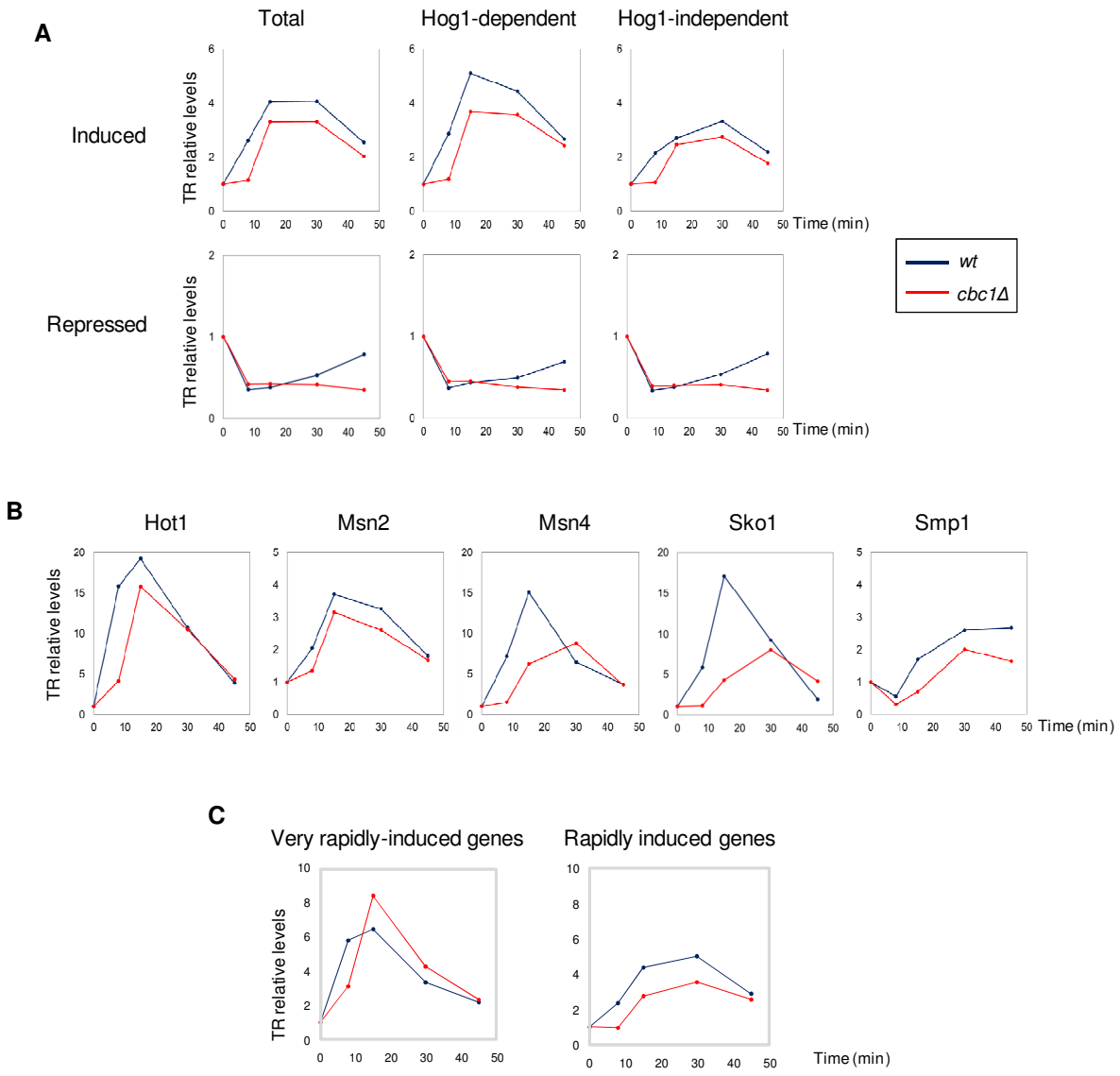


Figure 4.2 *CBC1* deletion influences TR and RA kinetics during osmotic stress of up- and downregulated genes and functions independently of Hog1. Genomic data were obtained from the median of three independent genomic run-on (GRO) experiments as described in Fig 4.1. **(A)** Graphical representations of the TR medians of all induced and repressed genes (left panel). Middle and left panels show clustering of genes that are Hog1-dependent and Hog1-independent, respectively, of both upregulated (upper panels) and downregulated genes (lower panels) in response to osmotic stress. Genes of each cluster were selected based on previous GRO experiments analysing wild type versus *hog1Δ* under stress and non-stress conditions (Romero-Santacreu, 2009). **(B)** Graphical representations of the median of upregulated genes that were divided into five distinct clusters based on their transcriptional dependency on TFs Hot1, Msn2, Msn4, Sko1 and Smp1. Gene classifications were obtained from YEASTRACT database (Teixeira *et al.*, 2014) or Saccharomyces Genome Database (Cherry *et al.*, 2012). **(C)** Medians of upregulated genes that were divided into two clusters, very-rapidly and rapidly induced genes, based on criteria described in Romero-Santacreu (2009).

4.3 Results

4.3.1 Defective recruitment of RNA polymerase II to osmo-induced genes in *cbc1Δ*

Recently, several studies have described Cbc1 to be involved in transcription by regulating pre-initiation complex (PIC) formation at galactose-inducible (*GAL*) genes (Lahudkar *et al.*, 2011) and through the recruitment of transcription elongation kinases (Lidschreiber *et al.*, 2013, Hossain *et al.*, 2013). To evaluate the molecular mechanisms employed by Cbc1 to induce the upregulation of osmostress-responsive genes during osmotic stress and its role in transcription initiation and elongation, we then specifically analysed two very rapidly induced genes: *STL1* whose gene induction is exclusively dependent on Hog1/Hot1 (Alepuz *et al.*, 2001, Bai *et al.*, 2015); and *GPD1* whose gene induction is partially HOG-dependent, specifically regulated by TFs Msn2/4 and Hot1, as well as marginally dependent on the **P**rotein **K**inase **A** (PKA) pathway (Rep *et al.*, 1999a, Boy-Marcotte *et al.*, 1998, Alepuz *et al.*, 2001). We also included a rapidly induced gene, *GRE3*, which encodes a protein that function in carbon source metabolism (Aguilera and Prieto, 2001) whose gene induction is dependent on Hog1 and Msn2/4 (Garay-Arroyo and Covarrubias, 1999). As seen in Figure 4.3, TR of *STL1*, *GRE3* and *GPD1* were rapidly induced following osmotic stress in the wild type strain, and in contrast, a delay was observed in the induction of TR of these genes in *cbc1Δ*. This delay in TR also corresponded to a delay in RA, which could be observed in GRO and confirmed by RT-qPCR data. Additionally, delayed recovery of RA levels after osmotic stress stimulation of the ribosomal protein (RP) gene *RPL30* was also confirmed by RT-qPCR (Figure 4.3 right panels). Next, to evaluate whether the delay in TR and RA induction was due to a direct defect in RNAPII occupancy at these genes, chromatin immunoprecipitation (ChIP) experiments were performed, and a delayed binding of RNAPII to both ORF and promoter of *STL1* and *GRE3* genes was observed in *cbc1Δ* during osmotic stress. Therefore, it can be concluded that the delayed recruitment of RNAPII to osmostress-genes in turn caused a delay in both TR and RA induction, which may directly contribute to the osmostress-sensitive phenotype observed in *cbc1Δ*.

To determine whether the delayed RNAPII recruitment in *cbc1Δ* was due to a change in total and/or active RNAPII protein amount, we measured, utilising western blotting, total and Ser2-phosphorylated RNAPII in wild type and *cbc1Δ* during osmotic stress treatment. Here, we observed no significant differences in total and the fraction of Ser2-phosphorylated RNAPII between wild type and *cbc1Δ* during treatment with osmotic stress (Figure 4.4). These results suggest that the delayed, and sometimes reduced, recruitment of RNAPII to osmostress genes observed in *cbc1Δ* was not due to differences in total or elongating RNAPII protein levels.

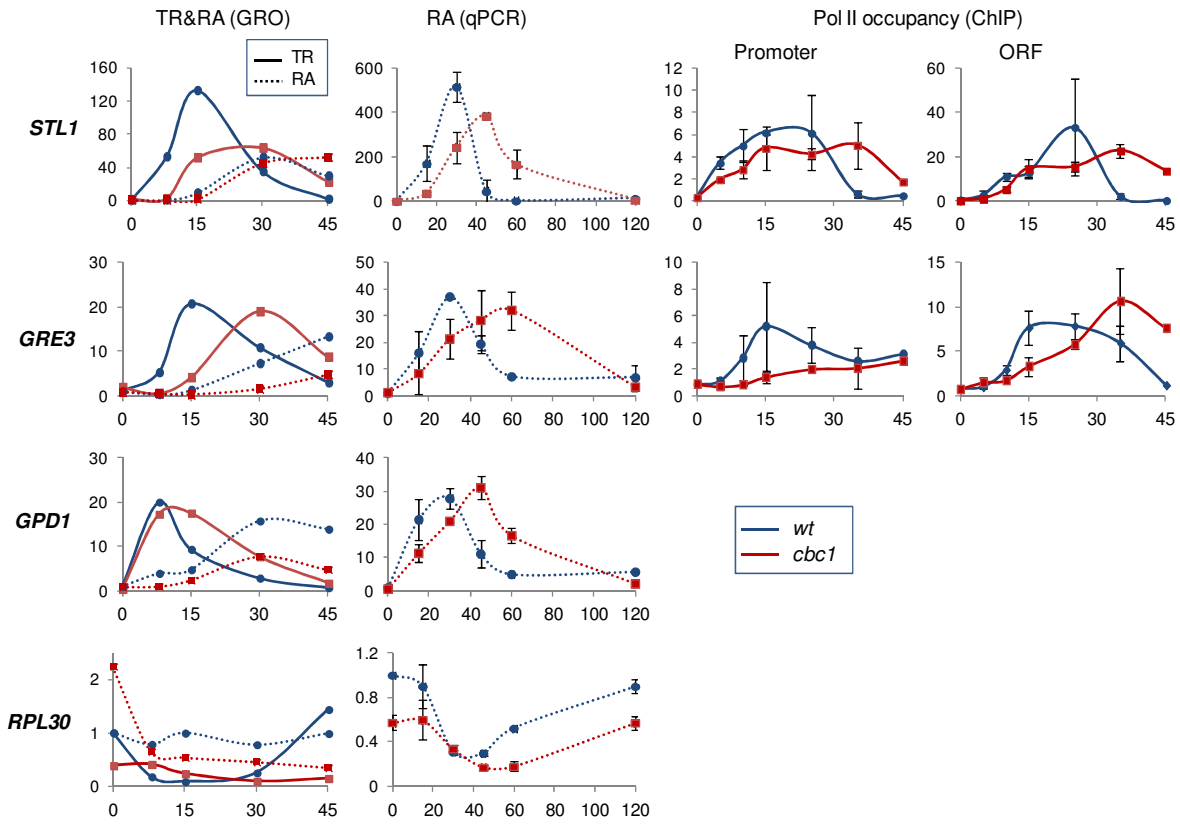


Figure 4.3 Recruitment of RNA polymerase II to upregulated genes is delayed in *cbc1Δ* mutant. The left panel shows averages of TR (solid line) and RA (dotted line) of 3 independent GRO experiments as described in Fig 4.1. Genes *STL1*, *GRE3*, *GPD1* and *RPL30* were analysed under 0.6 M KCl stress for the indicated times and non-stress conditions in wild type and *cbc1Δ*. All data were normalised against wild type non-stress and represented as fold-change. The middle panel shows RT-qPCR analyses of *STL1*, *GRE3*, *GPD1* and *RPL30* mRNA expression in wild type and *cbc1Δ* under 0.6 M KCl stress for the indicated times and non-stress conditions. Relative mRNA expression was calculated by first normalising against the reference gene *ACT1* of each sample and then represented as fold-change in respect to wild type non-stress of each experiment. Averages and standard deviations of independent experiments are represented graphically. The two right panels show ChIP analyses of RNA polymerase II (RNAPII) recruitment to the promoters and ORFs of *STL1* and *GRE3* in wild type and *cbc1Δ* under 0.6 M KCl stress for the indicated time and non-stress. Immunoprecipitation of RNAPII was performed using the antibody 8WG16. Promoters and ORFs were amplified by qPCR using specific primers, and signals were normalised against a non-coding region upstream of *FUS1*, and then represented as fold-change in respect to wild type non-stress of each experiment. Averages and standard deviations of independent experiments are represented graphically. For all graphs, blue line represents wild type and red line represents *cbc1Δ*.

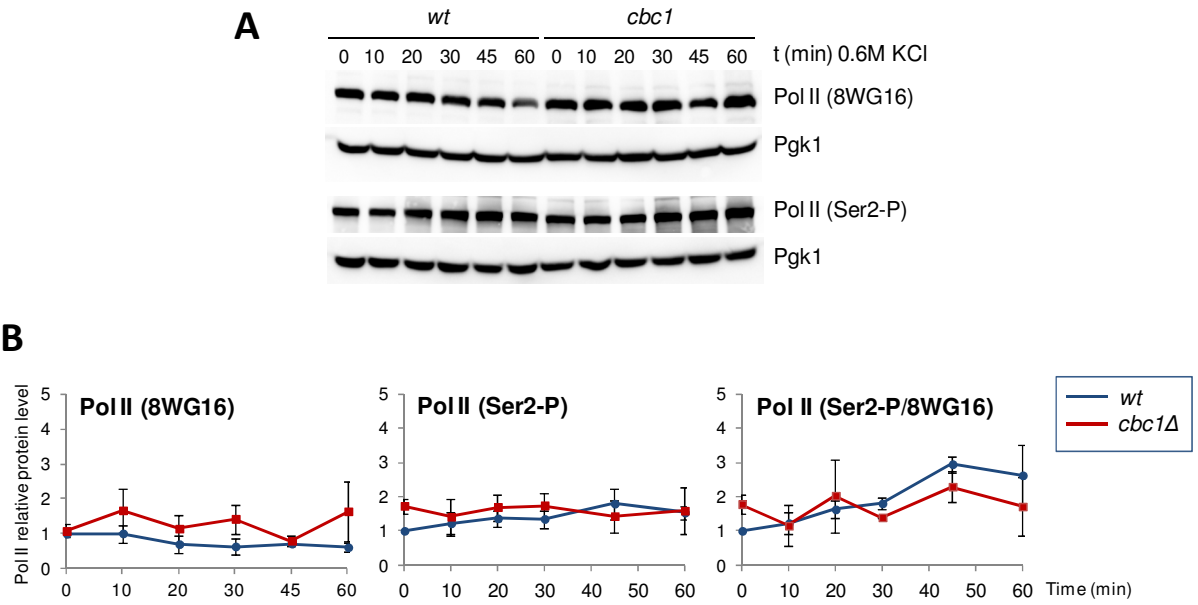


Figure 4.4 Total and elongating RNA polymerase II levels in *cbc1Δ* mutant. Western blot analyses of RNAPII protein expression during 0.6 M KCl treatment for 60 minutes. **(A)** Representative western blots showing protein expressions of total RNAPII and Ser2-phosphorylated RNAPII utilising 8WG16 and anti-Ser2-P antibodies, respectively. Each Western blot was probed with anti-Pgk1 antibody as a loading control. **(B)** Graphical representations of western blot band intensities from three independent experiments, where the blue and red lines represent wild type and *cbc1Δ* respectively. For each immunoblot, band intensities of RNAPII (total or Ser2-phosphorylated) were normalised against the signal of endogenous Pgk1 of each sample, which were then represented as fold-change in respect to wild type non-stress of each experiment. Ratios of phosphorylated RNAPII in respect to total RNAPII were calculated by dividing the relative signals of Ser2-P against 8WG16 for each time point and then normalised against wild type non-stress of each experiment.

4.3.2 Role of Cbc1 in transcription is dependent on Cbc2

As previously established, Cbc1, together with Cbc2, forms the cap-binding complex (CBC), and the two subunits bind to the mRNA cap structure synergistically, where neither subunit alone has sufficient affinity for the structure (Izaurralde *et al.*, 1994, Izaurralde *et al.*, 1995a). The m7GpppN cap-binding pocket resides in the Cbc2 subunit, however, Cbc1 causes a conformational change in Cbc2, which has been found to be required for CBC to bind to the cap structure with high affinity (Calero *et al.*, 2002, Worch *et al.*, 2009, Gonatopoulos-Pournatzis and Cowling, 2014). Moreover, the activities of both subunits are tightly linked and in some cases may be redundant, as *cbc1* and *cbc2* single deletion mutants show similar phenotypes (Das *et al.*, 2000, Hossain *et al.*, 2009). It is

therefore likely that the role of Cbc1 in transcription during osmotic stress is dependent on its ability to bind the m7GpppN cap structure, and hence dependent on the presence of Cbc2. Utilising a *cbc2Δ* deletion mutant, we first evaluated its ability to grow under osmotic stress, and as observed in Figure 4.5A, *cbc2Δ* showed similar growth defects as *cbc1Δ* under both NaCl and KCl osmotic stress. Upon evaluation of osmostress-responsive mRNA expression following osmotic stress, we observed a delay in RA induction in *cbc2Δ* compared to wild type (Figure 4.5B), where the gene expression profiles were similar to those observed in *cbc1Δ* (Figure 4.3). These results suggest that the role of Cbc1 in transcription of osmostress-responsive genes is dependent on its ability to bind the m7GpppN cap structure through Cbc2.

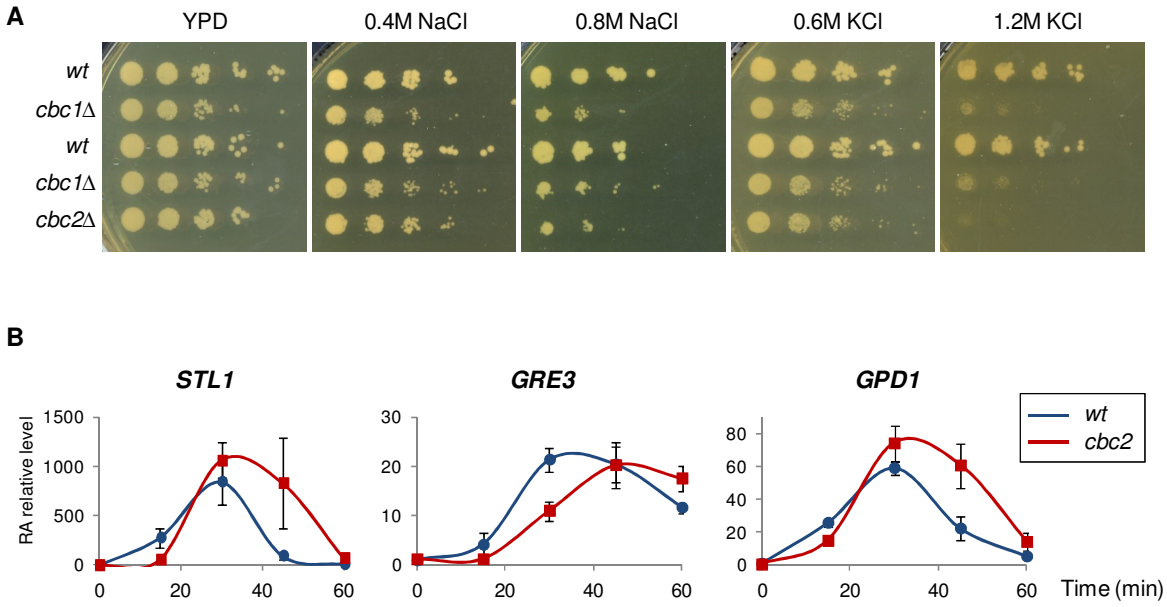


Figure 4.5 Cbc2 is necessary for Cbc1 regulation of transcription. (A) *cbc1Δ* and *cbc2Δ* strains showed similar growth phenotypes under both mild and severe osmotic stress. Serial dilutions of the indicated yeast strains were grown on YPD agar plates containing 0.4 M or 0.8 M NaCl, and 0.6 M or 1.2 M KCl. **(B)** RT-qPCR analyses of *STL1*, *GRE3*, and *GPD1* mRNA expression in wild type and *cbc2Δ* during 0.6 M KCl treatment for 60 mins. Relative mRNA expression was calculated by first normalising against the reference gene *ACT1* of each sample and then as fold-change of wild type non-stress of each experiment. Averages and standard deviations of independent experiments are represented graphically.

4.3.3 Cbc1 does not require Mot1, but recruits the transcription complex Hot1/Hog1 to osmostress promoters to aid PIC formation

It has been previously proposed that Cbc1 drives transcription of GAL genes by recruiting Mot1 transcription factor to *GAL* promoters, which in turn facilitates RNAPII binding (Lahudkar *et al.*, 2011). Mot1 has been described to act as a nucleosome remodeller and TBP regulator, and has been described to induce transcription at TATA-like promoters, including RP genes, and repress transcription at TATA promoters, which compasses majority of stress genes, under normal growth conditions (Zentner and Henikoff, 2013, Wollmann *et al.*, 2011, Topalidou *et al.*, 2004, Sikorski and Buratowski, 2009). It was therefore of interest to investigate whether Cbc1 modulates Mot1 binding at osmostress gene promoters to drive their transcription during osmotic stress response. Using a temperature-sensitive *mot1*^{ts} mutant, the inductions of osmo-mRNAs *STL1*, *GRE3* and *GPD1* were evaluated following Mot1 depletion by incubating at the restrictive temperature of 37 ºC and were compared with a *CBC1* deletion mutant also at the restrictive temperature. It could be observed in Figure 4.6 that the RA induction kinetics of osmo-mRNAs following osmotic stress were more rapid at 37 ºC compared to normal growth temperature of 30 ºC and this was in concordance with previous studies (Figure 4.6 compared to Figure 4.3) (Miguel *et al.*, 2013). Similarly to what was previously observed in Figure 4.3, the induction of osmo-mRNAs following osmotic stress was delayed in *cbc1Δ* in contrast to wild type at 37 ºC, indicating a defect in transcription induction in the absence of Cbc1 (Figure 4.6). However, the depletion of Mot1 did not change the induction profiles of the osmo-mRNAs, indicating that Mot1, unlike Cbc1, was not required for their rapid transcription following osmotic stress, and neither did it appear to function as a transcriptional repressor (Figure 4.6). Therefore it can be concluded that the function of Cbc1 in transcription activation of osmostress-responsive genes is not through the regulation of Mot1 binding to chromatin.

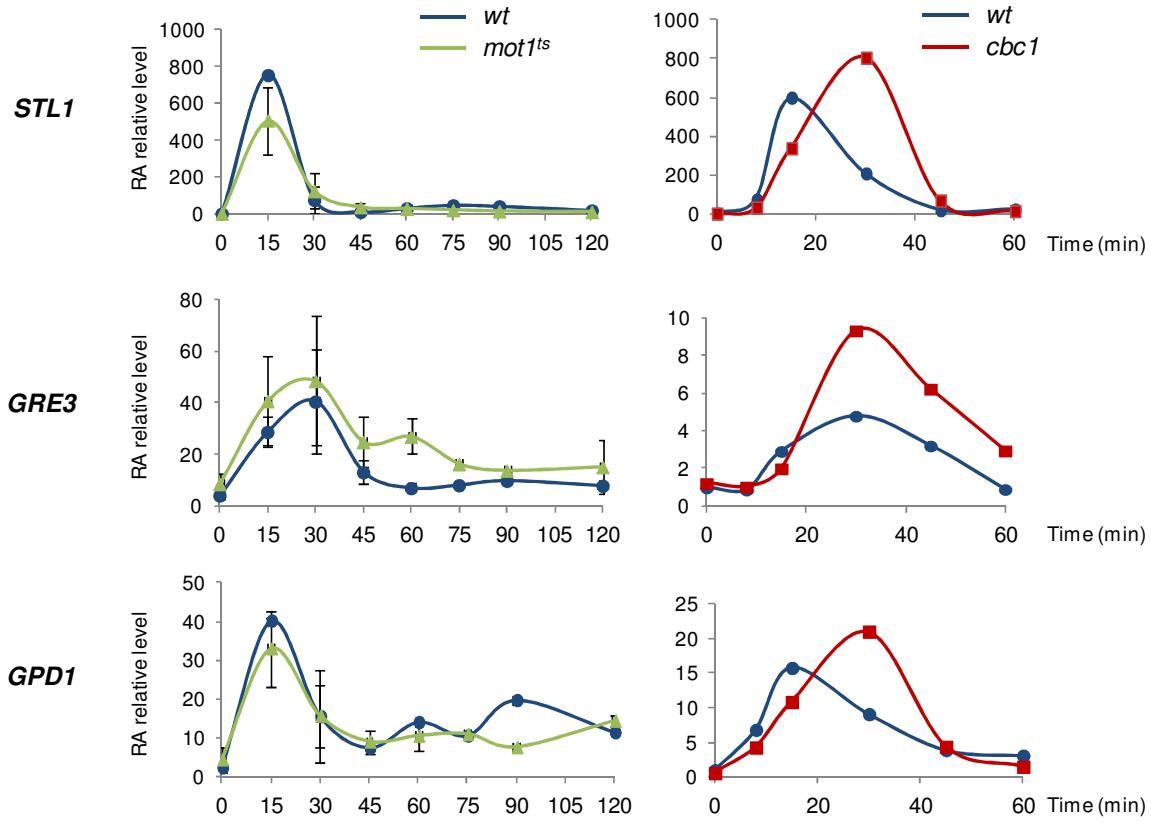


Figure 4.6 Function of Cbc1 in transcription regulation upon osmotic stress seems to be Mot1 independent. RT-qPCR analyses of *STL1*, *GRE3* and *GPD1* mRNA expressions in wild type (blue line), *mot1*^{ts} (green line, left panel) and *cbc1Δ* (red line, right panel). Strains were grown at the permissive temperature 23 ºC until mid-log phase, then transferred to the restrictive temperature 37 ºC for 1 hour and treated with 0.6 M KCl for the indicated time. Relative mRNA expression was calculated by first normalising against the reference gene *ACT1* of each sample and then as fold-change of wild type non-stress of each experiment. Averages and standard deviations of independent experiments are represented graphically in the case where more than one independent experiment has been performed.

Interestingly, in a previous study, Cbc1 has been shown to not play a role in the recruitment of the *GAL* transcriptional activator Gal4 and functions downstream of the transcription factor to directly mediate RNAPII recruitment and PIC assembly (Lahudkar *et al.*, 2011). Here, we investigated the possibility that the delay in RNAPII recruitment to osmostress-induced genes in *cbc1Δ* might be a consequence of a defect in the recruitment of specific activators, such as the transcription factor Hot1 which is required for the transcription of ~70 genes during osmotic stress (YEASTRACT database), and whose specific transcriptional regulon is Cbc1-dependent (Figure 4.2B). Upon osmotic stress stimulation, Hot1 is hyperphosphorylated by Hog1 and recruited to osmostress promoters, and there, the Hot1/Hog1 complex promotes PIC formation and reallocates RNAPII from

housekeeping genes to osmostress-responsive genes (Cook and O'Shea, 2012, Alepuz *et al.*, 2001, Alepuz *et al.*, 2003). Therefore, the Hot1/Hog1 transcriptional complex serves as an obvious candidate as the molecular link between Cbc1 and osmostress-dependent transcription. Utilising chromatin immunoprecipitation, we evaluated Hot1 occupancy at osmostress promoters in the presence and absence of Cbc1, and it could be observed in Figure 4.7A that Hot1 enrichment at *STL1* and *GPD1* promoters was significantly reduced and delayed in *cbc1Δ* following osmotic stress. This was coupled with reduced and delayed TBP occupancy at the same promoters in *cbc1Δ* (Figure 4.7A right panel).

Utilising the previously described M-track method (Section 3.1.2) (Zuzuarregui *et al.*, 2012, Zuzuarregui *et al.*, 2015), we then assessed the ability of Cbc1 to interact with the Hot1/Hog1 complex. A robust Cbc1-Hog1 interaction was observed both before and after osmotic stress, in which the Cbc1-Hog1 relative methylation signal was 10-fold higher than the background signal (Figure 4.8). The observation that the Cbc1-Hog1 interaction signal was higher in *hog1Δ* compared to a wild type strain (Figure 4.8) could be explained by the competitive binding of endogenous Hog1 to the plasmid-expressed methylase-fused Cbc1. Furthermore, Cbc1 binding to Hog1 did not dependent on Hog1 activation, since Hog1 phosphorylation was only observed after osmotic stress (shown as p38 signal), yet interaction signal did not show differences between non-stress and stress conditions (Figure 4.9).

All together, our results suggest that Cbc1 interacts with Hog1 to facilitate the recruitment of the Hot1/Hog1 activator complex to osmostress promoters, which results in the recruitment of TBP and initiation of transcription by RNAPII of these genes during osmotic stress. Therefore, contrary to previous results with *GAL* genes (Lahudkar *et al.*, 2011), Cbc1 does not appear to function through Mot1, but regulates the binding of gene-specific activators to promoters.

4.3.4 Cbc1 regulates Hog1/Hot1 dephosphorylation and Hot1 and TBP protein levels

Within the first few minutes of osmotic stress, Hog1 is rapidly phosphorylated following HOG pathway activation, which in turn rapidly phosphorylates Hot1. Although some Hot1 binds chromatin basally under non-stress conditions, osmotic stress-induced phosphorylation of Hot1 by Hog1 causes an accumulation of Hot1 at stress promoters (Alepuz *et al.*, 2003, Gomar-Alba *et al.*, 2013, Brewster *et al.*, 1993). It is therefore possible that the delayed and reduced Hot1 recruitment to osmostress promoters in *cbc1Δ* is a consequence of defective HOG signalling. Hence, to evaluate correct signal activation during osmotic stress, the phosphorylation status of Hog1 and Hot1 was analysed and we

observed no delay or reduction in the level of phosphorylation of either proteins in *cbc1Δ* during the first minutes of stress (Figure 4.7B). In contrast, a significant increase in Hot1 protein expression was observed in *cbc1Δ* (Figure 4.7B). These results indicated that the absence of Cbc1 did not cause a defect in HOG signalling activation, and additionally, the reduction in Hot1 binding to osomostress promoters was not a consequence of reduced Hot1 protein levels. On the other hand, an apparent delay in dephosphorylation kinetics of both Hog1 and Hot1 was observed in *cbc1Δ* during adaptation following osmotic stress (Figure 4.7B). We then analysed TBP protein expression in *cbc1Δ* and observed a significant reduction compared to wild type both before and after osmotic stress (Figure 4.7B bottom panel), and therefore we could not rule out that the decreased TBP enrichment at promoters in the absence of Cbc1 was not a consequence of reduced protein expression. However, given our observations, coupled with what has been previously described that Hot1/Hog1 recruits TBP to osmostress genes, it is more likely that in the absence of Cbc1, TBP recruitment profile changes as a direct consequence of defective Hot1/Hog1 recruitment, and therefore the reduction of TBP protein may be a due to reduced protein stability of unbound TBP. Furthermore, the stronger phosphorylation signals of Hot1 and Hog1 observed during osmotic stress recovery correlate with longer transcriptional induction kinetics of osmostress genes in *cbc1Δ* (Figure 4.3 and 4.7), suggesting that dephosphorylation may be related to transcription shutoff and/or chromatin binding of these TFs.

Altogether, our data show that the defective chromatin recruitment of Hot1/Hog1 observed in *cbc1Δ* is not a result of defective HOG signalling activation following osmotic stress, suggesting that Cbc1 itself directly mediates Hot1/Hog1 recruitment to promoters. Moreover, Cbc1 also plays a role, either directly and/or through changed chromatin-binding profiles, in Hot1 and TBP protein expression as well as the dephosphorylation kinetics of Hot1/Hog1 during osmostress recovery.

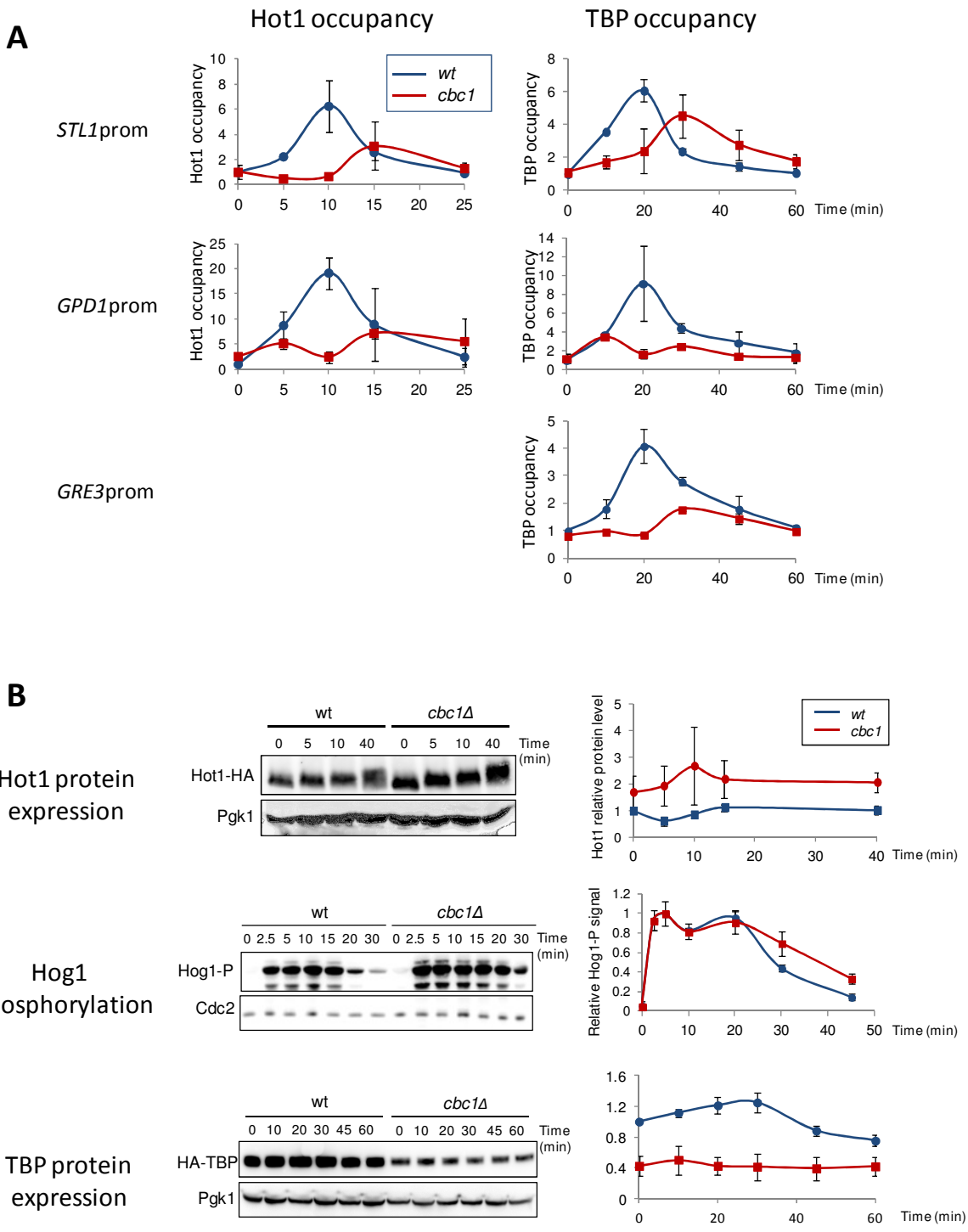


Figure 4.7 *CBC1* deletion does not affect rapid signalling upon osmotic stress but delays Hot1 recruitment and PIC formation at osmo-gene promoters. (A) Hot1 and TBP recruitment to osmo-promoters is deregulated in *cbc1Δ*. Graphical representations of ChIP analyses of Hot1 and TBP recruitment to the promoters of *STL1*, *GPD1* and *GRE3* in wild type and *cbc1Δ* without stress or stressed with 0.6 M KCl at the times indicated. Immunoprecipitations of genomically-tagged *HOT1-HA* (PAY730) and *TBP-HA* expressed in a centromeric plasmid (PA149) were performed using an anti-HA antibody. Promoters were amplified by

qPCR using specific primers, and signals were normalised against a non-coding region upstream of *FUS1*, and then represented as fold-change in respect to wild type non-stress of each experiment. Averages and standard deviations of independent experiments are represented graphically. **(B)** *CBC1* deletion does not delay signalling but changes Hot1 and TBP protein expression. Left panels show representative western blots depicting Hot1 (probed with anti-HA antibody), phosphorylated Hog1 (probed with anti-p38 antibody) and TBP (probed with anti-HA antibody) protein expressions before and after 0.6 M KCl treatment at the times indicated. Loading controls Pgk1 or Cdc28 were detected utilising anti-Pgk1 and anti-Cdc2 antibodies, respectively. Right panels show the average and standard deviations of band intensities of at least 3 independent experiments. Band intensities the protein of interest were normalised against the band intensities of loading controls of each sample, and all relative expressions were represented as a fold-change in respect to wild type non-stress of each experiment.

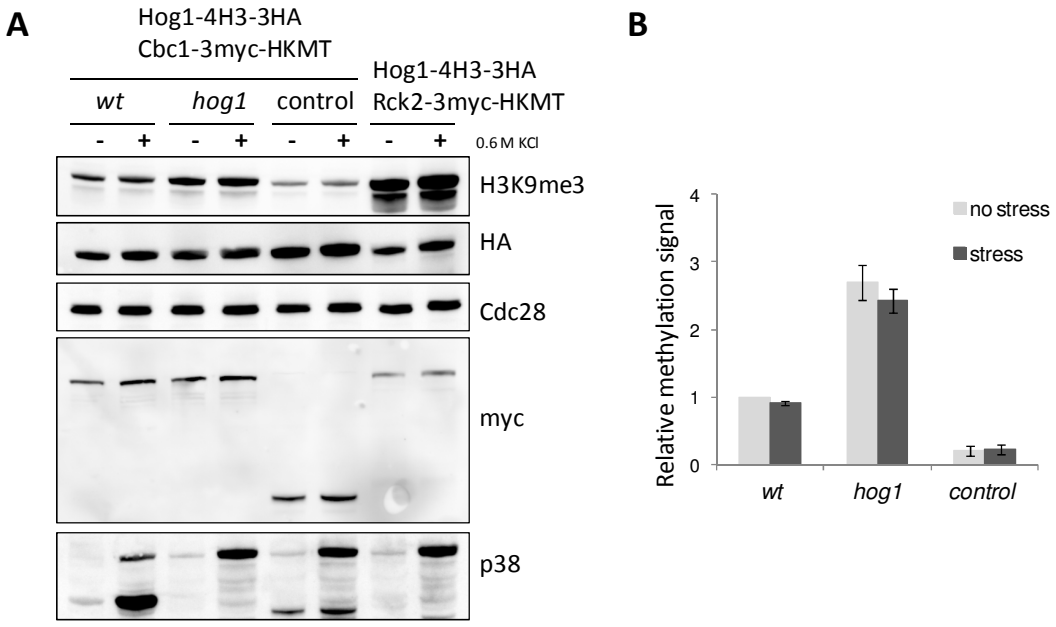


Figure 4.8 Cbc1 interacts with Hog1. (A) A representative western blot showing the interaction of *HOG1-4H3-HA* (CF1E5) and *CBC1-3MYC-HKMT* (P15) detected with anti-methylase antibodies (anti-meK9H3) in wild type and *hog1Δ* (PAY185) strains in the absence or presence 0.6 M KCl (15 minutes). Negative control corresponds to *hog1Δ* (PAY185) transformed with *HOG1-4H3-HA* (CF1E5) and a plasmid containing only the myc tag and methylase (PAZ187). Positive control corresponds to *hog1Δ* (PAY185) transformed with *HOG1-4H3-HA* (CF1E5) and its substrate *RCK2-3MYC-HKMT* (P12) which were known to interact (Bilsland-Marchesan *et al.*, 2000). Immunoblots were also developed with anti-HA antibody to detect *HOG1-4H3-HA* expression, as well as probed with anti-Cbc2 to detect the endogenous control Cbc28. Myc-tagged plasmids were detected with an anti-myc antibody, and phosphorylated Hog1, to observe HOG signalling, was detected with the anti-p38 antibody. **(B)** Quantification of the methylation signals normalised against HA signal and compared to the wild type in the absence of stress. The average and standard deviations (SD) of 2 independent experiments are shown.

4.3.5 Cbc1 modulates RP gene expression through the accumulation of Rap1 and PIC at RP promoters

Our genomic data showed that during osmotic stress most genes, not only induced genes, displayed a transcriptional defect in *cbc1Δ* mutant (Figure 4.1 and 4.2), and data from other authors have described the role of Cbc1 in the transcription of several genes under non-stress conditions, (Lahudkar *et al.*, 2011, Lidschreiber *et al.*, 2013, Hossain *et al.*, 2013), hence it is likely that the role of Cbc1 in transcription is not only restricted to osmotic stress genes, but have a more general role in regulating the transcription of a number of genes even under normal growth conditions. Utilising our genomic data under non-stress conditions (time 0), genes that displayed differential reduction in TR in *cbc1Δ* compared to wild type were most enriched (p-value 5.6e-9) in the GO functional group of ¨ribosome¨ (Figure 4.9A). Comparing the median TR of all RP genes for both *cbc1Δ* and wild type strains with global TR, it could be observed that while for global TR only a slight difference was observed between *cbc1Δ* and wild type (TR ratio *cbc1Δ*/wt of 0.9), the TR of RP genes was significantly and importantly reduced in *cbc1Δ* (TR ratio *cbc1Δ*/wt of 0.612), indicating a specific defect in RP transcription in a strain lacking Cbc1 (Figure 4.9B). RP gene transcription occupies approximately 10 % of all RNAPII activity under normal growth conditions, and their gene expression is rapidly downregulated, followed by recovery, during osmotic stress response (Garre *et al.*, 2013, Romero-Santacreu, 2009). A deletion in *CBC1* also changed the TR kinetics of RP genes during osmotic stress, as it could be observed in Figure 4.9C that the TR of RP genes rapidly decreased and then recovered after 45 minutes of stress in wild type, whereas in *cbc1Δ* the TR of RP genes was lower under normal conditions that presented a small reduction with osmotic stress, followed by almost no recovery. This result was in concordance with what was previously observed in Figure 4.3 for the specific RP gene, *RPL30*. Altogether, our results indicate that Cbc1 does not affect all genes equally, and for some specific groups of genes, such as RP genes, Cbc1 is required for their transcription both under normal conditions and during osmotic stress adaptation.

Our results have shown that Cbc1 mediates the high and timely expression of osmostress-induced genes by specifically facilitating the accumulation of the activator complex Hot1/Hog1 at corresponding promoters, which in turn enhances PIC formation and RNAPII recruitment (section 4.3.4), therefore it is entirely possible that Cbc1 may also mediate recruitment of the RP gene-specific TF Rap1 to RP promoters. Furthermore, reduced TBP protein expression has been observed in *cbc1Δ* (Figure 4.7B), which may directly affect RP gene expression. Hence, we investigated the role of Cbc1 in the regulation of Rap1 and TBP recruitment to two RP gene promoters, *RPL30* and *RPL33B*, under non-stress conditions and during osmotic stress. Firstly, a reduced occupancy of both Rap1 and TBP was observed at the RP promoters in *cbc1Δ* under non-stress conditions (Figure

4.10A). Secondly, following osmotic stress treatment, both Rap1 and TBP occupancy at RP promoters rapidly reduced in wild type cells, and then enhanced during recovery phase (Figure 4.10A). In contrast, lower occupancies of both factors were observed in *cbc1Δ* under non-stress conditions, with an apparent delay in recovery (Figure 4.10A).

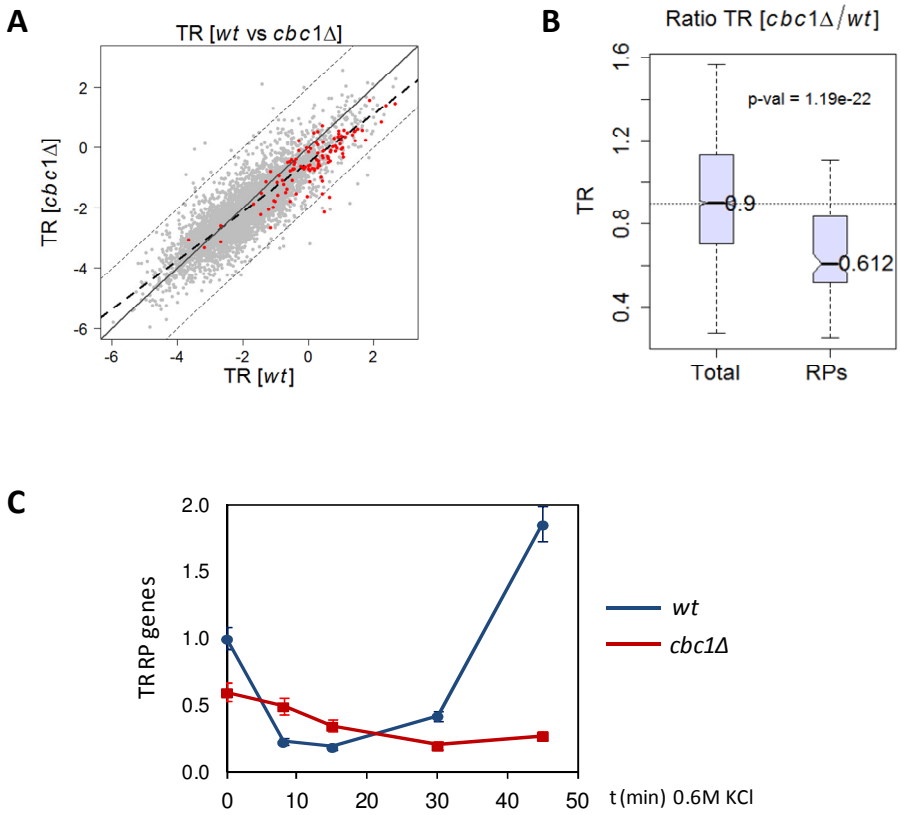


Figure 4.9 Transcription of RP genes under non-stress and osmotic stress conditions is strongly affected in *cbc1* mutant. (A) Plot of *cbc1Δ* TR against wt TR for 5868 genes analyzed by GRO under non-stress conditions (time 0). The bisector is indicated as a solid line and the trend line as a dotted line. Dots for RP genes are represented in red. **(B)** Box and whisker plot representations showing medians and quartiles of ratios between *cbc1Δ* TR and wt TR of total yeast genes or only RP genes under non-stress conditions. The median of each data set is denoted and the whiskers show the data extremes, where outliers are discarded. Total data is compared with RP data and the p-value obtained from t-test is denoted. **(C)** TR median of all RP genes under 0.6 M KCl stress for the indicated times and non-stress conditions in wild type and *cbc1Δ*. Error bars represent standard error of all RP genes analysed. All data were taken from 3 independent GRO experiments as described in Figure 4.1.

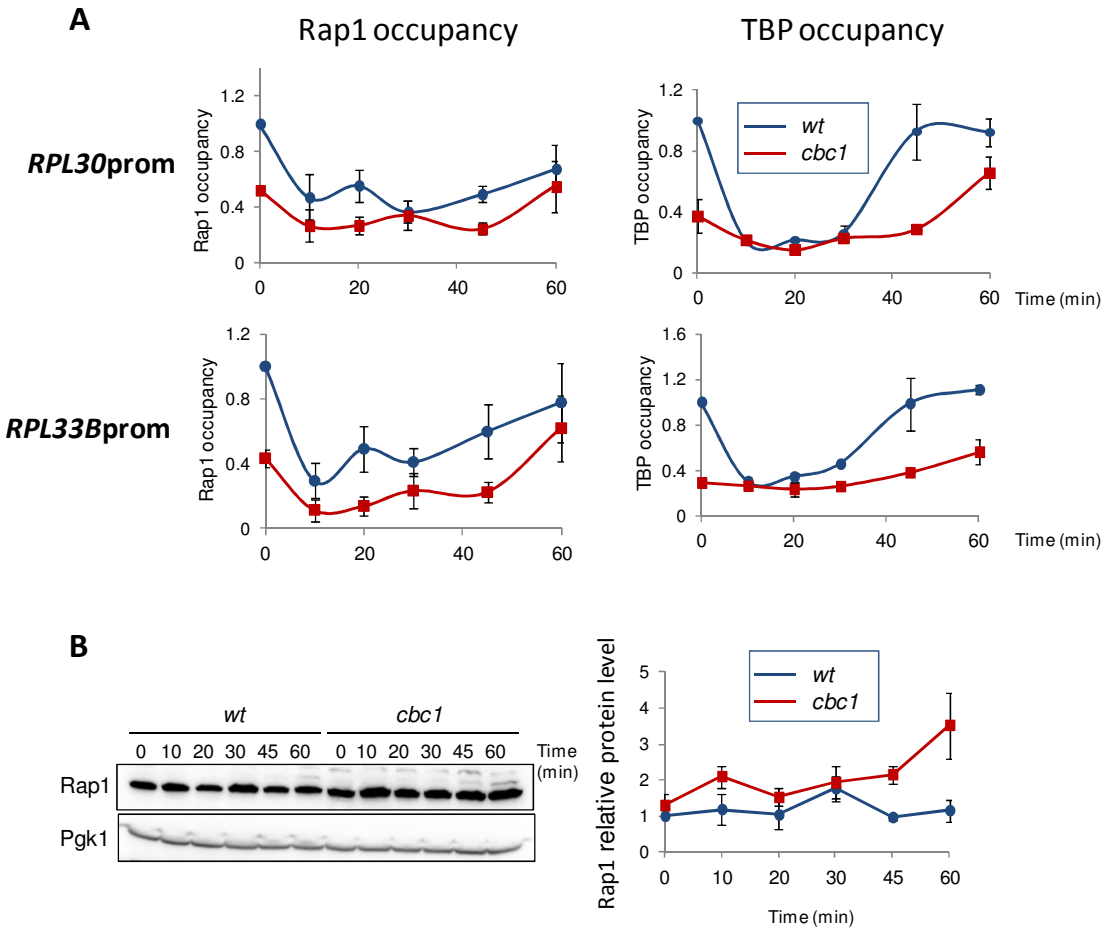


Figure 4.10 Cbc1 regulates recruitment and levels of Rap1 protein. (A) Reduced recruitment of Rap1 and TBP to promoters of RP genes in *cbc1Δ* during osmotic stress. Graphical representations of ChIP analyses of Rap1 and TBP recruitment to the promoters of RP genes *RPL30* and *RPL33B* in wild type (blue line) and *cbc1Δ* (red line) during 0.6 M KCl treatment for 60 minutes. Immunoprecipitation of endogenous Rap1 was carried out using an anti-Rap1 (clone y-300) antibody. Immunoprecipitation of TBP-HA expressed in a centromeric plasmid (PA149) was carried out using an anti-HA antibody. Promoters were amplified by qPCR using specific primers, and signals were represented as a percentage of input, and then normalised against wild type non-stress of each experiment. Averages and standard deviations of three independent experiments are represented graphically. **(B)** Protein expression of Rap1 is induced in *cbc1Δ*. Left panel shows a representative western blot probed with anti-Rap1 and anti-Pgk1 antibodies to detect endogenous protein expressions of Rap1 and loading control Pgk1, respectively, before and after treatment with 0.6 M KCl at the indicated times. Right panel shows the average and standard deviations of band intensities from three independent experiments. Band intensities of Rap1 were normalised against the band intensity of Pgk1 for each sample, and all relative expressions were represented as a fold-change in respect to wild type non-stress of each experiment.

Finally, we analysed Rap1 protein expression during osmotic stress and we observe no reduction in Rap1 protein in the absence of Cbc1 (Figure 4.10B). In fact, we observed a slightly higher Rap1 protein expression in *cbc1Δ* compared to wild type, indicating that the reduced binding of Rap1 to RP promoters was not a consequence of decreased protein expression (Figure 4.10B). In all, our results suggest that, similar to its function in osmostress-induced gene transcription, Cbc1 is required for the high expression of RP genes under non-stress conditions and their transcriptional recovery during osmotic stress by facilitating the accumulation of specific activator Rap1 and TBP at RP promoters. Morever, we also observed changes in protein levels of the transcription activator Rap1 in the absence of Cbc1.

4.3.6 Cbc1 modulates transcription of highly expressed genes and may be implicated in the response to various environmental stresses

To date, although Cbc1 has been described to function in the transcription of several genes, the transcriptional mechanisms employed by Cbc1 to determine transcript-specificity it is still not clear. Here, we hypothesised that Cbc1 might show functional bias towards highly expressed genes based on several observations: first, we showed that Cbc1 was required for the transcription of osmostress-responsive genes during osmotic stress and RP genes during exponential growth (Figure 4.3 and 4.9), in which in both instances the TR of these genes were highly induced; second, previous studies by other authors have described roles of Cbc1 for the transcription of highly expressed genes, such as *PMA1* and *ADH1*, or induced genes, such as *GAL1* (Lahudkar *et al.*, 2011, Lidschreiber *et al.*, 2013, Hossain *et al.*, 2013); third, our genomic data indicated that Cbc1 does not affect the transcription of all genes equally (Figure 4.1 and 4.9). Utilising our genomic data, we analysed the median of TR of 10 % highest and lowest expressing genes in wild type under non-stress conditions and compared to the corresponding TR median of these genes in *cbc1Δ*. We observed that, while *cbc1Δ* showed a slight but significant increase in TR for lowest expressing genes, a marked decrease in TR for highest expressing genes was observed (Figure 4.11). These results suggest that Cbc1, although it may regulate gene repression of low expressing genes, plays an essential role in maintaining high TR for genes that are highly transcribed.

Due to the general role of Cbc1 as a transcription activator and having specific roles in the rapid transcription of osmostress genes in response to KCl osmotic stress, it is therefore expected that Cbc1, together with Cbc2, may have similar roles in the response to other environmental stresses, where rapid and strong upregulation of genes are required for survival and adaptation. Analysing

cellular growth of *cbc1Δ* and *cbc2Δ* mutants under different environmental stresses, we observed that, indeed, both *cbc1Δ* and *cbc2Δ* mutants showed similar growth defects under NaCl, $CaCl_2$, cold and heat stress, with the growth defects being more pronounced with harsher stresses (Figure 4.12). These results suggest that the cellular function of CBC is more general and may play a role in the regulation of transcription in response to various environmental stresses.

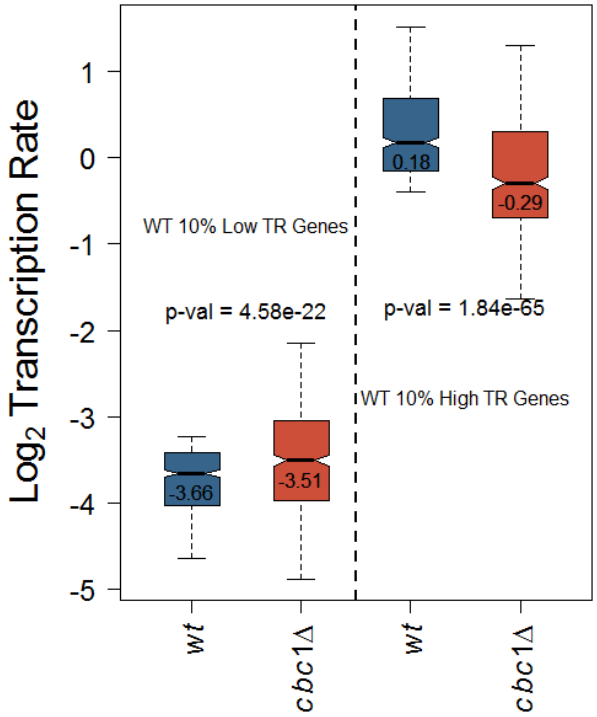


Figure 4.11 Cbc1 show functional bias towards highly expressed genes. The box and whisker plots represent the median and quartiles of the data belonging to the genes with the lowest 10 % TR (left) and the highest 10 % TR (right), which were sorted from low to high in regards to the wild type TR levels under normal. The numbers inside the boxes denotes the median value and the whiskers shows the data extremes (excluding the outliers which lie beyond 1 times the inter quartile range). The wild type TR (blue boxes) was compared to the TR of the same genes in *cbc1Δ* mutant (red boxes) and the p-values from t-test are shown.

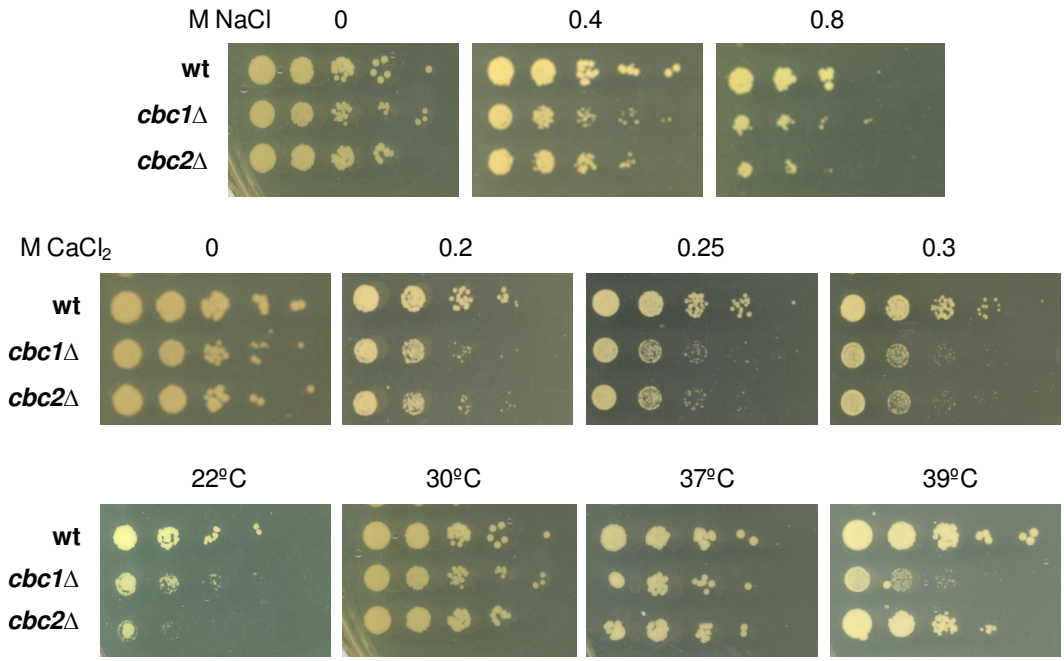


Figure 4.12 Cbc1 and Cbc2 are necessary for adaptation to other stresses. *cbc1Δ* and *cbc2Δ* strains show similar growth phenotypes under sodium, calcium, cold and heat stress. Serial dilutions of the indicated yeast strains were grown on YPD agar plates containing 0, 0.2, 0.25 and 0.3 M of $CaCl_2$ or 0, 0.4 and 0.8 M NaCl and plates were incubated at 30 ºC. For cold and heat stress, serial dilutions of the indicated yeast strains were grown on YPD agar plates and were incubated at 22 ºC, 30 ºC, 37 ºC and 39 ºC for 48 hours.

4.4 Discussion

RNAPII transcription is a complex process involving diverse, yet distinct, stages, such as initiation, elongation and termination, which respond to intracellular signalling in such a dynamic manner that the mRNA being synthesized is simultaneously modified and imprinted for its subsequent life (Venters and Pugh, 2009, Haimovich *et al.*, 2013). Cross-talk between factors involved in all these processes will determine the speed, intensity and length of transcription for each particular mRNA under each particular cellular condition (Moore and Proudfoot, 2009, Perez-Ortin *et al.*, 2013). In this context, interaction between factors of different stages of mRNA life and the existence of factors with multifunctional roles will be necessary to achieve this complex coordination.

The cap-binding protein Cbc1, which forms part of the cap-binding complex (CBC) with Cbc2, associates with the 5´ mRNA cap structure co-transcriptionally and has been identified as an important multifunctional factor coordinating different steps of co-transcriptional RNA processes,

including pre-mRNA splicing (Zhang and Rosbash, 1999, Gornemann *et al.*, 2005, Pabis *et al.*, 2013) and regulation of RNA degradation (Grudzien *et al.*, 2006, Schwartz and Parker, 2000). Recent studies have also described Cbc1 to regulate several stages of transcription, including PIC formation and recruitment of RNAPII to promoters during transcription initiation, and efficient recruitment of the RNAPII kinases, Bur1 and Ctk1, to establish high levels of CTD Ser2-phosphorylation during transcription elongation of several genes (Lahudkar *et al.*, 2011, Lenasi *et al.*, 2011, Lidschreiber *et al.*, 2013). Our work takes a further step to show that Cbc1 mediates the accumulation of gene-specific activators at promoters, which in turn recruit PIC components and RNAPII, and we have identified a physical interaction between the cap-binding protein and the central signalling factor of the osmotic stress response, the MAPK Hog1, which itself acts as a chromatin-binding activator by promoting the rapid recruitment of RNAPII upon osmotic shock (Alepuz *et al.*, 2001, Alepuz *et al.*, 2003). Therefore, our results, together with what has been previously described, demonstrate that Cbc1 connects RNA processing to all steps of transcription, including the accumulation of the signal-induced transcription activators at gene promoters.

4.4.1 Mechanism of Cbc1 regulation of transcription initiation

In this Chapter, we provide evidence for the role of Cbc1 in regulating transcription initiation, more specifically in the accumulation of transcription activator at gene promoters to induce transcription, obtained from both stress and non-stress results. First, we observed that Cbc1 was required for the transcriptional response to osmotic stress, as a deletion in *CBC1* caused a change in global TR profiles. More specifically, TR and RA induction of osmostress-induced genes were seen to be delayed and/or reduced in *cbc1Δ*, which was coupled with a reduction in TR and RA recoveries of repressed genes in response to osmotic stress (Figure 4.1-4.3). Second, our analyses revealed that Cbc1 was required for the recruitment of the Hot1/Hog1 activator complex to the osmostress promoters and we detected a physical interaction between Cbc1 and Hog1 (Figures 4.7 and 4.8). Third, Cbc1 mediated the recruitment of RNAPII and formation of PIC at the promoters during osmotic stress, which might be a consequence of the role of Cbc1 in recruitment of the activator complex (Figures 4.3 and 4.7). Fourth, no delay in Hot1 and Hog1 phosphorylation by the HOG signalling pathway was observed in *cbc1Δ* mutant; therefore the delayed accumulation of the Hot1/Hog1 complex at promoters was not a consequence of defective signalling (Figure 4.7). Fifth, under normal growth conditions, Cbc1 was required for the high expression of RP genes and for the accumulation of their gene-specific activator Rap1 and PIC at the promoters (Figures 4.9 and 4.10). And finally, during osmotic stress, Cbc1 appeared to play a minor role in the rapid downregulation of

RP expression; however it was required for the recovery of transcription by accumulating Rap1 and TBP at the RP promoters (Figures 4.9 and 4.10).

The Cbc1/Cbc2 complex binds the RNA 5′ m^7GpppN cap during transcription elongation when the pre-mRNA is approximately 25 nucleotides in length (Section 1.2.3) (Shandilya and Roberts, 2012, Gonatopoulos-Pournatzis and Cowling, 2014), indicating that CBC does not play a role in the pioneer rounds of transcription. However it may regulate subsequent rounds of transcription once bound to the cap by facilitating the accumulation of transcription activator at the promoter and enhancing RNAPII recycling. Indeed, this is also supported by the observation that the deletion of *CBC1* does not completely abolish transcription of Cbc1-dependent genes, but instead delays and reduces induction of these genes. The accumulation of sequence-specific activators at promoters may be mediated by Cbc1 through the stabilisation of activator/DNA interaction or the interaction between the activator and other components of the PIC. Additionally, we have documented a reduced binding of TBP to osmostress-induced promoters and to RP promoters under stress and non-stress conditions in the absence of Cbc1 (Figures 4.7 and 4.10), and it has been described that stable binding of TBP at promoters facilitates the reinitiation of transcription (Poorey *et al.*, 2010). Therefore, a lower level of TBP at promoters caused by the deletion of *CBC1* could explain the inability to reach high transcription rates in the *cbc1Δ* mutant. However, since the binding of TBP should not affect activator binding to promoters, our observations suggest that reduced TBP binding is a consequence of reduced accumulation of activator at promoters. Yet, it is entirely possible that Cbc1 may regulate the binding of both activator and TBP in mechanistically distinct manners.

The previous work by Lahudkar *et al.* conducted using several galatose-induced *GAL* genes shows that CBC interacts and recruits Mot1, which has been shown to regulate TBP binding to promoters, and positively regulates transcription of *GAL* genes (Lahudkar *et al.*, 2011, Zentner and Henikoff, 2013), and previously it has also been shown that Mot1 binds stress-regulated promoters (Geisberg and Struhl, 2004). However, kinetics of osmostress mRNA upregulation in conditions of Mot1 depletion did not show delayed induction, whereas under same conditions, depletion of Cbc1 provoked a clear alteration in osmo-mRNA kinetics (Figure 4.6). Therefore it is likely that the transcriptional contribution of Mot1 to the expression of specific genes is not equal, where it is required for *GAL1* expression but not for osmostress-responsive genes.

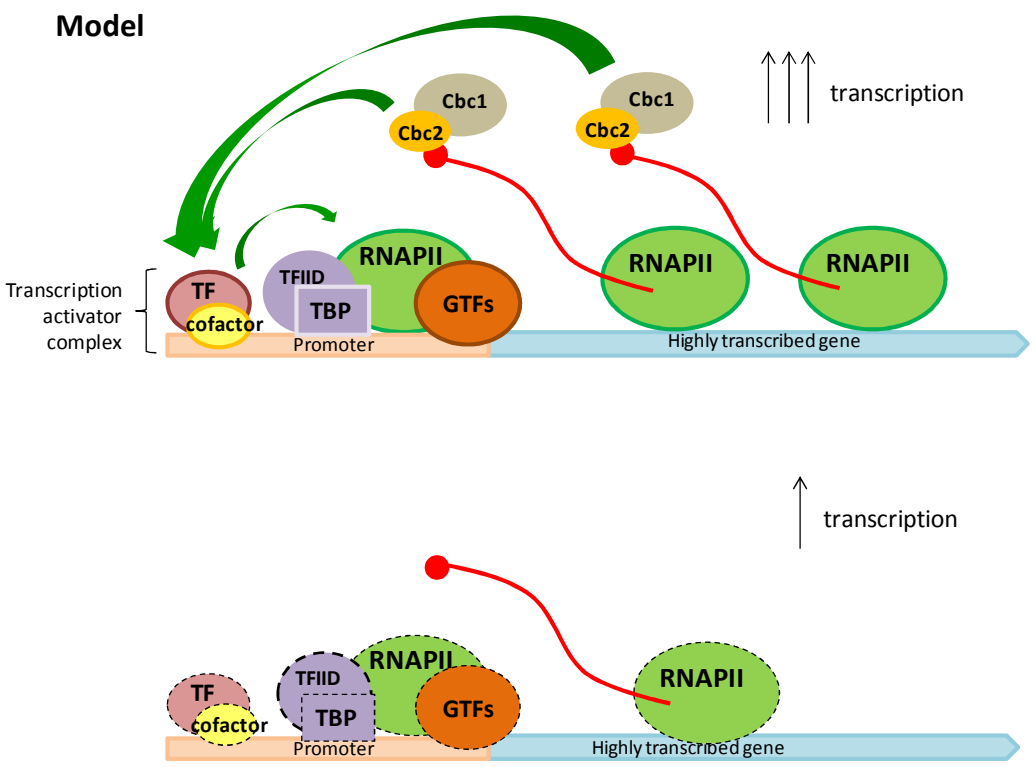


Figure 4.13 Cbc1 rapidly and strongly induces transcription of highly transcribed genes. CBC binds mRNA cap during transcription elongation, and once bound, Cbc1 mediates the promoter-recruitment of the transcription activator complex, consisting of gene-specific transcription factor and/or cofactors, which in turn induces PIC formation and RNAPII recruitment to carry out high and rapid transcription. On the contrary, in the absence of Cbc1, highly transcribed genes are not efficiently induced and show delayed and/or reduced accumulation of activators and PIC at the promoter. Furthermore, the absence of Cbc1 results in altered protein stability of transcription activators. Green arrows indicate positive feedback, and shapes with dotted black lines indicate a decrease in protein recruitment.

In our study, we assume that Cbc1 acts on the transcriptional machinery once bound to the 5' cap of the mRNA that is being synthesised. Cbc1 binds to the cap through its dynamic interaction with the cap-binding subunit Cbc2, and this interaction is essential for the binding of CBC to the cap (Calero *et al.*, 2002). Several lines of evidence suggest that Cbc1 functions in transcription initiation through its binding to the capped mRNA: first, we have shown that a *CBC2* deletion mutant displays similar stress-sensitive phenotypes and gene expression defects as *cbc1Δ* (Figures 4.5 and 4.12), which is also supported by a previous study showing similar induction of *GAL* genes for *cbc1Δ* and *cbc2Δ* mutants (Lahudkar *et al.*, 2011); second, in *GAL* genes, regulation of PIC formation by CBC seems to occur after mRNA capping, since depletion of the capping enzymes, Cet1 and Ceg1, also render

defects in TBP and RNAPII recruitment (Lahudkar *et al.*, 2011). Third, the deletion of Cbc1 has been previously shown to significantly reduce the stability of Cbc2, whereas the stability of Cbc1 is not dependent on the presence of Cbc2, indicating that in a *cbc2Δ* mutant there is sufficient Cbc1 protein amount but is unable to bind the 5' mRNA cap (Wong *et al.*, 2007). However, given that neither monomeric Cbc1 nor Cbc2 is able to bind the mRNA cap (Izaurralde *et al.*, 1994, Izaurralde *et al.*, 1995a), it is therefore more likely that the molecular functions of the two subunits of CBC are synergetic and inter-dependent and linked to their association with the mRNA cap structure.

4.4.2 Cbc1 specifically enhances transcription of highly expressed genes

Interestingly, our work presents Cbc1 as a factor that shows functional bias towards highly expressed genes, as such that it is required for the high expression of osmostress-induced genes during osmotic stress treatment and RP genes under normal conditions and during the recovery phase of osmotic stress response. Furthermore, previous studies showed that Cbc1 regulates transcription of highly induced *GAL* genes and of two constitutive genes, *PMA1* and *ADH1*, with high levels of expression under normal conditions (Lidschreiber *et al.*, 2013, Hossain *et al.*, 2013, Lahudkar *et al.*, 2011). Most importantly, we show that, under non-stress conditions, transcription of the highest transcribed genes is significantly reduced in *cbc1Δ* mutant, and on the contrary, transcription of the lowest transcribed genes is even slightly augmented in *cbc1Δ* (Figure 4.11). These observations suggest that although Cbc1 binds all mRNAs, it does not affect RNAPII trasnscription equally in all genes. Previous expression analysis of RNA levels by microarrays have shown that Cbc1 deletion leads to a two-fold altered expression of a subset of yeast genes (around 6 %) (Hossain *et al.*, 2009), and in mammalian cells depletion of the Cbc1 homolog, Cbp80, results in the deregulation of around 400 genes (Narita *et al.*, 2007). We propose that the specificity of Cbc1-mediated transcriptional regulation depends on the gene transcription rate and the velocity of its induction kinetics under induced conditions, and therefore, not restricted to one or two specific activators. In fact, although Cbc1 is observed to mediate Hot1/Hog1 promoter recruitment to a subset of genes, Cbc1 is also found to be required for the transcriptional upregulation of Hog1-independent genes during osmotic stress (Figure 4.2A), suggesting that Cbc1 may function to positively recruit other gene-specific activators to their corresponding promoters of upregulated or highly expressed genes. Indeed, this is the case for establishing the high transcription of RP genes, which is mediated by Cbc1 through the accumulation of Rap1. Furthermore, although in our work we show a physical interaction between Hot1/Hog1 complex and Cbc1, further investigation is

needed to understand how Cbc1 is able to regulate the accumulation of different gene-specific activators on promoters.

Taken together, we provide a model in which highly transcribed genes, containing high density of RNAPII, recruits more CBC to bind to the mRNA 5´ cap, and once bound, CBC then aids subsequent rounds of transcription initiation and formation of PIC through the accumulation at promoters of gene-specific activators, which in turn increase the PIC formation to further induce mRNA expression of genes in a positive feedback loop.

4.4.3 Impact of Cbc1 on the protein expression of activators

We have observed that deletion of *CBC1* appears to increase or decrease protein expression of the Cbc1-dependent chromatin binding activators Hot1, Rap1 and TBP (Figures 4.7 and 4.10). In the case of Hot1 and Rap1, a higher protein level is observed in a *cbc1Δ* mutant, and therefore the reduced promoter association of these factors is not caused by reduced protein expression. For TBP, a significant lower protein level is observed in *cbc1Δ*, which could explain the reduced TBP and RNAPII promoter enrichment. Alternatively, it is possible that the decreased promoter accumulation of these transcription activators in absence of Cbc1 in turn causes a change in their protein stabilities, as such that decreased promoter association results in increased Hot1 and Rap1 stability. Similarly, TBP protein may be rendered more unstabled due to a decrease in its chromatin association as a consequence of decrease Hot1/Hog1 recruitment in *cbc1Δ*. Our hypothesis is supported by previous studies showing that the ubiquitin protease Ubp3, which reverses the ubiquitination of TBP during transcriptional activation (Chew *et al.*, 2010), is recruited by Hog1 to osmostress-inducible promoters where it modulates transcription, suggesting that Ubp3 could protect promoter-bound TBP from degradation (Sole *et al.*, 2011). Moreover, a connection between recruitment of gene-specific activators to chromatin and enhanced degradation has been established by other studies (Molinari *et al.*, 1999, Rosonina *et al.*, 2012), and the attenuation of stress response has been connected to nuclear protein modification and degradation of such activators as Msn2 (Bose *et al.*, 2005, Chi *et al.*, 2001). Furthermore, our results also reveal a delay in HOG signalling downregulation in *cbc1Δ*, as Hot1 and Hog1 remain phosphorylated for longer following osmotic stress exposure, which correlate with decreased promoter binding of these factors (Figure 4.7), suggesting that dephosphorylation occurs on chromatin-bound proteins.

4.4.4 Biological relevance of Cbc1 function in gene expression

Based on our results, we present a model in which Cbc1 is required for adequate cellular transcription, in which its absence may cause growth defects under normal and/or stress conditions. Furthermore, we observe that transcription of ribosomal protein genes is reduced by around 40 % in *cbc1* mutant in respect to wild type (Figure 4.9) and lower RP expression has been shown to correlate with lower growth rate both under non-stress and stress conditions (Levy *et al.*, 2007, O'Duibhir *et al.*, 2014). Accordingly, we have observed a longer duplication time in *cbc1* mutant in respect to wild type (2.1 and 1.5 hours, respectively) in rich media under normal conditions. Moreover, we have shown that *cbc1Δ* mutants show growth defects and lower viability under osmotic stress (Garre *et al.*, 2012), however, the function of Cbc1 upon osmostress goes beyond its role in transcription, since we have previously documented that Cbc1 associates with polyribosomes in active translation and, moreover, the absence of Cbc1 reduces the percentage of osmostress mRNAs engage in active translation (Garre *et al.*, 2012). Additionally, in *Arabidopsis*, the cap-binding proteins Cbp80 and Cbp20 modulate the response to osmotic stress through the regulation of pre-mRNA splicing, and hence gene expression, of several genes involved in sugar and proline metabolism (Kong *et al.*, 2014). Yeast Cbc1 has also important roles in splicing and specifically in the processing of the introns of RP genes (Bragulat *et al.*, 2010), whose pre-mRNAs are also highly regulated in response to osmotic stress (Garre *et al.*, 2013). Together, these results suggest that Cbc1 has multifunctional roles during osmotic stress, and acts as a key factor coordinating different levels of gene expression. The response to other environmental stresses also requires high and timely transcription of protective genes coupled with downregulation and later recovery of house-keeping genes, such as RP genes (Canadell *et al.*, 2015). Our observation that cellular growth under various stresses requires Cbc1 and Cbc2 (Figure 4.12), suggest that the multifunctional roles in gene expression of the mRNA cap-binding proteins are necessary to attain an adequate response to stress.

In conclusion, we have made several interesting observations in regards to the function of Cbc1 in transcription. First, Cbc1 is required for the high and timely induction of osmostress-responsive genes by directly recruiting the Hot1/Hog1 activator complex to promoters, which then subsequently enhances TBP and RNAPII binding. Second, Cbc1 is specifically required for RP gene transcription under non-stress condtions as well as during transcriptional recovery following osmotic stress adaptation by directing Rap1 and TBP association to RP promoters. Third, Cbc1 shows functional bias towards highly expressed or induced genes compared to lowly expressing genes. Finally, Cbc1, together with Cbc2, is required for the response to other environmental stresses.

Chapter 5

Fertility and polarised cell growth dependents on eIF5A for translation of polyproline-rich formins

5.1 Background

The evolutionarily conserved essential eIF5A is the only protein known to undergo hypusination, a unique post-translational modification, involving two enzymic steps catalysed in S. cerevisiae by the deoxyhypusine synthase Dys1 and the deoxyhypusine hydroxylase Lia1 (Park *et al.*, 1993, Abbruzzese *et al.*, 1989). Both eukaryotic eIF5A and its bacterial ortholog, EF-P, were first identified as translation factors for their ability to carry out translation in *in vitro* systems (Kemper *et al.*, 1976, Glick and Ganoza, 1975). Additionally, endogenous hypusinated eIF5A was observed to bind with actively translating 80S ribosomes in an RNA-dependent manner (Jao and Chen, 2006). However, depletion of eIF5A in yeast only causes a 30 % decrease in protein synthesis rate, which does not explain its essential nature in yeast and mammalian cells (Kang and Hershey, 1994, Hanauske-Abel *et al.*, 1994b). eIF5A was later defined as a translation elongation factor as depletion of eIF5A causes polysome accumulation as well as increasing transit time of ribosomes along mRNAs (Saini *et al.*, 2009, Dias *et al.*, 2012). Most recently, the activity of the bacterial ortholog EF-P is found to be required to alleviate ribosome stalling and stimulate peptide bond formation between proline residues, being essential for the translation of proteins with three or more consecutive proline residues (Ude *et al.*, 2013, Doerfel *et al.*, 2013). In *S. cerevisiae*, eIF5A has also been demonstrated to be required for the translation of polyPro-containing reporters with three or more consecutive proline residues both *in vivo* and *in vitro* (Gutierrez *et al.*, 2013). Although the molecular role of eIF5A is beginning to be elucidated, its essentiality in cellular processes and its biological significance are yet to be uncovered (see section 1.6).

Several lines of evidence have led us to hypothesise that eIF5A may have a role in yeast fertility through the specific translation of proteins with polyPro sequences involved in mating. First, the hypusine-derivative spermidine, a member of the polyamine family, is indispensable for fertilisation efficiency in all eukaryotes (Lefevre *et al.*, 2011). In *S. cerevisiae*, spermidine is required for the formation of the shmoo during sexual reproduction, and has also been shown to be essential for effective fertilisation in *C. elegans* (Bauer *et al.*, 2013). Second, eIF5A has also been implicated to play a role in polarized cellular growth. eIF5A knockout in mice causes early embryonic lethality, and in *C. elegans*, mutation of eIF5A produces slow larval growth, structural abnormalities and sterile adults (Nishimura *et al.*, 2012, Hanazawa *et al.*, 2004). Furthermore, in yeast, depletion of eIF5A has shown to change actin dynamics (Chatterjee *et al.*, 2006). Third, recent studies have described both the bacterial homolog EF-P and the yeast eIF5A to be essential for the specific translation of genes containing three or more consecutive proline residues by alleviating ribosome stalling (Ude *et al.*,

2013, Doerfel *et al.*, 2013, Gutierrez *et al.*, 2013). Finally, we have conducted a search for the number and functional roles of polyPro proteins in *S. cerevisiae*, and found that many polyPro proteins fall under the functional categories of shmoo formation and site of polarised growth (Figure 3.1). Based on our search results and previous studies, we therefore proposed that hypusinated eIF5A may be necessary for the translation of proline-rich proteins implicated in yeast fertility. To explore this hypothesis, we utilised temperature-sensitive mutants to deplete eIF5A protein expression, and assessed the cellular ability to form shmoo, localise components of the polarisome and induce pheromone-dependent genes. Furthermore, we evaluated the translational dependency of polyPro proteins on the protein expression of eIF5A.

Overall, our studies confirm that eIF5A functions in the translation of polyPro proteins, and for the first time, we provide a functional role for eIF5A in yeast mating.

5.2 Results

5.2.1 Pheromone-induced shmoo formation is inhibited in eIF5A mutants

To investigate the possible functional role of yeast hypusinated eIF5A, we first searched the number of polyPro proteins in *S. cerevisiae* and their functional roles. As shown in Figure 5.1A, 549 yeast proteins were found to contain at least one motif of three consecutive proline residues, followed by a decreasing number of proteins with increasing number of consecutive prolines. A further search using Gene Ontology (GO) tools revealed four significant over-represented categories, including “site of polarized growth” and “mating projection (shmoo)” (Figure 5.1B). Furthermore, only two yeast proteins, Bnr1 and Bni1, contain 10 or more consecutive prolines. These two proteins are evolutionary conserved formins that function in the regulation of actin cytoskeleton during budding (Pruyne *et al.*, 2004, Imamura *et al.*, 1997), with Bni1 also involved in shmoo formation during mating (detailed in section 1.4) (Evangelista *et al.*, 1997). From these data, we investigated whether eIF5A is involved in shmoo formation through the regulation of Bni1.

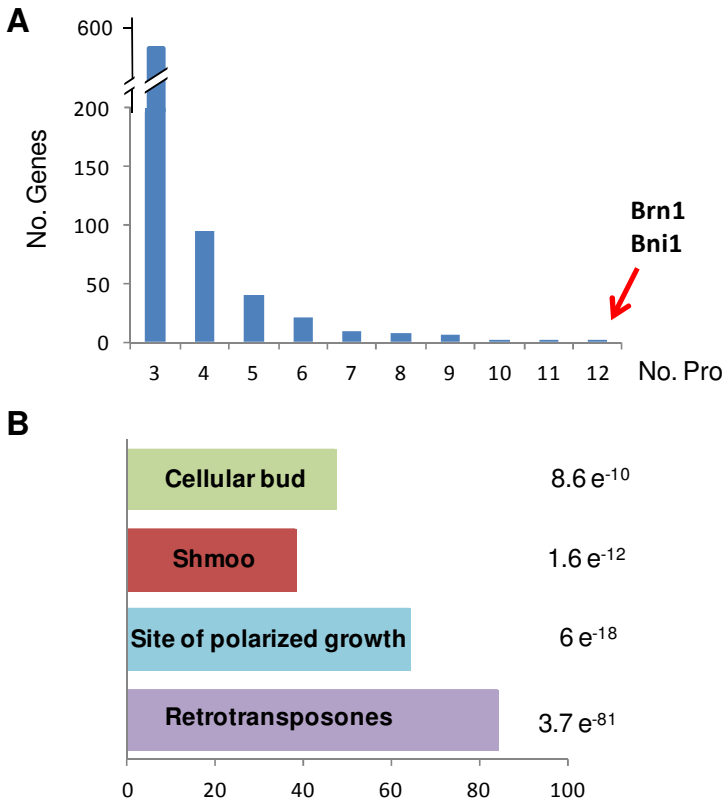


Figure 5.1 Number and functional categories of *S. cerevisiae* proteins containing three or more consecutive prolines. (A) Number of yeast proteins containing polyproline stretches with the indicated consecutive prolines. Formins Bni1 and Bnr1 are the only yeast proteins with polyproline stretches with 10 or more prolines. **(B)** GO functional categories significantly over-represented in 549 yeast genes containing a single polyPro stretch with at least three consecutive prolines. Bars represent the number of genes found in each category and the p-value is indicated for each category.

Firstly, we evaluated the ability to form shmoo projections under pheromone-treatment of mutants in genes *SPE2* (biosynthesis of spermidine), *LIA1* (deoxyhypusine hydroxylase of eIF5A), mutants in the formins *BNR1* and *BNI1*, and mutant in the non-essential eIF5A gene, *TIF51B*. *lia1Δ* was unable to form shmoos in the presence of α-factor, whereas, as expected in a rich media where spermidine is available, *spe2Δ* showed a percentage of shmoo formation similar to the wild type (Figure 5.2A). Since the only known target of Lia1 is eIF5A, these results suggest that hypusination of eIF5A is necessary for shmoo formation and that perhaps the essential requirement of spermidine relies on eIF5A activation by hypusination. Furthermore, *tif51BΔ* was able to shmoo, and, as it has been described, the formin Bni1, but not Bnr1, is essential for shmooing (Figure 5.2A).

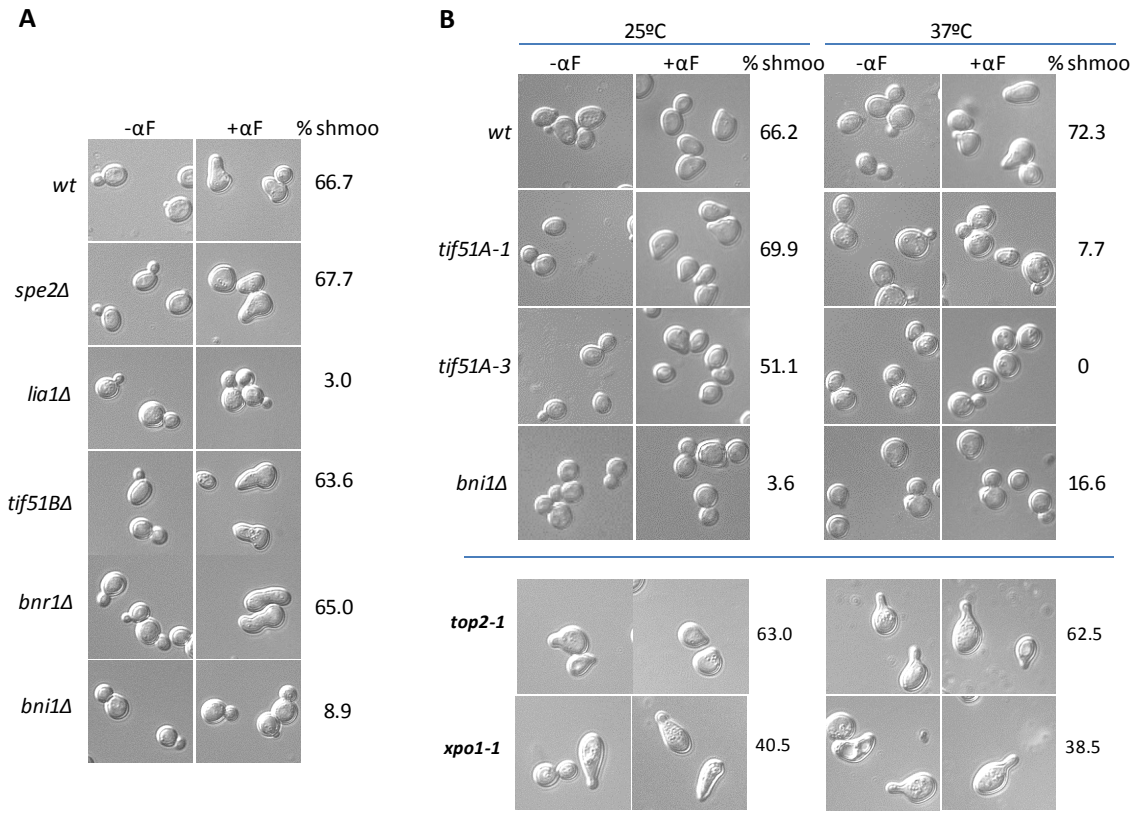


Figure 5.2 Yeast shmoo formation requires hypusinated eIF5A. Representative DIC images of wild type and mutants with or without treatment of 10 µg/ml α-factor for 2 h. **(A)** Null mutants were maintained in 30 ºC, and **(B)** temperature-sensitive mutants were maintained at 25 ºC or transferred to 37 ºC for 4 h. Mutants containing temperature sensitive alleles in the essential proteins topoisomerase II (*top2-1*) and karyopherine Crm1/Xpo1 (*xpo1-1*) were used as controls. Quantification of percentage of cells containing shmoos in samples treated with α-factor is indicated. Approximately 300 cells were manually counted for each sample from at least two independent experiments.

Secondly, to evaluate the shmoo forming ability of yeast cells lacking in eIF5A, we used temperature-sensitive strains that harboured mutations in the *TIF51A* gene as previously described (Valentini *et al.*, 2002, Li *et al.*, 2011). Mutants *tif51A-1* and *tif51A-3* contain a single (Pro83 to Ser) and double (Cys39 to Tyr, Gly118 to Asp) mutation respectively in eIF5A protein. Both mutants showed a decrease in viability after 8 hours incubation at 37 ºC, with a more severe growth defect in *tif51A-3* (see Figure 5.3A, B and D). Accordingly, eIF5A protein was mostly depleted after 4 h of incubation at 37 ºC in both mutants (Figure 5.3D and E). Treatment with 10 µg/ml α-factor induced shmoo formation in eIF5A mutants and wild type at permissive temperature (25 ºC) (Figure 5.2B). However, at restrictive temperature (37 ºC), *tif51A-1* yield very low shmooing (7.7 %) and *tif51A-3* was unable to shmoo, with most cells in both strains appearing ellipsoidal. As previously described, *bni1Δ*

showed a low percentage of shmoo formation at both temperatures (Figure 5.2B). As positive control in shmoo formation at 37 ºC, we also used two other temperature-sensitive mutants in essential genes (*top2-1* mutation in topoisomerase II and *xpo1-1* mutation in exportine Crm1/Xpo1) and observed that, in both, the percentages of shmoo formation were similar at permissive and restrictive temperatures (Figure 5.2B). Taken together, these results indicate that hypusinated eIF5A is required for successful shmoo formation.

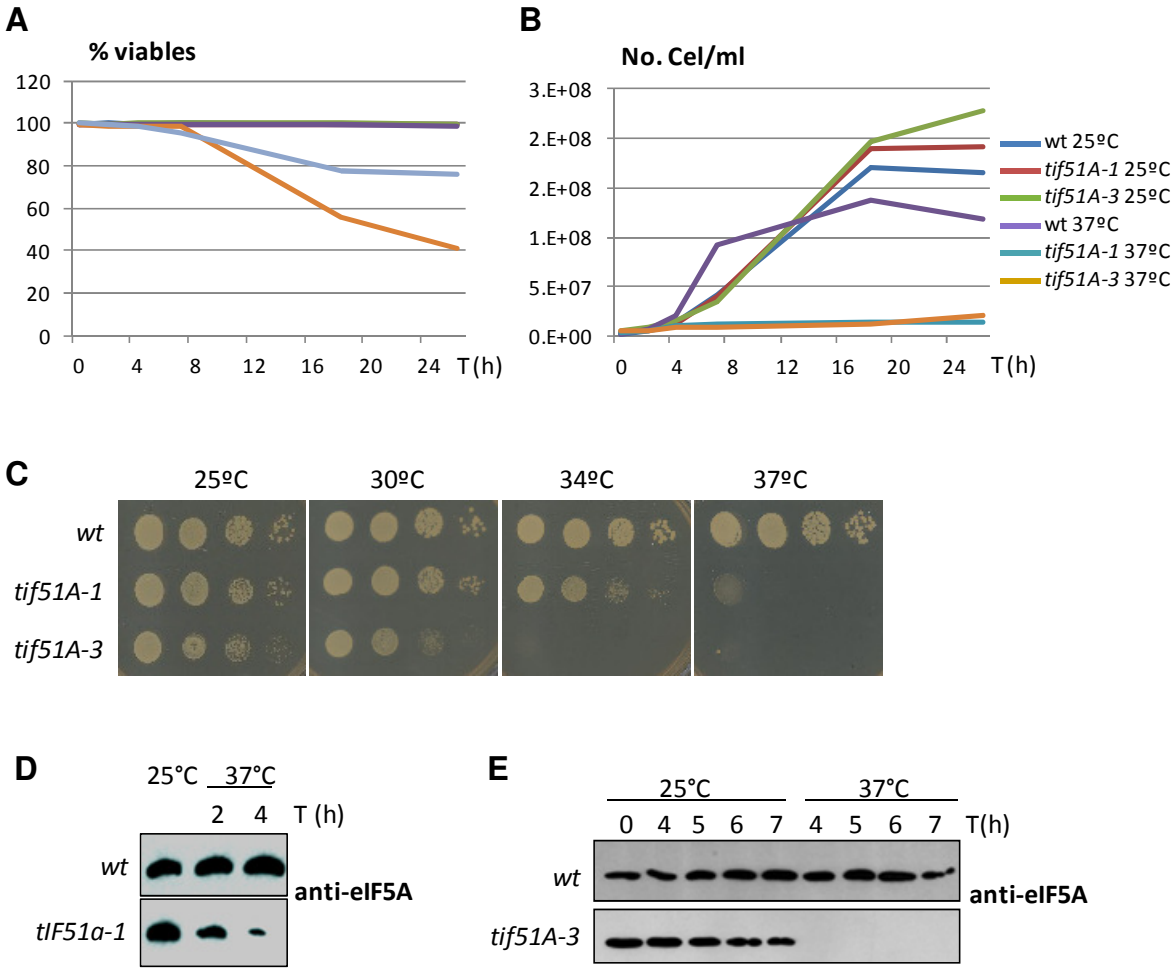


Figure 5.3 Depletion of the eIF5A protein results in lethality. (A, B) Percentage of viable cells and number of cells per ml in wild type, *tif51A-1* and *tif51A-3* grown in YPD at 25 ºC or after incubation at non-permissive 37 ºC at the times indicated. **(C)** Exponentially growing cultures at 25 ºC of wild type, *tif51A-1* and *tif51A-3* were plated onto YPD medium (10-fold serial dilutions) and incubated at 25 ºC, 30 ºC, 34 ºC and 37 ºC. **(D and E)** Immunoblots showing eIF5A protein depletion in *tif51A-1* and *tif51A-3* after incubation at 37 ºC for the indicated times. eIF5A protein expression was also visualized in wild type under the same conditions.

5.2.2 eIF5A is required to shmoo-localise Spa2, induce *FUS1* gene and assemble actin cables during pheromone stimulation

To further investigate the basis for the defect in shmoo formation in *tif51A* mutants, we tracked, by flouresence microscopy, the localisation of Spa2, a known component of the polarisome that aggregates at the shmoo tip during mating through its interaction with Bni1 (Liu *et al.*, 2012, Fujiwara *et al.*, 1998). Upon pheromone treatment at 25 ºC, wild type and *tif51A* mutants showed Spa2-GFP accumulation at the shmoo tip, meanwhile Spa2 was delocalised in *bni1Δ* (Figure 5.4). Inactivation of eIF5A by incubation at 37 ºC inhibited polarised localisation of Spa2 in *tif51A-1* and *tif51A-3* mutants (Figure 5.4).

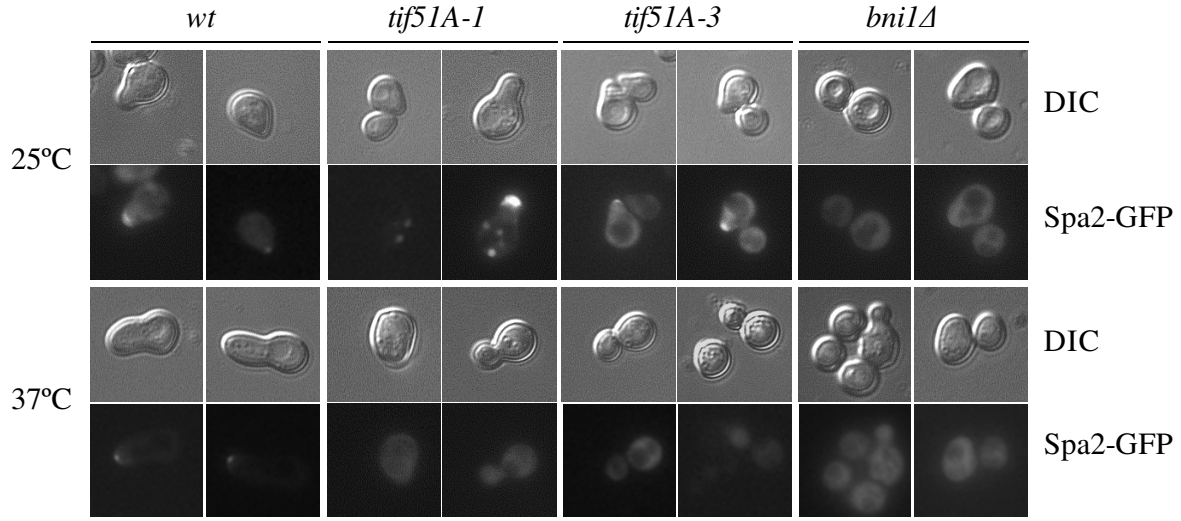


Figure 5.4 Localisation of the polarisome protein Spa2 at the shmoo tip in response to pheromone requires eIF5A. Representative GFP and their corresponding DIC images of wild type, *bni1Δ*, *tif51A-1* and *tif51A-3* mutants overexpressing Spa2-GFP. Cells were maintained at 25 ºC or transferred to 37 ºC for 4 h. Cells were examined after treatment with 10 µg/ml α-factor for 2 h.

Furthermore, in order to determine the full activation of the MAP kinase Fus3 with α-factor, we examined the expression of the *FUS1* gene, which encodes a pheromone-induced transmembrane protein involved in cell fusion (Trueheart and Fink, 1989). A *FUS1-lacZ* fusion protein was induced under pheromone treatment in wild type and to a lower level in *tif51A* mutants at permissive temperature. However, no *FUS1-lacZ* induction was observed in eIF5A mutants at restrictive temperature (Figure 5.5A). In *bni1Δ*, *FUS1* gene was not induced at 37 ºC, and surprisingly, *FUS1*

induction was observed at 25 ºC (Figure 5.5A). In contrast to the observed defects in *FUS1* induction, *tif51A* mutants were able to induce an osmotic stress gene (*STL1*) fused to *Lac*Z during osmotic stress treatment, indicating that, at experimental conditions, *tif51A* mutants maintain the ability to induce signalling pathways in response to external stimuli (Figure 5.5B).

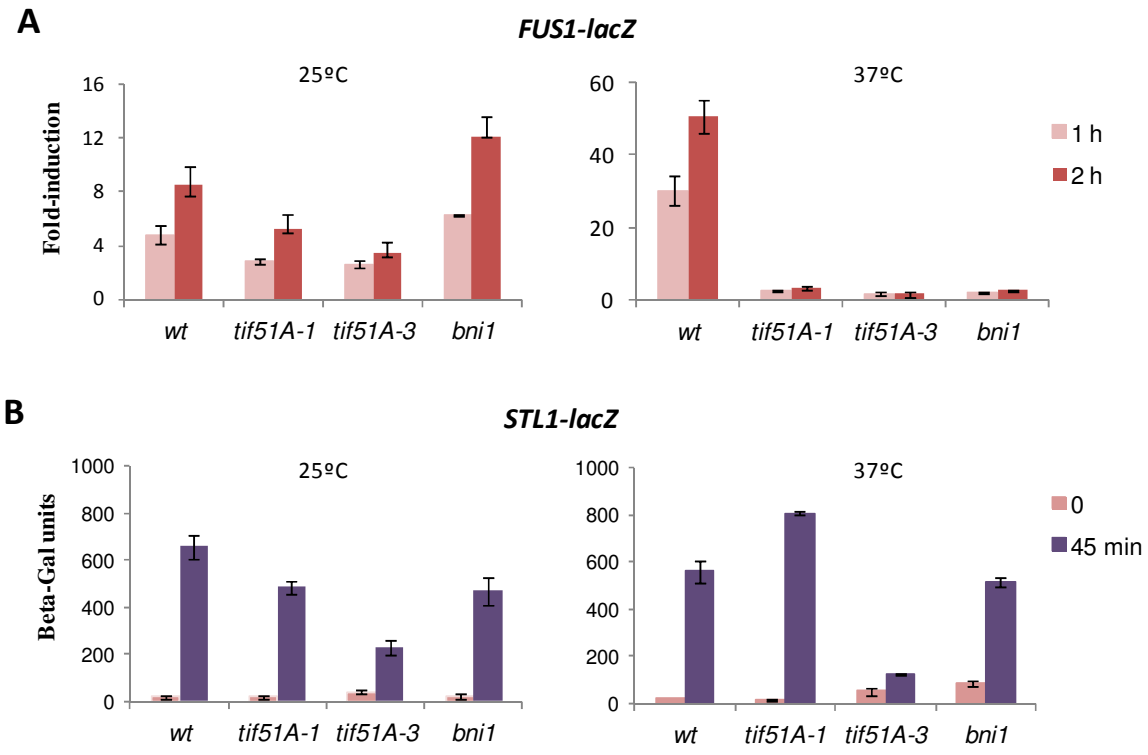


Figure 5.5 Expression of *FUS1-lacZ* in response to pheromone requires eIF5A. Expression of *FUS1-LacZ* **(A)** or *STL1-LacZ* fusion **(B)** in wild type and mutants. Cells were grown at 25 ºC or 4 h at 37 ºC and then treated with 10 µg/ml α-factor for either 1 h or 2 h **(A)** or stressed with 0.6 M KCl for 45 min **(B)**. Error bars represent the standard error of the mean of three or more biological replicates.

It has been previously described that depletion of eIF5A causes changes in the actin cytoskeleton by unknown mechanisms (Chatterjee *et al.*, 2006, Zanelli and Valentini, 2007). We therefore investigated the role of eIF5A in pheromone-induced polarisation of actin cables by Phalloidine-staining of actin before and after treatment with pheromone, which was then visualized using fluorescent microscopy. At 25 ºC in the absence of α-factor, eIF5A mutants showed a normal pattern of actin with patches and cables (Figure 5.6). Addition of α-factor at permissive temperature induced

the formation of actin cables from the shmoo tip in *tif51A-1* and wild type cells, and actin was visualised mainly as patches in *tif51A-3*. However, at 37 ºC with α-factor, both eIF5A mutants were unable to form actin cables, but contained delocalised actin patches. Consistent with previous reports (Matheos *et al.*, 2004), actin was observed as intense delocalized patches in all conditions in *bni1* mutant. Therefore, eIF5A is necessary for the formation of actin cables upon pheromone stimulation.

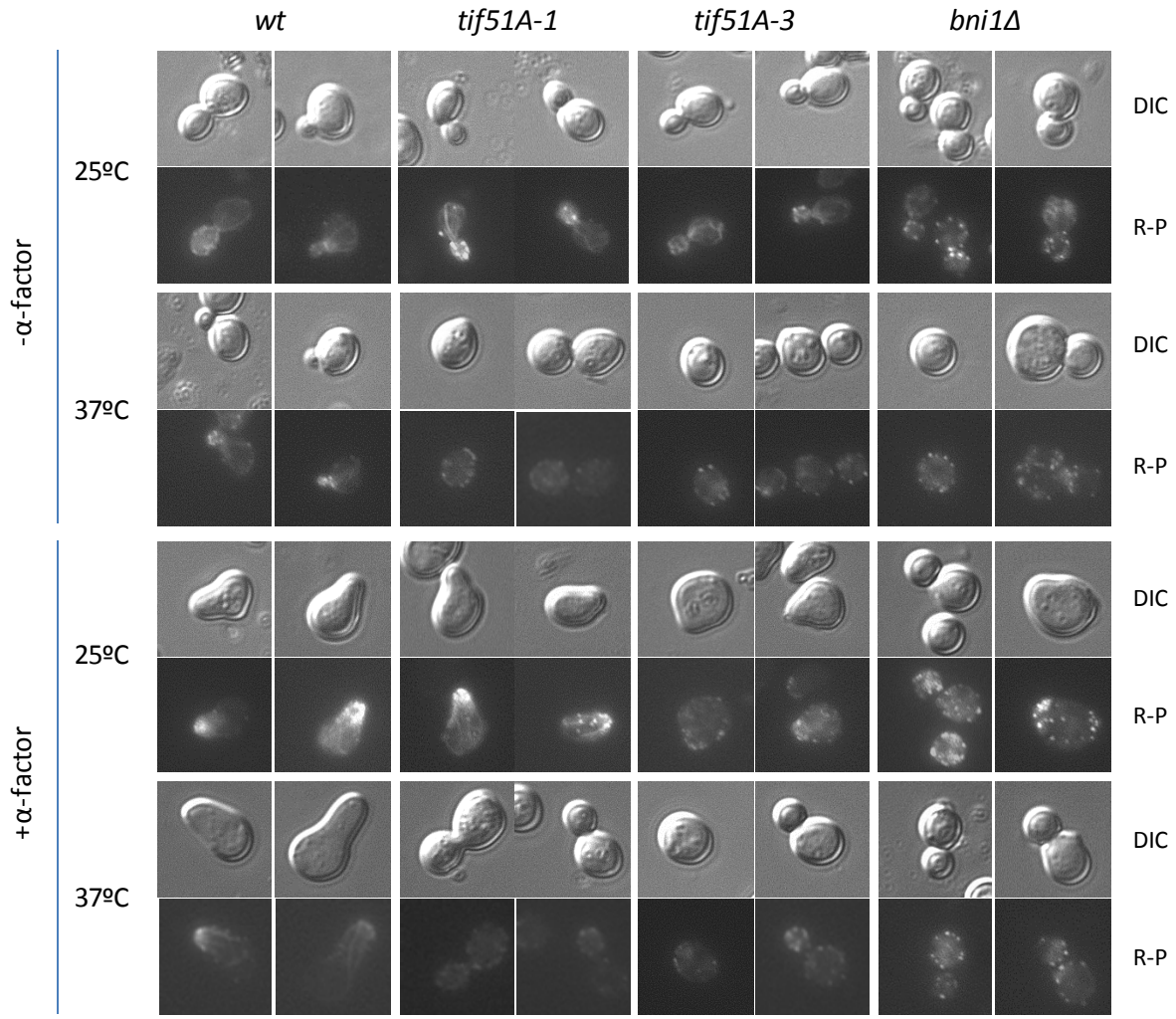


Figure 5.6 Polymerisation and localisation of actin cables require eIF5A during shmoo formation. Representative fluorescence and their corresponding DIC images of wild type, *tif51A-1*, *tif51A-3* and *bni1Δ* showing F-Actin staining by phalloidin (Alexa Fluor 488®) at 25 ºC or after incubation for 4 h at 37 ºC, in the presence or absence of treatment with 10 μg/ml α-factor for 2 h.

Taken together, these results indicate that eIF5A controls different outcomes of pheromone signalling, including induction of pheromone-responsive genes and localisation of components of the polarisome at the shmoo. Given the essential role of actin in the induction of cell polarisation during mating (Slaughter *et al.*, 2009), these downstream effects could be a direct result of the essentiality of eIF5A in actin polymerization, which may occur through the regulation of Bni1.

5.2.3 eIF5A is required for the translation of the polyproline formin Bni1

The formin Bni1 is a large protein containing three segments of 10, 12 and 5 consecutive proline residues in the formin homology domain 1 (FH1), the domain required for assembly of actin filaments (Figure 5.7) (Sagot *et al.*, 2002). Since it has been shown that the bacterial ortholog of eIF5A, EF-P, enhances the synthesis of proteins containing polyPro stretches (Ude *et al.*, 2013, Doerfel *et al.*, 2013), we hypothesised that yeast eIF5A may be involved in the translation of the polyPro motifs of Bni1, and that low levels of Bni1 in eIF5A mutants is the basis for their inability to shmoo. To test this, we obtained two genomic HA tagged versions of *BNI1* in wild type and *tif51A-1*; a full-length *BNI1* with HA after the coding region (*BNI1-HA*) and a truncated *BNI1* with HA fused before the first polyPro stretch (*BNI1ΔCt-HA*) (Figure 5.7A). The shift of wild type cells from 25 ºC to 37 ºC slightly reduced the protein levels of Bni1-HA and Bni1ΔCt-HA. However, full-length Bni1-HA level was importantly reduced in *tif51A-1* cells after 2 hours of incubation at 37 ºC, and was almost undetectable after 6 hours. Conversely, we only observed a partial reduction in truncated Bni1ΔCt-HA protein levels in *tif51A-1* after the shift to 37 ºC, and this level was sustained up to 6 h (Figure 5.7B). We then analysed *BNI1-HA* and *BNI1ΔCt-HA* mRNA expression in the same cell cultures by RT-qPCR and calculated the protein/mRNA ratios as an indication of translation efficiency, which was normalised to the translation efficiency of a control gene encoding hexokinase 2 (*HXK2)*, a protein with no polyPro motifs. After temperature shift, *tif51A-1* showed an important decrease in the translation efficiency of full length *BNI1*, reaching almost zero after 6 h. In comparison, the translation efficiency of C-terminus truncated *BNI1* lacking the polyPro motifs was sustained from 2 to 6 hours upon eIF5A depletion (Figure 5.7C). These results suggested that eIF5A controls the protein expression of the Bni1 formin and is required for the translation of the polyPro motifs. To further demonstrate that the polyPro motifs are specifically responsible for the translation dependency of *BNI1* on eIF5A, we obtained an additional *bni1* mutant, in which only a short genomic region of the FH1 domain containing the polyPro stretches was deleted, maintaining intact the rest of the *BNI1* C-terminus (*BNI1ΔPro-HA*) (Figure 5.7A). In *tif51A-1*, the translation efficiency of Bni1ΔPro also reduced at 37 ºC but was sustained at 4 and 6 hours of incubation at restrictive

temperature, with similar efficiencies observed in the wild type strain (Figure 5.7B and C). Hence, while the translation of Bni1 containing polyPro motifs is dependent on eIF5A, a Bni1 mutant without these motifs is not dependent on eIF5A. This strongly suggests that eIF5A is involved in the specific translation of the proline-rich regions of Bni1.

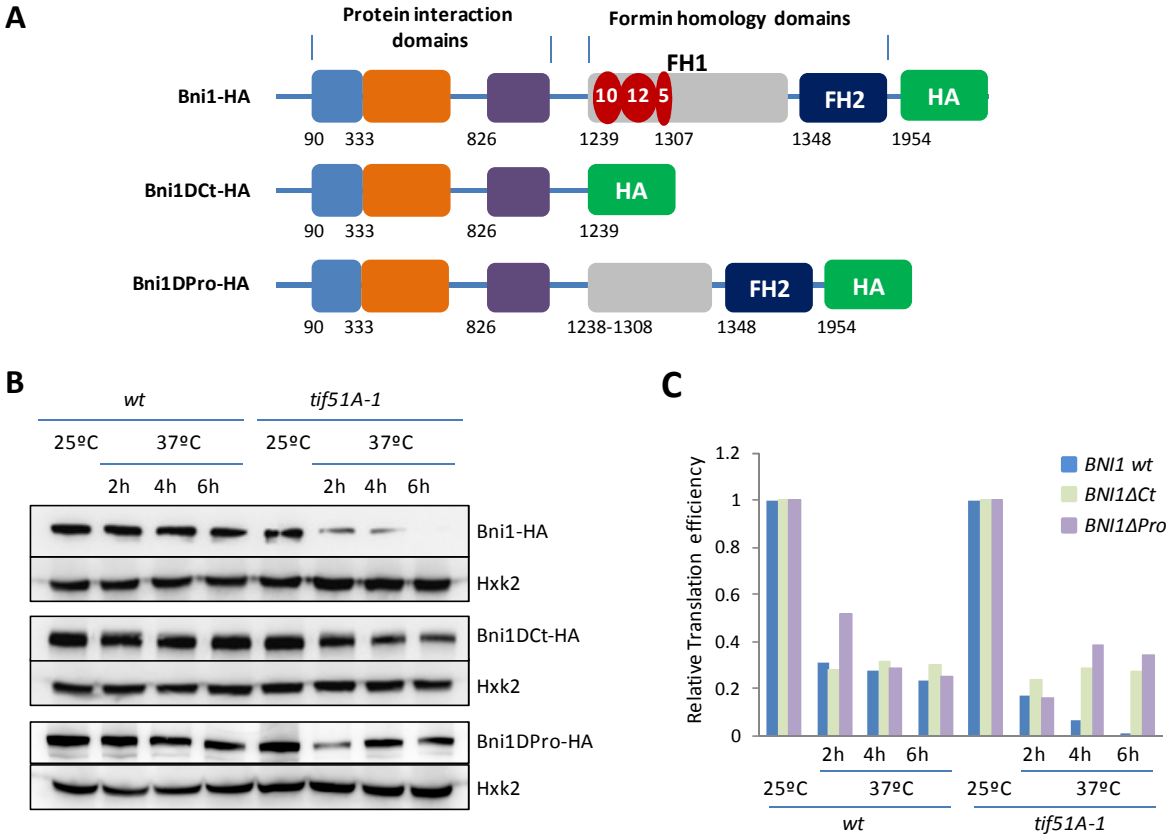


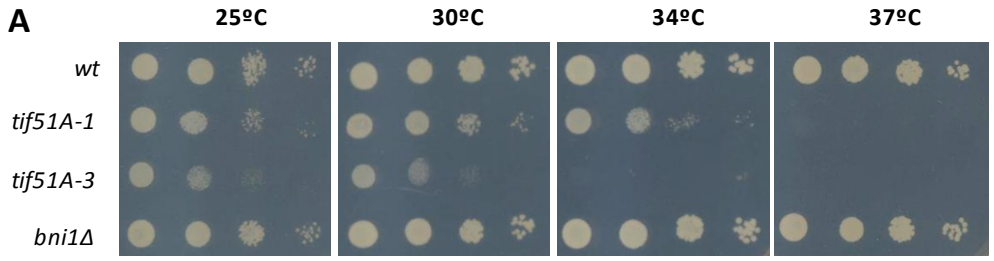
Figure 5.7 Translation of the proline stretches of the formin *BNI1* requires eIF5A. (A) Schematic diagrams showing C-terminal HA genomic tagging of full-length (*BNI1-HA*), C-terminal truncated (Δ1240-1954, *BNI1ΔCt-HA*) and proline-deleted (Δ1239-1307, *BNI1ΔPro-HA*) Bni1. Formin homology (FH) and protein interaction domains are indicated, as well as the polyPro stretches with the number of consecutive prolines represented in red. The numbers below the boxes indicate the first amino acid of each domain or stretch. **(B)** Immunoblots with antibodies against HA and hexokinase 2 protein (Hxk2) to show expression of full-length Bni1, C-terminal truncated Bni1, proline-deleted Bni1 and Hxk2 in wild type and *tif51A-1* at 25 ºC or 37 ºC at the indicated times. **(C)** Translation efficiency of *BNI1-HA, BNI1ΔCt-HA* and *BNI1ΔPro-HA* relative to translation efficiency of *HXK2* in wild type and *tif51A-1*. Protein/mRNA ratios for Bni1, Bni1ΔCt-HA, Bni1ΔPro-HA and Hxk2 were calculated by Western **(B)** and quantitative PCR from same samples. Translation efficiencies of *BNI1-HA, BNI1ΔCt-HA* and *BNI1ΔPro-HA* are calculated relative to translation efficiency of *HXK2* and represented as a fraction against 25 ºC for each strain.

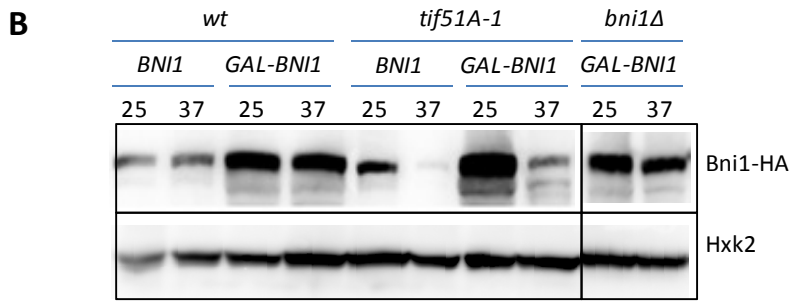
It is noteworthy to mention that the above results could have an alternative interpretation, where the differences in protein amount seen between Bni1 wild type and Bni1 mutants are due to different protein degradation rates , and it could be that eIF5A is involved in the stabilisation of only the wild type Bni1 protein. Although we cannot exclude this possibility, the fact that the specific elimination of the Bni1 polyPro stretches renders the protein eIF5A-independent deems the interpretation that the eIF5A elongation factor is involved in Bni1 translation rather than in the regulation of protein stability to be more reasonable. Bni1 protein stability has been studied in a previous work in which it was demonstrated that local cell wall/membrane damage results in the PKC-regulated proteosomal degradation of Bni1 (Kono *et al.*, 2012). Moreover, it was described that the Bni1 N-terminal region (amino acids 1-642), which does not contain the polyPro motifs, is responsible for this regulated degradation that can be provoked by heat-shock at 39 ºC (Kono *et al.*, 2012). Perhaps, our observation that Bni1 protein levels slightly decrease in all strains during the shift from 25 ºC to 37 ºC (Figure 5.7B) could be explained by the regulation of Bni1 degradation during a mild heat-shock.

5.2.4 Over-expression of Bni1 protein partially restores the shmoo formation defect of eIF5A mutants

Finally, we addressed the possibility of suppressing the eIF5A mutant shmoo defect by increasing cellular levels of Bni1. To do so, we used a multicopy plasmid containing a *BNI1-HA* fusion under the control of the strong *GAL* promoter (Kono *et al.*, 2012). Given the unessential nature of Bni1 to cell viability, the lack of this protein cannot explain the essentiality of eIF5A, and therefore, as expected, the growth defect at restrictive temperature of *tif51A-1* and *tif51A-3* mutant cells was not recovered when *GAL-BNI1* was expressed in media containing galactose (Figure 5.8A). In over-expression conditions, we observed a high increase in Bni1 protein levels in wild type and in *tif51A-1* at permissive temperature with respect to the genomic endogenous Bni1 levels, however, Bni1 protein level increased only slightly in *tif51A-1* at restrictive temperature (Figure 5.8B). This result further confirms that eIF5A is necessary for the production of Bni1 protein, even when over-expressing the *BNI1* gene. Nevertheless, a slight increase in Bni1 protein was obtained in eIF5A mutants in restrictive temperature, therefore we studied if this increase could improve the shmoo formation of eIF5A mutants. As expected, a complete complementation of the shmoo formation defect in *bni1* mutant was observed by inducing *GAL-BNI1*. We also observed, at restrictive temperature, higher percentages of shmoo formation in *tif51A-1* (19.5 %) and *tif51A-3* (6.9 %; Figure 5.8C) in respect to what was observed in these strains without *BNI1* over-expression (7.7 % and 0 %, respectively;

Figure 5.2B). This partial suppression of the shmoo defect seen in eIF5A mutants by a slight increase in Bni1 protein levels further supports that the shmooing defect in these mutants is due to the low levels of Bni1 formin. Additionally, since increasing Bni1 protein levels does not produce a total restoration of shmoo formation ability in eIF5A mutants, it suggests that more Bni1 protein would be necessary for a full complementation or that other proteins involved in shmoo formation might be under the translational control of eIF5A.





C

	% shmoo			
	wt	*tif51A-1*	*tif51A-3*	*bni1Δ*
25°C	64.7	60.6	43.1	56.1
37°C	60.3	19.5	6.0	52.8

Figure 5.8 Over-expression of the formin *BNI1* partially restores the shmoo formation defect of eIF5A mutants. (A) Over-expression of *BNI1* does not restore the growth defect of eIF5A thermo-sensitive mutants at restrictive temperature. Growths of the indicated yeast strains containing a plasmid expressing *BNI1* under the *GAL* promoter were tested in Sgal-URA plates incubated at the indicated temperatures. **(B)** Immunoblots with antibodies against HA and hexokinase 2 protein (Hxk2) to show the expression of genomic Bni1 (*BNI1*) or the over-expression of Bni1 under the *GAL* promoter (*GAL-BNI1*) at 25 ºC or 37 ºC for 4 h. **(C)** Shmoo formation is partially restored in eIF5A mutants. The indicated strains containing a *GAL-BNI1* plasmid were grown at 25 ºC in Sgal-URA media and then were maintained at 25 ºC or transferred to 37 ºC for 4 h. Quantification of percentage of cells forming shmoos in samples treated of 10 µg/ml α-factor for 3 h are indicated. Approximately 300 cells were manually counted for each sample from at least two independent experiments.

5.3 Discussion

5.3.1 Role of eIF5A in polarised cell growth and fertility

The budding yeast is often used as a model organism to study molecular interactions that generate cell polarity in response to external stimuli. During the yeast mating response, pheromones produced by partner cells are detected by specific cell-surface receptors, and as a result, a mating project, the shmoo, is formed towards the pheromone gradient. Remarkably, many of the genes that control cell polarity are conserved between yeast and more complex eukaryotes, making them great candidates for genetic study.

Formins are a group of highly conserved eukaryotic proteins that are involved in several actin-based processes, including cell polarisation, cytokinesis, and embryonic development (Ridley, 1999, Zeller *et al.*, 1999). In *S. cerevisiae*, formin Bni1 is required for the formation of actin cables to form a shmoo in response to pheromone. Characteristically, Bni1 contains long stretches of consecutive prolines located in the formin homology domain 1 (FH1), which has been conserved amongst eukaryotic formins (Pruyne *et al.*, 2002, Sagot *et al.*, 2002). In this thesis, we describe the role of translation elongation factor eIF5A and its activating enzyme Lia1 in shmoo formation during mating through the translation of the proline stretches in Bni1. First, we show that a mutant lacking in eIF5A is defective in all downstream events of polarised cell growth, including polarised localisation of polarisome component Spa2 at the shmoo tip, induction of the fusion gene *FUS1*, and the polarisation of actin filaments (Figures 5.2-5.6). Since Bni1 is known to be activated by pheromone-responsive MAP kinase Fus3, and, concurrently, Bni1 is required for fully activation of Fus3 by mediating its polarised localisation at the shmoo (Matheos *et al.*, 2004, Qi and Elion, 2005), we went on to show that the defect in shmoo formation in eIF5A mutant is a direct consequence of dramatically low levels of Bni1 protein upon eIF5A depletion (Figures 5.7 and 5.8). Utilising three genomically-tagged BNI1 versions: a full-length wild type Bni1 whose translation is sensitive to eIF5A, and two Bni1 mutants, containing either the deletion of the C-terminus (Bni1ΔCt), and by effect deleting the polyPro stretches or the specific deletion of the three polyPro motifs (Bni1ΔPro), whose translations are eIF5A-independent (Figure 5.7), our results strongly suggest that eIF5A is required for the specific translation of proline-rich sequences. Our data concords with previous findings (Doerfel *et al.*, 2013, Gutierrez *et al.*, 2013) to establish a clear role for eIF5A in the translation of poly-proline motifs; however, for the first time, we discovered a connection between its molecular function as a translation factor with a physiological role in yeast mating, as well as identifying a specific target Bni1 formin.

It is worth mentioning that the defects in shmooing (Figure 5.2) and actin cable formation (Figure 5.6) are more severe in the eIF5A mutants than in *bni1Δ*. Additionally, the restoration of shmoo formation is only partial when over-expressing Bni1 in eIF5A mutants (Figure 5.8). These results suggest that Bni1 may not be the only polyPro potein involved in shmoo formation and actin organisation that is under the translational control of eIF5A. In fact, the GO functional category of ¨mating projection¨ was significantly represented in the group of proteins with three or more consecutive prolines (Figure 5.1). More specifically, 38 proteins out of 119 in this functional category are polyPro proteins, and five of these proteins contain at least six consecutive prolines. Moreover, five yeast proteins that function in actin cable formation and cytoskeleton organisation contain polyPro motifs with at least seven consecutive prolines, including the only other yeast formin Bnr1. At first described as a functional homolog of Bni1, it was found that these two formins have different cellular localisations and contribute to different sets of actin cables (Buttery *et al.*, 2007, Pruyne *et al.*, 2004). In fact, we observed that a mutant lacking only Bnr1 is not defective in shmoo formation, unlike *bni1Δ* (Figure 5.2), indicating that while Bni1 plays a role in yeast mating, its counterpart Bnr1 may selectively play a role in yeast budding. Therefore, Bnr1 being a putative target of eIF5A, our results may provide evidence for another functional role for eIF5A in yeast budding. In all, the formin Bnr1 as well as the other polyPro proteins are putative targets of eIF5A that provide interest for further investigation.

5.3.2 Role of eIF5A in polyPro translation

Our study is not the first to describe a role for eIF5A in the translation of polyPro proteins. It has been originally described that the bacterial ortholog EF-P alleviates ribosome stalling in polyPro motifs, and the lysinylation of EF-P is required for its function (Doerfel *et al.*, 2013, Ude *et al.*, 2013). It has also been recently demonstrated in yeast that partial inactivation of eIF5A impairs expression of reporters and native proteins containing polyPro sequences *in vivo* (Gutierrez *et al.*, 2013). Furthermore, in the absence of eIF5A, translating ribosomes stall on with the second or third Pro codon in the P sites, suggesting that eIF5A promotes synthesis of the Pro-Pro peptide bonds to convert diPro to higher order polyPro sequences (Gutierrez *et al.*, 2013). The functional significance of polyPro motifs are starting to be visualised, as consecutive proline repeats form a unique secondary structure termed poly-L-proline type II (PPII) helix, which is a structural element of fibrillar proteins as well as holding functional significance in protein-protein and protein-nucleic acid interactions (Adzhubei *et al.*, 2013). Proline-rich regions are predominately localised in solvent-exposed or intrinsically disordered regions which are normally involved in intermolecular

interactions, including actin cytoskeleton organisation and signal transduction (Srinivasan and Dunker, 2012, Berisio and Vitagliano, 2012). A recent study by Mandal *et al.*, describing the role of eIF5A in eukaryotic evolution through functional classification of polyPro proteins, has observed that consecutive polyPro repeats show the highest conservation among all amino acid repeats (Mandal *et al.*, 2014). Furthermore, a marked increase in the frequencies of PPP and PPG motifs in higher organisms suggest a potential role for eIF5A in eukaryotic evolution (Mandal *et al.*, 2014). More importantly, although bacterial EF-P is shown to function in polyPro translation, it is not essential for cellular function and nor are its modification enzymes (Doerfel *et al.*, 2013, Ude *et al.*, 2013, Navarre *et al.*, 2010). In yeast, eIF5A is essential, however, the gene encoding the deoxyhypusine hydroxylase *LIA1* is not (Park *et al.*, 2006). In contrast, eIF5A and all its modification enzymes are required for the development of multi-cellular eukaryotes (Patel *et al.*, 2009, Sievert *et al.*, 2014). The increasing stringency in the requirement for the hypusinated form of eIF5A in the evolutionary tree may have evolved along with the increase in proPro proteins with critical functions in higher eukaryotes.

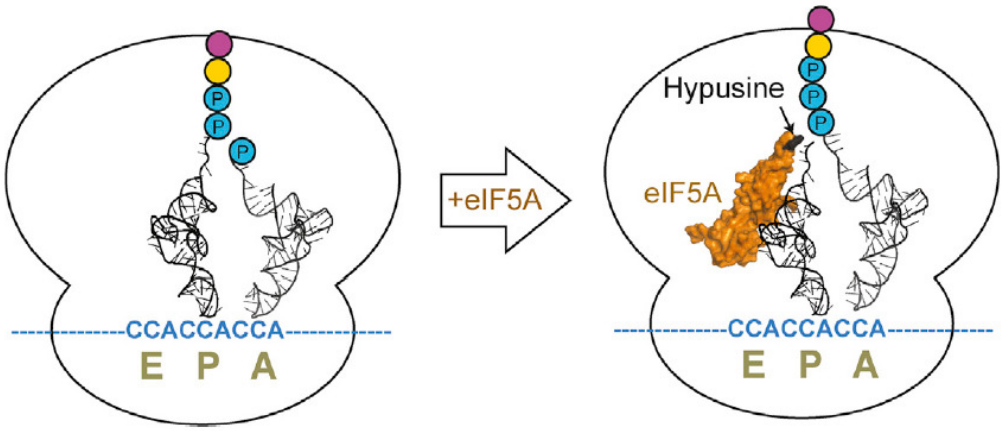


Figure 5.9 Schematic model of eIF5A stimulating polyPro synthesis (Gutierrez *et al.*, 2013). Ribosome stalls on the polyPro sequence with diPro attached to the P site and Pro-tRNA attached to the A site (left). Binding of eIF5A (indicated by an arrow), containing the post-translation modification hypusine (indicated), leaves the hypusine chain adjacent to the Pro-tRNA which helps to promote peptide bond formation with the diPro.

5.3.3 Implications for higher eukaryotes

In humans, two eIF5A isoforms exist that share 84 % identity with each other and 65 % identity with yeast eIF5A, which are encoded by two genes, *EIF5A1* and *EIF5A2* (Jenkins *et al.*, 2001). *EIF5A1* is constitutively expressed in all human cells, and is especially abundant in proliferating cells. In fact, eIF5A-1 mRNA expression is driven by the oncogene c-myc and is over-expressed in cancer tissues (Caraglia *et al.*, 2013). In contrast, *EIF5A2*, encoding the eIF5A-2 protein, is differentially expressed in parts of the brain and testis, however, was found to be up-regulated in selective ovarian and colorectal cancer cell lines (Clement *et al.*, 2006). Furthermore, the differential over-expression of eIF5A-2 has been described to be associated with cellular transformation and metastasis of several cancer types including ovarian, colon, liver and bladder cancer (Guan *et al.*, 2004, Xie *et al.*, 2008, Yang *et al.*, 2009a, Luo *et al.*, 2009), making this protein particularly attractive as a chemotherapeutic target. Both human eIF5A isoforms are able to become hypusinated when expressed in yeast and can functionally substitute yeast eIF5A in yeast strains where *TIF52A* and *TIF51B* are disrupted (Clement *et al.*, 2006, Schnier *et al.*, 1991), indicating high levels of conservation from yeast to humans.

Similarly, formins are also members of a highly conserved family of proteins, in which most members contain two C-terminal formin homology domains, FH1 and FH2, that participate in the control of cytoskeletal remodelling (DeWard *et al.*, 2010). FH1 domain is particularly proline-rich, and it has been described that the number of polyPro stretches present is directly proportional to the rate profiling-mediated elongation of actin (Paul and Pollard, 2008), indicating the significance of its conservation in the evolutionary tree. Furthermore, several human formins have been directly linked to human diseases; for example, the mammalian formin mDia1 has been suggested as a tumour suppressor due to its association with APC in colorectal cancer, as well as being implicated in the immune response by mediating T cell adhesion and migration (reviewed in (DeWard *et al.*, 2010)), and Fmnl2 is upregulated in colorectal cancers and it is associated directly with increased lymph node metastasis (Zhu *et al.*, 2008). Most importantly, actin and microtubule cytoskeleton play several prominent roles in cancer and disease, including tumour morphogenesis, cancer metastasis and cellular migration, where cell movements and alterations in cell shape require spatial and temporal reorganisation of the cell cytoskeleton, (reviewed in (Hall, 2009, Fife *et al.*, 2014)). Nonetheless, cancer therapeutics targeting actin have been largely unsuccessful due to cardiotoxicity. Our results indicate a direct molecular function for eIF5A in actin organisation and polarised cell growth through the translation of proline-rich formins in yeasts; it is very possible to envision that this role can be extended to higher eukaryotes to better understand their molecular

connection in the development of cancer and metastasis, and to aid the search for novel therapeutic targets.

On the other hand, several lines of evidence suggest that the role of eIF5A in yeast mating could be extended to fertility in higher eukaryotes. Firstly, the polyamine spermidine is indispensable for mammalian reproduction at several stages, including spermatogenesis, oogenesis, embryogenesis, and implantation (Bauer *et al.*, 2013). In yeast, being the only known substrate for eIF5A hypusination (Park *et al.*, 2010), and similarly, eIF5A hypusination being described as the single most important activity requiring spermidine (Chattopadhyay *et al.*, 2008b), it is very possible that eIF5A also plays a role in mammalian fertility. Secondly, gamete fusion and fertilisation during reproduction requires dynamic interactions between cytoskeletal components such as actin (in the form of microvilli) (Primakoff and Myles, 2007), and actin polymerisation is directly mediated by formins (Zeller *et al.*, 1999). Finally, it has been described that the mammalian formin homology gene, formin-2, is required for the progression through metaphase during meiosis and mouse oocytes lacking in this gene is unable to form the first polar body (Leader *et al.*, 2002).

In conclusion, this thesis provides the first evidence of the functional role of eIF5A in yeast mating through the translation of polyPro formins. Since both eIF5A and formins are highly conserved from yeast to humans, this work can be the basis to further investigate their connection in the context of human diseases and fertility.

Conclusions

Chapter 3

- In response to osmotic stress, signalling components of the HOG pathway come in close contact with each other to elicit efficient and appropriate signal transduction. Here, we have demonstrated that all Ste11 interactions require the adaptor protein Ste50, and occur under non-stress conditions which increase with osmotic stress.

- The transient Ste11 interaction with its substrate Pbs2, which increases with osmotic stress, requires all upper components of the pathway and their dissociation is negatively regulated by Hog1.

- The Ste11-Sho1 interaction does not require the Sho1-SH3 domain, but requires the adaptor Ste50 and osmosensors Msb2/Hkr1.

- We have characterised a novel dynamic interaction between Ste11 and Msb2, in which Msb2 acts as a membrane concentrator of Ste11, requiring adaptors Ste50 and Opy2. However, the presence of Sho1 is not absolutely required for the Ste11-Msb2 interaction.

- Comparing the binding affinities of Ste11 with several signalling components, we have seen that Ste11 robustly interacts with Sho1 and Msb2 both under stress and non-stress conditions, and these two interactions are stronger than the interaction between Ste11 and its substrate Pbs2.

- Multiple negative feedbacks mediated by downstream components, Hog1 and Pbs2, regulate protein levels of Ste11 and Msb2 as well as Ste11 protein interactions with Pbs2, Sho1 and Msb2.

Chapter 4

- During osmotic stress response, the cap-binding protein Cbc1 is required for the reprogrammation of global transcription, and specifically it is required for the high and timely expression of osmostress-responsive genes.

- Cbc1 is found to recruit the transcription activator complex Hot1-Hog1, through an interaction with Hog1, either direct or in close proximity, to appropriate promoters, which in turn enhances PIC formation and recruits RNAPII.

- Cbc1 also plays a role in ribosomal protein (RP) gene expression under non-stress and during the recovery from osmotic stress through the recruitment of RP-specific transcription factor (TF) Rap1 and formation of PIC.

- We have found that Cbc1 has specific roles in driving the transcription of highly expressed genes compared to lowly expressed genes.

- The function of Cbc1 appears to require Cbc2, and both factors are implicated not only in osmotic stress, but in heat, cold and calcium stress.

- Our results also hint that Cbc1 may regulate protein expression of TBP and Hot1, and additionally it appears that Cbc1 has roles in the shutoff of Hog1 and Hot1 signalling during osmotic stress recovery.

Chapter 5

- The evolutionarily conserved translation factor eIF5A, described to promote the translation of proteins containing poly-proline (polyPro) motifs, has a number of potential genetic targets in yeast functioning in various molecular processes, in which four functional categories are over-represented: cellular bud, shmoo, site of polarised growth, and retotransposons.

- In this work, eIF5A is found to play a role in the yeast pheromone response, where activated eIF5A, by hypusination, is required for the formation of the sexual projection shmoo towards a mating partner, the localisation of polarisome components at the shmoo tip, polarised actin assembly, and induction of cell fusion genes.

- eIF5A is found to necessary for the translation of Bni1, a formin containing stretches of 10, 12 and 5 consecutive proline residues, which is involved in polarised cell growth during shmoo formation. Furthermore, the exogenous overexpression of Bni1 also partially suppresses the shmooing defect observed in eIF5A mutants.

- More specifically, the translation of the poly-proline motifs in Bni1 is eIF5A-dependent, and this translation dependency is lost upon deletion of the poly-prolines.

Publications

- **Tianlu Li,** Nikki De Clercq, Daniel A. Medina, Elena Garre, Per Sunnerhagen, José E. Pérez-Ortín, and Paula Alepuz, ¨The cap-binding protein Cbc1 is required for the high and timely expression of genes by stabilizing gene-specific activators and the pre-initiation complex at promoters¨, in draft.

- Aurora Zuzuarregui*, **Tianlu Li*,** Christina Friedmann, Gustav Ammerer, and Paula Alepuz, ¨Msb2 is a Ste11 membrane concentrator required for full activation of the HOG pathway¨, *Biochim Biophys Acta.* 2015 June; 1849(6): 722-30. (IF=5.44)
  *These authors contributed equally to this work

- **Tianlu Li,** Borja Belda-Palázon, Alejandro Ferrando, and Paula Alepuz. "Fertility and polarized cell growth depends on eIF5A for translation of polyproline-rich formins in *Saccharomyces cerevisiae*", *Genetics.* 2014 Aug; 197: 1191-1200. (IF=4.87)

- Ana Miguel, Fernando Montón, **Tianlu Li**, Fernando Gómez-Herreros, Sebastián Chávez, Paula Alepuz, and José E. Pérez-Ortín. "External conditions inversely change the RNA polymerase II elongation rate and density in yeast", *Biochim Biophys Acta*. 2013 Nov; 1829(11): 1248-55. (IF=4.66)

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