SCF^{CDC4} REGULATES Msn2 AND Msn4 DEPENDENT GENE EXPRESSION TO COUNTERACT Hog1 INDUCED LETHALITY

Alexandre Vendrell Arasa

PhD Thesis UPF,

Barcelona, 2008

Memòria presentada per a optar al títol de Doctor per la Universitat Pompeu Fabra (UPF)

Treball dirigit pel Dr. Francesc Posas Garriga

Unitat de Senyalització Cel.lular

Departament de Ciències Experimentals i de la Salut (CEXS)

Programa de Doctorat en Ciències de la Salut i de la Vida de la Universitat Pompeu Fabra

A l'Elsa.

A la meva família i amics.

Bé, ara ve quan la maten. Encara que us ho trobeu al principi, això és el final de la tesi. O com a mínim , el que s'ha escrit al final. Per això, primer de tot, voldria demanar disculpes. Discuples per si em deixo algú, i per si alguna frase acaba sonant desafortunada. Espero que ningu se m'ofengui, penseu que ara mateix estic bastant, com us ho diria, espès. Bé, vamos al lío.

Voldria agrair al meu director de tesi, en Francesc Posas, el fet d'haver-me deixat fer la tesi al seu grup. Igualment, a la Laia, per haver fet de mestra quan jo només era un estiduantito de 2on que feia més nosa que servei.

Després, com no, voldria recordar a la primera fornada de Posas. Primer la Laura, quina troballa! Vam començar picats perquè em va prendre el lloc a la poiata (ja t'he perdonat) i ham acabat essent amics. Molt més que amics, per mi com una germana gran que no deixa que me "descarrie". Per tot el que has fet per mi, moltes gràcies, mai ho oblidaré. Poc després, va arribar en Xavi De Montblanc (com els bons vins té denominació d'origen). Xato, sempre seré el teu gai transexual. Les estones que hem passat al lab (i les que ens queden per passar!), han estat genials. La Meri també va ser un bon fitxatge. La mamífera-chipera incansable. Sempre recordaré les tardes-nits (més nit que tarda) que vam passar als laboratori. Tu eres qui m'animava a no deixar-ho estar amb la teva presència, encara que potser no t'ho semblés. Per acabar d'adobar-ho, va arribar la Glòria., la Tremenda. Ai, xaia, xaia, quin fart de riure amb les teves històries d'aquelles, tu ja m'entens. Has estat una persona molt especial, de qui he après molt, i m'ha aguantat un bon munt de "rallades", moltes gràcies! I la Nuri, ai la Nuri! Mi compañera de fatigas, y eso que en la carrera te caia mal!!! Jajaja! Cuantas cosas habemos pasao, eh? Esperemos que vayamos a mejor, no? Nunca olvidaré tantos ratos compartidos. I finalment, les dues jefas que em van guiar durant bona part dels meus anys d'estudiant i doctorat. La primera va ser la Paula, cuanto ha llovido desde entonces! Espero que sigas siendo tan brillante como cuando estabas por aquí. I la segona la Monste M, mi maestra proteómica. Qué tiempos, todo el día con el churro en la mano.

Tu modo de trabajo, ordenado y metódico fue un gran ejemplo. Y tu eterna sonrisa una buena manera se sobrellevar los disgustos de la ciencia. Ep! Veus, ja em deixava algú! Una persona que també va començar al lab des dels inicis, i del que realment, he après moltíssim del món dels llevats. I és que en Pep és un pou de ciència, un arcano, cuya sabiduría no conoce límites. Gràcies per tots els teus consells, tant "espirituals" com laborals. Fins aquí els que, almeys per mi, van formar part de la meva "primera tesi". La que vaig fer com a estudiant, i a on, vaig aprendre molt, sobretot de la dinàmica de treball d'un grup humà que convivia 12 hores al dia (o més, quines èpoques!).

Això no vol dir que jo no hagi fet una tesi de veritat, que també. En aquesta segona etapa, també hi hagut molta gent important, que voldria recordar. Vam ampliar la família. Primer amb la Laiaprimer amb la secció de mamífers: la Montse B, una persona molt persona, sempre preocupada pels demés; el Manel, un tio com pocs.; el Dani, amb el que formem el "rincón del inversor", no sé con quien compartiré ahora el pulso del mercado, eres un tío genial; i la Isabel, la gallega más simpática de todo el laboratorio, nunca olvidaré las risas que nos hemos echado, me has animado en muchos momentos malos, eres la leche! I l'últim fitxatge, l'Albert, no hem coincidit gaire, però ha esta guai parlar de "gotitas" amb tu, jo crec que promets!

També van arribar reforços a cicle: en Gilad, qué tío! has sido un gran ejemplo por tu capacidad de sacrificio sin quejarte, a pesar de que no todo fuera bien; en Miquel Àngel, l'home de "sa roqueta" que ens porta la tranquilitat quan la cosa es posa chunga; el Javi, también para mí un gran ejemplo, un tío hecho y derecho de la estepa castellana, tus consejos siempre llegan en buen momento, muchas gracias por tu paciencia! A última hora s'ha incorporat l'Alba, espero que tinguis molta sort a Can Posas.

I també va arribar més gent a la part tècnica, tantes vegades poc valorada. Va venir la Marisa, la técnico más flamenca del lab, tu pon orden que si no, esto es una jungla!; la Laia petita, petita para unas cosas, pero muy muy grande para otras!, eres una persona para la que también tendré un gran recuerdo; el Jonathan, el figura, desde que te fuiste, ya no es lo mismo, cuando se levanta el telón y no estás tú, la cosa falla; i la

Sandra, no hem coincidit gaire, però m'has donat un cop de mà en tot el que t'he demanat, merci per tot.

La família també es va ampliar a transcripció, el núcleo duro del lab, a on es talla el bacallà. Va entrar el estudiantito, el nostre Messi particular, el nen de Torrelles, el Sergi. Anar de festa amb tu ha estat tota una experiència. I a nivell humà, un 10! Un amic de debò, pa lo bueno, pa lo malo, y para Hog, que eso sí que tiene mérito, espero que fem alguna altra aventura junts. I va venir la Carme, ai Carmeeeeeeeee! The revolution. Et trobaré molt a faltar, ets una persona com n'hi ha poques, no deixis mai ser així de franca!

Una altra part important de la meva vida al lab han estat "los vecinos": Ester-churripé, Ana, Alberto, Malapé, Merce, Alice, Blanca, Isabelita, Natalia, sin unos vecinos como vosotros esto no habría sido lo mismo. El ambientaso que se creaba a partir de las 6 no tenía precio, Sabina incluido.

Hi ha hagut també molta gent de fora del lab que ha participat molt d'questa tesi. Un munt d'amics han estat "ahí" perquè això acabés bé. Els amics del SAFA, los irrecductibles, amics amb majúscules: Carlos, desde los 3 años aguantándonos tiene que significar algo, cásate conmigo!; Dani, el gran Dan, el ying de mi yang, siempre con tu humor a punto para darle la vuelta a todo, me has ayudado mucho a aguantar todo esto; Chus, mi confesor, tengo que admitirlo, eres una de las personas más importantes en mi vida, hablar contigo siempre me hace ver todo diferente, aun estando en el lejano Granollers; Jordi Pastor, aun ahora en la lejanía, nunca olvidaré tu manera de ser; y Monchi, la energía, esas fiestas de fin de año fueron inolvidables.

També molts amics de la carrera han participat d'aquest projecte: la Guio, l'Anna, l'Ari, el Ramon, el Gabriele han estat molt importants en tot això, espero que la distància dels post-docs no ens faci perdre el contacte! Us estimo molt a tots! I la Mónica, Joana, Susana, Ali, a vosotras también os llevo en el corazón. I la Montse, la

meva mestra del voleibol, a ella li haig d'agrair el tenir la sev amistat, i una altra cosa, que al final per mi ha estat molt important: que em fiqués dins el mundillo del volei. Per tot això i molt més, gràcies.

Ara, i per anar acabant, la família. Els que més reben! Primer els meus pares. A la meva mare agrair-li tot el seu suport, la seva fe en que me'n sortiria quan jo ja no podia més. I pel recolzament logístic, amb els seus tupperss, una cosa que a vegades no he valorat suficient. I al meu pare, voldria dir-li que és l'artífex de tot això. Tu has estat el que des de ben petit, em va transmetre el plaer per pensar, per investigar, per saber, la culpa de tot això és teva. Per això gràcies. Als meus cosins, Montse, Joan i Pep, i tiets, també gràcies per fer-me sentir un fill més. I finalment, dins la meva família, hi ha hagut algú molt important, que ja no hi és, i que també va ser decisiva perquè ara sigui doctor. Moltes gràcies iaia, per haver viscut com vas viure.

I finalment, quedes tu. Al final, sempre has estat tu. Tu has estat la que realment ha estat al peu del canó. Has sido tu, Elsa, la que más ha sufrido esta tesis, la que me ha ayudado a soportar la frustación que supone la investigación. Desde que entraste en mi vida, me has enseñado a ser mejor persona cada día, a vivir, a superar los miedos y los fantasmas que a veces me atenazan. Y espero que estés conmigo en las aventuras que aún nos quedan por vivir.

Bé, ara si que això és el final de la tesi, quatre anys intensos i inoblidables... i durs, possiblement els més durs de la meva vida. Esperem que a partir d'ara vingui lo bo...

Àlex.

Sustained Hog1 activation leads to an inhibition of cell growth. In this work, we have observed that the lethal phenotype caused by sustained Hog1 activation is prevented by SCF^{CDC4} mutants. The prevention of Hog1-induced cell death by SCF^{CDC4} mutation depends on the lifespan extension pathway. Upon sustained Hog1 activation, SCF^{CDC4} mutation increases Msn2 and Msn4 dependent gene expression that leads to a *PNC1* overexpression and a Sir2 deacetylase hyperactivation. Then, hyperactivation of Sir2 is able to prevent cell death caused by sustained Hog1 activation.

We have also observed that cell death upon sustained Hog1 activation is due to an induction of apoptosis. The apoptosis induced by Hog1 is decreased by SCF^{CDC4} mutation. Therefore, lifespan extension pathway is able to prevent apoptosis by an unknown mechanism.

L'activació sostinguda de Hog1 porta a una inhibició del creixement cel·lular. En aquest treball, hem observat que el fenotip de letalitat causat per l'activació sostinguda de Hog1 és parcialment inhibida per la mutació del complexe SCF^{CDC4}. La inhibició de la mort causada per l'activació sostinguda de Hog1 depèn de la via d'extensió de la vida. Quan Hog1 s'activa de manera sostinguda, la mutació al complexe SCF^{CDC4} fa que augmenti l'expressió gènica depenent de Msn2 i Msn4 que condueix a una sobreexpressió del gen *PNC1* i a una hiperactivació de la deacetilassa Sir2. La hiperactivació de Sir2 és capaç d'inhibir la mort causada per l'activació sostinguda de Hog1.

També hem observat que la mort cel·lular causada per l'activació sostinguda de Hog1 és deguda a una inducció d'apoptosi. L'apoptosi induïda per Hog1 és inhibida per la mutació al complexe SCF^{CDC4}. Per tant, la via d'extensió de la vida és capaç de prevenir l'apoptosi a través d'un mecanisme desconegut.

SUMMARY

“Allò que no ens mata, ens fa més forts”

Friedrich Nietsche

Adaptation to environmental stress requires changes in many aspects of the cell biology. In eukaryotic cells, stress-activated protein kinases (SAPKs) play an essential role for proper cell adaptation to extracellular stimuli. In *Saccharomyces cerevisiae*, changes in the extracellular osmotic conditions are sensed by the Hog1 MAPK pathway, the functional homolog of the mammalian stress-activated MAP kinases JNK and p38 pathways. The Hog1 MAPK elicits the program for cell adaptation to osmostress that includes modulation of metabolism, gene expression, translation and cell-cycle progression. However, adaptive responses not properly regulated result in decreased cell viability. Thus, sustained activation of the Hog1 MAPK is detrimental for cell growth.

At the beginning of this PhD project, the mechanisms by which sustained activation of Hog1 was inhibiting cell growth remained unknown. However, previous results in the laboratory showed that mutant strains on the SCF^{CDC4} ubiquitination complex survive at the continuous activation of Hog1. Our main objective was to characterize the molecular mechanisms by which SCF^{CDC4} mutations prevented inhibition of cell growth by sustained Hog1 activation.

Here we show that SCF^{CDC4} mutation prevents inhibition of cell growth caused by sustained Hog1 activation through hyperactivation of the Msn2 and Msn4 transcription factors. Upon sustained Hog1 activation, SCF^{CDC4} mutation leads to overexpression of Msn2 and Msn4 dependent genes due to delayed Msn2 and Msn4 degradation. *PNC1*, a key activator element of the lifespan extension pathway, is induced by the upregulation of Msn2 ans Msn4 transcription factors and *PNC1* deletion avoids *cdc4-1* suppression of Hog1 lethality. Similarly, deletion of *SIR2* and no other deacetilases genes eliminates the effect of *cdc4-1.* Thus, activation of the lifespan extension pathway is crucial to suppres Hog1 lethality.

On the other hand, we have shown that sustained activation of Hog1 induces apoptosis depending on the presence of O_2. SCF^{CDC4} mutation prevents apoptosis induced by Hog1, probably by decreasing ROS formation. The Hog1 dependent inhibition of mitochondrial respiration could be the mechanism by which Hog1 induces ROS formation.

Overall, our results define a new role for Hog1 in regulation of cell viability and, on the other hand, a connexion between the lifespan extension pathway and apoptosis.

TABLE OF CONTENTS

“”Les escales sempre han de començar a escombrar-se per dalt”

Proverbi romanès

INTRODUCTION

"Quin sentit té córrer quan estem en el camí equivocat?"

Proverbi alemany

1.1. OSMOTIC STRESS

1.1.1 Yeasts and their environment

Cells constantly evaluate and respond to their external environment in order to maximize the probability of survival and proliferation. Of particular importance are the mechanisms cells use to respond to sudden and adverse changes in environmental conditions, commonly referred to as cell stresses.

Yeasts such as *Saccharomyces cerevisiae*, are ubiquitous unicellular fungi and hence eukaryotic microorganisms. Living as saprophytes on substrates such as fruits and flowers, these single cell organisms are exposed to a highly variable environment with respect to the availability and quality of nutrients, temperature, pH, radiation, access to oxygen, and water activity (Hohmann and Mager, 1997; Hohmann, 2002). Water activity is of special importance, as it is necessary for maintaining an appropriate cell volume and favorable conditions for biochemical reactions. If yeast cells experience a hyperosmotic shock (or osmotic upshift), there is a rapid water outflow and cell shrinking. Oppositely, a hypoosmotic shock (or osmotic downshift) leads to a rapid water influx, cell swelling and hence increased turgor pressure. Survival mechanisms need to operate within the first seconds after a sudden osmotic shift because passive water loss or uptake occurs very fast (Brown, 1976; Blomberg and Adler, 1992). In the case of a hyperosmotic shock, water loss leads to an increased concentration of biomolecules and ions in the cell, which eventually results in an arrest of cellular activity. However, yeast cells have developed an active mechanism to adapt to high external osmolarity by sensing the osmotic changes and performing the appropriate responses to maintain optimal cellular activity. The mechanisms of osmoadaptation can vary depending on the strength of the stress signals. Accumulation of chemically inert osmolytes, such as glycerol, allows the cells to balance the internal osmolarity with that of the external environment and plays a central role in the process of osmoadaptation (Gustin et al., 1998; Hohmann, 2002; de Nadal et al., 2002). As a consequence, yeast

cells can be metabolically active and proliferate over a wide range of external water activity.

1.1.2 Signalling Pathways Involved in Osmoadaptation

The field of yeast osmoadaptation has received much wider scientific interest with the discovery in of the HOG pathway (**H**igh **O**smolarity **G**lycerol response pathway). Nonetheless, changes in medium osmolarity also have been shown to activate other signaling pathways in yeast: the protein kinase A pathway and the phosphatidylinositol-3,5-bisphosphate.

The influence of cAMP-dependent protein kinase (PKA) on protein expression during exponential growth under osmotic stress suggests that low PKA activity causes a protein expression pattern resembling that of osmotically stressed cells, and thus, PKA is a major determinant of osmotic shock tolerance (Norbeck and Blomberg, 2000). However, this pathway mediates a general stress response observed under essentially all stress conditions, such as heat shock, nutrient starvation, high ethanol levels, oxidative stress and osmotic stress (Marchler et al., 1993; Ruis and Schuller, 1995). Therefore, protein kinase A most probably does not respond directly to osmotic changes. In fact, it is not fully understood how the activity of protein kinase A is regulated by stress.

On another hand, the production of phosphatidylinositol-3,5-bisphosphate seems to be stimulated by an osmotic upshift. This molecule could serve as a second messenger in an osmotic signalling (Dove et al., 1997), although its clear function is still to be resolved.

By far the best-characterized osmoresponsive system in eukaryotes is the HOG pathway. It is a MAP kinase cascade consisting of a well-conserved eukaryotic signal transduction module and involved in the process of osmoadaptation (Gustin et al., 1998; Hohmann, 2002; de Nadal et al., 2002). The HOG pathway is activated within less than one minute by osmotic upshift, being essential for cells to rapidly adapt and survive in high-osmolarity medium (Brewster et al., 1993). Thus, the cellular role of the HOG pathway is indeed to orchestrate a significant part of the response of yeast cells to high osmolarity.

1.2 MAP KINASE PATHWAYS

MAP Kinase pathways are highly conserved signaling modules in both higher and lower eukaryotes, where they allow cells to quickly respond and adapt to environmental stresses, hormones, growth factors, and cytokines (Kyriakis and Avruch, 2001). MAP kinase pathways control cell growth, morphogenesis, proliferation and stress responses, and they are involved in many disease processes.

1.2.1 Modular organization

The mitogen-activated protein (MAP) kinase pathways are organized in modules containing at least three types of protein kinases, which transmit signals by sequential phosphorylation events in a hierarchical way (Figure 1). The MAPKKK activates a MAPKK by phosphorylation on serine and threonine residues within a conserved loop at the N-terminal lobe of the kinase domain. Subsequently, the MAPKK phosphorylates the MAP kinase on a threonine (sometimes serine) and tyrosine residue, which are located adjacent to each other separated by a single aminoacid (Thr/Ser-X-Tyr). This phosphorylation site is located in the activation loop of the catalytic domain; dual phosphorylation on threonine and tyrosine is needed for activation of the MAP kinase. Typically, phosphorylation stimulates transfer of a portion of the activated MAP kinase from the cytosol to the nucleus, where it phosphorylates targets on serine/threonine followed by a proline. MAPKs are able to phosphorylate many substrates including transcription factors, other protein kinases, cytoskeleton-associated proteins and ionic transporters.

In its inactive state, the C-terminal catalytic kinase domain of a MAPKKK is locked by the N-terminal regulatory domain. Activation may occur by phosphorylation or interaction with other proteins, a process that often involves small G-proteins. The activation mechanisms and sensor systems upstream of MAP kinase pathways are diverse and include receptor-tyrosine kinases (i.e. in

animal systems), G-protein-coupled receptors, phosphorelay systems, and others.

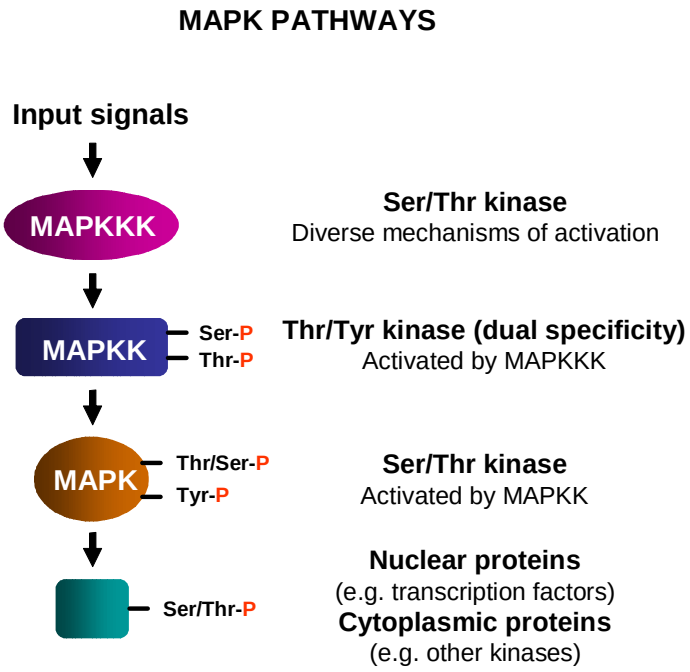

Figure 1. Schematic diagram of a MAPK pathway module. The core module of a MAPK pathway is composed of three kinases; a MAPK kinase kinase, a MAPK kinase and a MAPK, that are sequentially activated by phosphorylation.

Different MAPK pathways within the same organism often share protein kinases which are regulated by multiple signalling inputs. This situation results in highly complex network systems of signaling pathways. Given this complexity, signalling specificity is achieved by scaffolding proteins. Scaffold proteins bind and sequester selected MAPK pathway components, thereby favouring the rapid signal transmission through the cascade and preventing unwanted crosstalk between MAPK modules. Some scaffold proteins can function as simple adaptors, but in some MAPK pathways the signalling components themselves perform scaffolding functions, such as the yeast MAPKK Pbs2 (Hohmann, 2002).

1.2.2. Yeast MAPK pathways

S. cerevisiae has five typical MAP kinases. Based on genetic analyses as well as studies on the transcriptional readout upon physiological, pharmacological

and genetic stimulation, the five MAP kinases are allocated to five distinct MAP kinase cascades (Hohmann, 2002) (Figure 2):

(i) The mating pheromone response pathway (MAP kinase Fus3)

(ii) The pseudohyphal development pathway (Kss1)

(iii) The HOG pathway (Hog1)

(iv) The protein kinase C (PKC) or cell integrity pathway (Slt2/Mpk1)

(v) The spore wall assembly pathway (Smk1)

These MAP kinase pathways are required for directed cell growth (bud formation, mating projections, and pseudohyphal growth), remodeling of the cell surface associated with growth (cell wall integrity, cell integrity, and HOG pathway), and maintenance of the appropriate turgor pressure (HOG and cell integrity pathways). The Smk1 MAPK regulates sporulation (Krisak et al., 1994). Overall, yeast MAPK pathways have roles in response to developmental and external stimuli, as well as on cell cycle control.

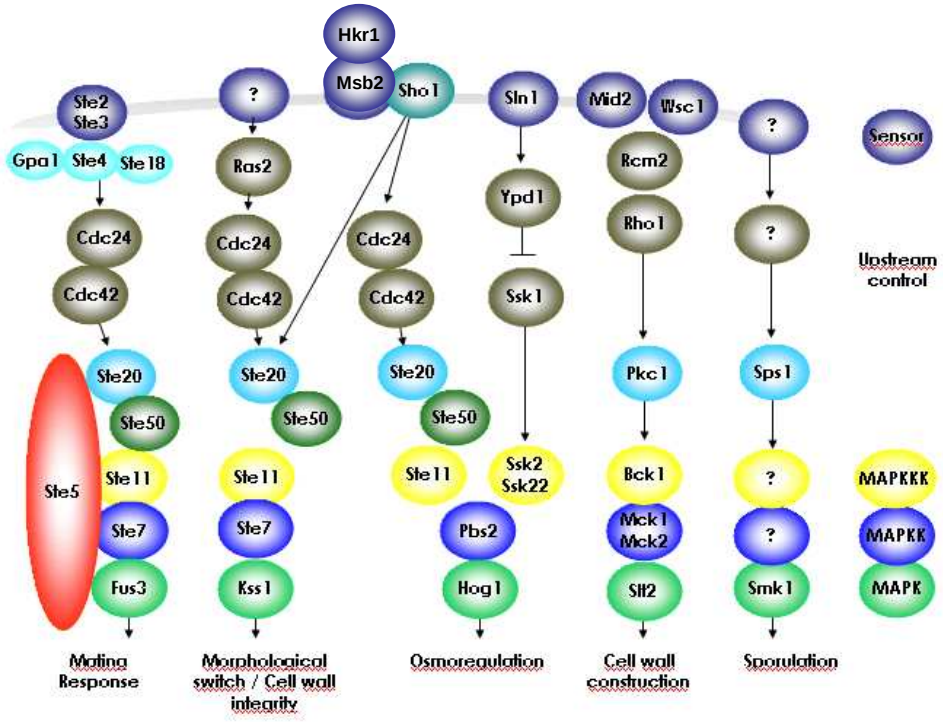

Figure 2. *S.cerevisiae* MAP kinase pathways. *S. cerevisiae* contains five MAPK pathways: mating response, filamentation-invasion, osmoregulation, cell wall integrity and sporulation pathways. Some of the elements are shared between pathways.

1.3. THE HOG PATHWAY

1.3.1 Components and organization

The HOG pathway is the best-characterized and understood osmoresponsive system in eukaryotes. Exposure of cells to increased extracellular osmolarity results in rapid activation of the yeast MAPK Hog1, which elicits the program for cell adaptation required for survival under these conditions. Activation of this pathway leads to a set of osmoadaptive responses, including metabolic regulation, cell cycle progression, translation and gene expression regulation (de Nadal et al., 2002; Sheikh-Hamad and Gustin, 2004). The Hog1 MAPK is a prototype of the Stress-Activated Portein Kinases (SAPK) family, equivalent to the mammalian p38 and c-Jun N terminal kinases. The functional conservation between the HOG pathway and the p38 pathway is illustrated by the fact that the corresponding human gene complements the yeast mutants in the HOG pathway (Galcheva-Gargova et al., 1994).

Schematically, the central core of the yeast HOG pathway comprises a layer of MAPKKKs, Ssk2, Ssk22 (Maeda et al., 1995) and Ste11 (Posas and Saito, 1997) that are responsible for the activation of the MAPKK Pbs2. Once activated, Pbs2 phosphorylates and activates the Hog1 MAPK (Figure 3).

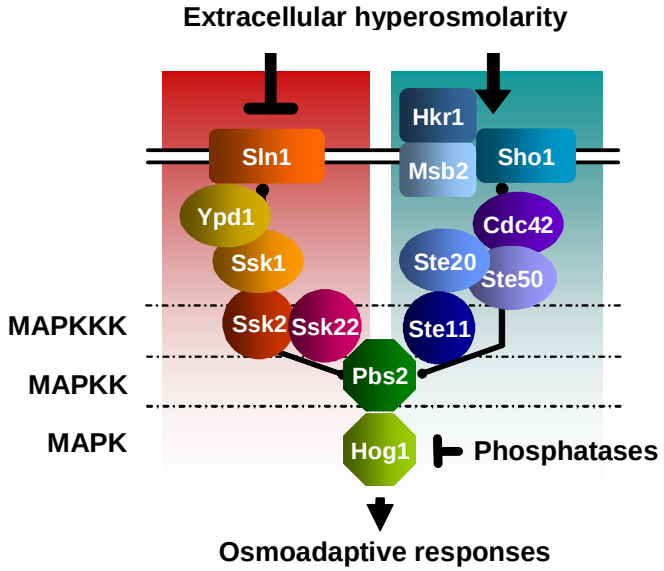

Figure 3. Schematic diagram of the yeast HOG pathway. Two major independent upstream osmosensing mechanisms lead to the activation of specific MAPKKKs and converge on a common MAPKK, Pbs2. Under osmostress, activated Pbs2 activates the MAPK Hog1, which induces a set of osmoadaptive responses.

The pathway is activated predominantly by two independent mechanisms that lead to the activation of either the Ssk2 and Ssk22 or the Ste11 MAPKKKs, respectively. The first mechanism involves a 'two-component' osmosensor, composed of the Sln1-Ypd1-Ssk1 proteins. The second mechanism involves an osmosensor comprised by three transmembrane proteins (Sho1, Hkr1 and Msb2), the MAPKKK Ste11, the Ste11-binding protein Ste50, the Ste20 p21-activated kinase (PAK) and the GTPase Cdc42 (Posas et al*,* 1998; Maeda et al, 1995; reviewed in de Nadal et al, 2002; Tatebayashi et al*,* 2007) (Figure 3).

1.3.2. Osmostress sensors

2.3.2.1. The Sln1 branch

The Sln1 branch involves a "two-component" osmosensor: a sensor molecule and a response-regulator molecule. Typically, a sensor protein has an extracellular input domain and a cytoplasmatic histidine kinase domain. On the other hand, a response-regulator is a cytosolic protein containing a receiver domain and a DNA binding domain. Upon activation, the sensor protein phosphorylates a histidine residue within its kinase domain and transfers this phosphate group to an aspartic acid in the receiver domain of the response-regulator molecule, resulting on its function activation.

In particular, the Sln1 branch is composed by the osmosensor Sln1, the phosphorelay protein Ypd1, and the response regulator protein Ssk1. At normal conditions, the osmosensor Sln1 autophosphorylates itself, this phosphate being sequentially transferred to Ssk1. Phosphorylation activates the response regulator Ssk1 which, in turn, represses the activity of two redundant MAPKKs: Ssk2 and Ssk22. At high osmolarity, the Sln1 histidine kinase is inhibited, resulting in an accumulation of unphosphorylated Ssk1, which then interacts with Ssk2/Ssk22 MAPKKKs to activate the HOG cascade (Posas et al., 1996). Thus, *SLN1* and *YPD1* gene disruption causes lethality, due to the resulting constitutive activation of the HOG pathway (Maeda et al., 1994).

2.3.2.2. The Sho1 branch

The Sho1 branch is involved in an alternative sensor mechanism that leads to Pbs2 activation independently of the Sln1 branch (Maeda et al., 1995).

Sho1 branch involves an osmosensor complex, formed by two mucin-like transmembrane proteins, Hkr1, and Msb2, and Sho1, a protein that contains four transmembrane domains and a COOH-terminal cytoplasmic region with a Src homology 3 (SH3) domain (Tatebayashi et al., 2007). The complex consists of Sho1, Hkr1, Msb2 and Pbs2. Sho1 SH3 domain interact with a proline-rich region in the N terminus of Pbs2 (Maeda et al., 1995). The complex also contains the PAK Ste20, the Rho-like G protein Cdc42 (Raitt et al., 2000; Reiser et al., 2000) and the MAPKKK Ste11 (Posas and Saito, 1997; O'Rourke and Herskowitz, 1998) as well as Ste50, required for Ste11 function (Jansen et al., 2001; O'Rourke and Herskowitz, 1998; Posas et al., 1998).

In high osmolarity conditions, Msb2 and Hkr1 induce Sho1 to generate an intracellular signal. Then, Sho1 binds Pbs2 and recruits it to the cell surface. Then Cdc42 is also recruited and activated. Cdc42 activates the PAK-like kinase Ste20, and, in addition, binds to the Ste11-Ste50 complex, to bring activated Ste20 to its substrate Ste11 (Truckses et al., 2006; Tatebayashi et al., 2006). Activated Ste11 activates Pbs2, which in turn, activates Hog1 (Posas and Saito, 1997).

On the other hand, the MAPKKK Ste11 is used in three functionally distinct MAPK cascades in yeast (Posas and Saito, 1997) (see Figure 2). The ability of Ste11 to function in separate pathways requires stable associations with pathway-specific proteins. For example, Ste11 interacts with the pheromone response pathway-specific scaffold protein Ste5 (reviewed in Hohmann, 2002). In the HOG pathway, the MAPKK Pbs2 serves as a scaffold protein, interacting with Ste11 (Posas and Saito, 1997) and Sho1 (Maeda et al., 1995).

Genetic evidences suggest that the upstream branches of the HOG pathway operate independently of each other; blocking one branch of the pathway still allows rapid Hog1 phosphorylation and cells are fully resistant to high osmolarity. Although these observations suggest redundant functions of the two branches, it is unlikely that the cell maintains two different complex pathways to activate Pbs2. It has been proposed that different sensitivities of the two branches may allow the cell to respond over a wide range of osmolarity changes (Maeda et al., 1995). Data suggest that Sln1 is more sensitive than the

sensor of the Sho1 branch. It also appears that the Sho1 branch operates in an on-off fashion, while the Sln1 branch shows an approximately linear dose response up about 0.4 M NaCl.

1.3.3. Signalling through the HOG pathway

Downstream of the sensor systems, any of the MAPKKKs Ssk2/Ssk22 and Ste11, is able to activate the MAPKK Pbs2 by phosphorylation on Ser514 and Thr518. Pbs2 is a cytoplasmic protein and appears to be excluded from the nucleus, and thus, phosphorylation of its substrate, the Hog1 MAPK, occurs in the cytosol. Dual phosphorylation on the conserved Thr174 and Tyr176 activates Hog1 (Brewster et al., 1993; Schuller et al., 1994). Phosphorylation on both sites is necessary and sufficient to cause a rapid and marked concentration of Hog1 in the nucleus, while under normal conditions Hog1 appears to be distributed between the cytosol and the nucleus (Ferrigno et al., 1998; Reiser et al., 1999). The catalytic activity of Hog1, however, is not required for transfer to the nucleus, since a catalytically inactive mutant of Hog1 is transferred to the nucleus very much like the wild-type. Both phosphorylation and nuclear localization of Hog1 are transient effects: under mild osmotic stress (0.4M NaCl) Hog1 phosphorylation peaks within 1 minute and disappears within about 30 minutes. However, the more severe the osmotic shock, the longer it takes until active Hog1 is dephosphorylated and deactivated (Rep et al., 1999a; Van Wuytswinkel et al., 2000).

Nuclear accumulation of Hog1 suggests that an important part of Hog1 functions take place in the nucleus. However, a portion of activated Hog1 remains in the cytosol where it mediates regulatory effects. Among the best documented of such effects is the activation of the protein kinase Rck2 (Bilsland-Marchesan et al., 2000; Teige et al., 2001), which controls translation efficiency (Teige et al., 2001), and the phosphorylation of ion transporters, essential for the rapid reassociation of proteins, previously dissociated from chromatin due to osmotic stress (Proft and Struhl, 2004).

1.3.4. Modulation and feedback control of the HOG pathway

The HOG pathway is controlled by specific feedback events, as indicated by the transient phosphorylation and activation of the MAP kinase (Maeda et al., 1994; Jacoby et al., 1997; Tamas et al., 2000) and the modulation of the responses depending on the strength of the osmostress input.

One of these feedback mechanisms include two phosphotyrosine phosphatases (Ptp2 and Ptp3), as well as three phosphoserine/threonine phosphatases (Ptc1 to Ptc3). Protein phosphatases are critical for HOG pathway regulation for various purposes: i.e., to reduce the basal activity to prevent initiation of undesirable response in the absence of relevant stimuli; to prevent excessive MAPK activation upon stimuli; and to resume normal cell growth after adaptive responses.

Specifically, Ptp2 and Ptp3 interact directly with phosphorylated Hog1 and dissociate rapidly from the kinase after dephosphorylation. Ptp2 seems to be more important for Hog1 dephosphorylation than Ptp3, possibly because Ptp2 is predominantly nuclear, as is activated Hog1, while Ptp3 is located in both the cytosol and the nucleus (Mattison et al., 1999). However, since in the *ptp2*Δ *ptp3*Δ double mutant the level of tyrosine-phosphorylated Hog1 is still responsive to osmotic shock, additional dephosphorylation mechanisms must exist (Jacoby et al., 1997; Wurgler-Murphy et al., 1997). Ptc1 inactivates the pathway by dephosphorylating the MAPK phosphothreonine *in vitro* (Warmka et al., 2001) indicating that Ptc1 is perhaps the main phosphatase that acts on the phosphothreonine in Hog1.

The Hog1 downregulation after activation is very well modulated by, at least, four redundant phosphatases. It is because Hog1 inactivation is very important for cell viability. Actually, simultaneous deletion of *PTC1* and *PTP2* is lethal. There are several mechanisms that lead to sustained Hog1 activation in addition to phosphatase mutation: i.e. expression of a constitutively active MAPKK (allele called $Pbs2^{DD}$) and the *SLN1* deletion. The use of transient expression of these alleles has been instrumental to study HOG properties. For instance, $PBS2^{DD}$ expression under the *GAL1* promoter or , *sln1*Δ with a plasmid expressing Hog1 phosphatases under the *GAL1* promoter.

The way in what Hog1 induces cell death remains unknown. This question has been addressed by mutation of some downstream elements of the Hog1 pathway that suppressed *PBS2*^{DD} or *sln1*Δ lethality. Only two mutations, *rck2*Δ and *hot1*Δ, showed a slight increased survival rate. But the main lethal effect remained unknown.

This is the main subject of this thesis: to describe a mechanism to promote cell survival under sustained Ho1 activation.

1.4. PHYSIOLOGICAL ROLES OF Hog1

Once activated, Hog1 elicits the program for cell adaptation to osmotic stress, which includes modulation of several aspects of cell biology essential for cell survival. Some of the biological processes regulated by Hog1 are the control of gene expression, cell cycle progression, protein synthesis and metabolic adaptation (Figure 4).

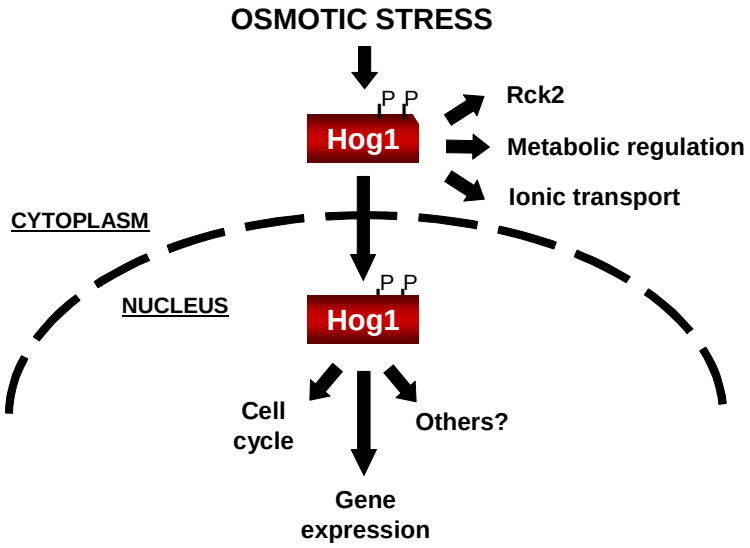

Figure 4. Functions of the MAPK Hog1. Once Hog1 is phosphorylated and activated, it controls several aspects of the cell biology, such as cell cycle regulation, modulation of gene expression, metabolic adaptation and regulation of protein synthesis.

1.4.1. Metabolic adaptation

As mentioned above, one of the roles of Hog1 upon osmotic stress is metabolic adaptation (Figure 4). Among the targets of the HOG pathway are

important transcription factors such as Sko1 and Msn2/Msn4. These transcription factors induce the expression of osmolyte-synthesizing genes, i.e. *GPD1* (encoding glycerophosphate dehydrogenase 1) and *TPS2* (encoding trehalose phosphate phosphatase) (Ruis and Schuller, 1995). The resulting increase in the levels of the compatible osmolytes glycerol and trehalose leads to the replacement of excessive inorganic ions and the restoration of intracellular electrolyte homeostasis in situations of hyperosmotic stress. Moreover, activation of the HOG pathway upon osmotic stress leads to phosphorylation and activation of the 6-phosphofructo-2-kinase (Pfk2) (Dihazi et al., 2004). This activation causes a stimulation of the upper part of glycolisis, a precondition for glycerol accumulation. Yeast cells containing *PFK2* accumulate three times more glycerol than cells lacking *PFK2*, which are not able to grow as efficiently under hypertonic stress (Dihazi et al., 2004).

1.4.2 Regulation of protein synthesis

In response to increases in external osmolarity, there is a transient decrease in protein synthesis (Norbeck and Blomberg, 1998; Uesono and Toh, 2002) caused by a decrease in amino-acid uptake, repression of ribosomal protein genes and a decrease in translation efficiency. The HOG pathway appears not to be involved in the initial inhibition of translation, but rather in the reactivation of translation under stress as an adaptation mechanism (Uesono and Toh, 2002). The yeast Rck2 kinase, which is a member of the calmodulin protein kinase family, is targeted by Hog1 (Bilsland-Marchesan et al., 2000; Teige et al., 2001). Rck2 affects translation by directly regulating the elongation factor EF-2, as it phosphorylates and thereby inhibits this translation elongation factor (Bilsland-Marchesan et al., 2000; Teige et al., 2001). An overall reduction of protein synthesis may be compatible with a transient inhibition of cell growth and proliferation caused by osmotic stress. However, expression of genes encoding functions required for stress adaptation is stimulated and their translation has to be ensured. This suggests that mechanisms must exist that allow the preferential translation of subsets of mRNAs under certain conditions, but little is known about the molecular bases underlying this phenomena.

1.4.3. Regulation of cell cycle progression

Progression through the cell cycle is critically dependent on the presence of nutrients and stress stimuli. In response to osmostress, Hog1 mediates a transient cell-cycle arrest to allow cell adaptation (Escoté et al., 2004; Clotet et al., 2006).

In *S. cerevisiae*, cell cycle is divided in four phases: S-phase (DNA synthesis), M-phase (mitosis), and G_1 and G_2. At Start, yeast cells decide if begin a new cycle, conjugate with another cell, or sporulate. Transitions between G_1/S and between G_2/M are strongly regulated to provide a successful cell division. This regulation is possible due to diverse checkpoints that monitor proper completion of each stage of the cell cycle. Thus, cell cycle progression can be delayed until the execution of an unfinished step allowing the cells to begin a new cycle. In yeast, Cdc28 is the unique CDK (Cyclin Dependent Kinase) that controls cell cycle progression, and cyclins are the proteins responsible for binding and activating the CDK. Cdc28 cyclins can be G_1 cyclins (Cln1, Cln2 and Cln3), that regulate events during the interval between mitosis and DNA replication, and B-type cyclins (Clb1- Clb2, Clb3-Clb4) and finally Clb5-Clb6, that control DNA replication until cytokinesis. Cdc28 activity can be regulated through the synthesis and degradation of cyclins, through association with CDK inhibitors (Sic1 and Far1), and through phosphorylation and dephosphorylation of Cdc28 by Swe1 and Mih1.

In Clotet et al. (2006), it is proposed a novel regulatory mechanism of the G_2 checkpoint that allows cells to integrate stress signals to modulate cell cycle. Hog1 controls G_2 progression by a dual mechanism: the downregulation of Clb2 levels, as well as the direct phosphorylation of the Hsl1 kinase. Upon osmotic stress, Hog1 phosphorylates Ser1220 of Hsl1, which is the checkpoint kinase (Shulewitz et al., 1999; Cid et al., 2001) promoting delocalization of Hsl7 from the bud neck. This prevents Swe1 from being recruited to the bud neck and from being phosphorylated, which leads to Swe1 accumulation and G_2 arrest. The Hog1 MAPK also controls the G_1 transition in response to osmotic stress. Similarly to the mechanism for G_2 regulation, this consists in a dual mechanism that

involves regulation of cyclin expression and the targeting of the cell cycle regulatory protein Sic1 (Escoté et al., 2004).

1.4.4. Regulation of gene expression

In *S. cerevisiae*, genome-wide transcription studies revealed that a large number of genes (~ 5-7%) show significant changes in their expression levels after a mild osmotic shock and that the Hog1 MAPK plays a key role in much of this global gene regulation (Posas et al., 2000; Rep et al., 2000; Gasch et al., 2000; Causton et al., 2001). Osmostress-regulated genes are implicated in carbohydrate metabolism, general stress protection, protein biosynthesis and signal transduction.

1.5. ROLE OF Hog1 IN TRANSCRIPTION

1.5.1. MAP kinases and transcription.

As mentioned above, one of the main functions of MAPKs in response to different stresses is the regulation of gene expression. There is no a unifying mechanism by which MAPKs modulate gene expression. The best-understood mechanism is the direct phosphorylation of promoter-specific transcription factor targets (Karin and Hunter, 1995; Kyriakis and Avruch, 2001). This mechanism can regulate the target activity by several mechanisms, including control of protein levels, regulation of binding to DNA, nucleocytoplasmic shuttling and by altering their ability to transactivate (Yang et al., 2003).

1.5.2. Gene expression regulated by Hog1

Increases in extracellular osmolarity result in changes in the expression of a large number of genes. Better understanding of the yeast response to osmotic stress has been achieved from the use of DNA microarrays, which have permitted to perform genome-wide analysis of the transcriptional response under any type

of stress. In the last few years, several independent studies have analyzed the global transcriptional response of *S. cerevisiae* to osmotic stress (Posas et al., 2000; Rep et al., 2000; Gasch et al., 2000; Causton et al., 2001; O'Rourke and Herskowitz, 2004). From these studies two conclusions can be drawn. First, different stress conditions (such as time of exposure to salt and osmolyte concentration) result in a different pattern of expression. For instance, exposure to high concentration of salt results in a delayed transcriptional response. Second, the Hog1-mediated signalling pathway plays a key role in global gene regulation under saline stress condition, since the response in *hog1*Δ cells is different from that observed in wild-type cells. In fact, approximately the 75% of genes induced after 10 minutes of exposure to 0.4M NaCl are fully or strongly dependent on the Hog1 MAPK (Posas et al., 2000). A relationship between the intensity of the response and the involvement of the Hog1 kinase can be drawn from the observation that genes considered very highly induced are more dependent on Hog1 than genes only moderately induced. Thus, although in some cases the HOG pathway is not the unique relevant signalling pathway involved, its central role in the global response to osmotic stress is clearly established.

The main groups of genes highly induced after exposure to 0.4M NaCl for 10 minutes (Posas et al., 2000) are genes encoding proteins involved in carbohydrate metabolism (mainly, sugar transport and phosphorylation, and glycerol, trehalose and glycogen metabolism), protein biosynthesis (mainly ribosomal proteins), ion homeostasis, signal transduction and aminoacid metabolism.

1.5.3 Transcription factors under the control of Hog1

As mentioned before, it has been reported that SAPKs can modify gene regulation by direct phosphorylation of transcription factors, both activators and repressors. In yeast, five transcription factors have been proposed to be controlled by the Hog1 MAPK: the redundant zinc finger proteins Msn2 and Msn4 (Schuller et al., 1994), Hot1 (which does not belong to a known family of transcription factors) (Rep et al., 1999b), the bZIP protein Sko1 (Proft et al., 2001) and the MADS box protein Smp1 (de Nadal et al., 2003) (Figure 5).

Each factor seems to be controlling a small subset of the osmoresponsive genes and thus, deletion of a particular transcription factor has a very limited

effect on general osmostress gene expression. Still remains the possibility that additional transcription factors are required for gene expression upon stress.

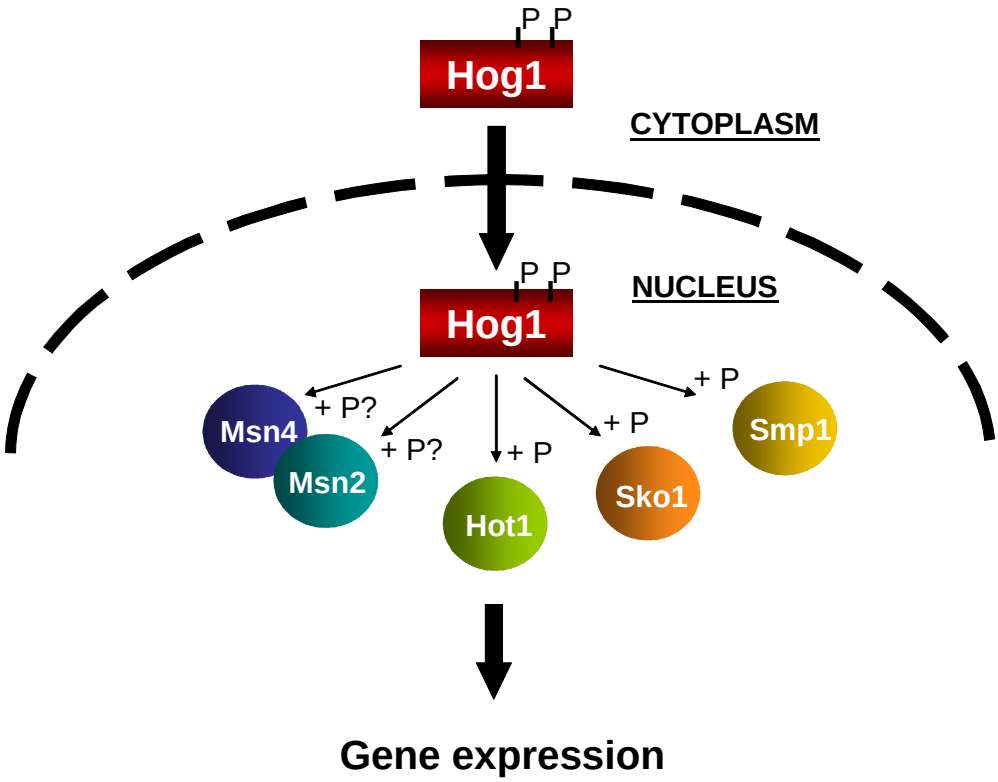

Figure 5. Transcription factors under the control of the Hog1 MAPK. Five transcription factors are regulated by Hog1, although only three of them (Hot1, Sko1 and Smp1) are described as direct *in vivo* substrates of Hog1.

1.5.3.1. Smp1

Smp1 is a member of the MEF2C family of transcription factors. It had been previously reported in higher eukaryotes that this family of transcription factors can be targeted by the mammalian p38 MAPK (McKinsey et al., 2002). In yeast, Smp1 activator is directly phosphorylated by Hog1 on several residues within its transactivation domain. This phosphorylation by the MAPK is essential for Smp1-mediated gene expression (de Nadal et al., 2003). Smp1 plays an important role not only in osmostress responses, but also in a new function for the Hog1 MAPK required for cell survival in the stationary phase (de Nadal et al., 2003).

1.5.3.2. Hot1

Hot1 (**H**igh-**O**smolarity-induced **T**ranscription) is a transcription factor related to Msn1 that was identified in a two-hybrid screening for proteins interacting with Hog1 (Rep et al., 1999b). It has been reported that Hot1 controls a small subset of genes, such as *STL1*, *GPD1* and *GPP2*, involved in the transport and production of glycerol (Rep et al., 1999a). Hot1 is phosphorylated by Hog1 in response to osmotic stress, but this phosphorylation appears not to be relevant for Hot1 DNA binding, recruitment of RNA polymerase II (Pol II) complex or transcriptional activation.

However, interaction of Hot1 with Hog1 is critical for recruitment of the MAPK to Hot1-dependent promoters and essential for their transcriptional induction upon stress (Alepuz et al., 2001; Alepuz et al., 2003). Assembly of the general transcription machinery at Hot1-dependent promoters depends on the presence of Hot1 and active Hog1 MAPK. Then, Hog1 interacts with the RNA Pol II and with general components of the transcription machinery, forming the initiation complex. Thus, anchoring of active Hog1 to promoters by the Hot1 activator is essential for recruitment and activation of RNA Pol II.

The recruitment of Hog1 to the promoters is a general mechanism to induce transcription. Hog1 recruitment is also mediated by the rest of transcription factors regulated by Hog1, as Msn1 and Msn2/Msn4. In addition, the mammalian p38 also interacts with the RNA Pol II, which might suggest a conserved mechanism for regulation of gene expression by SAPKs among eukaryotic cells (Alepuz et al., 2001; Alepuz et al., 2003).

1.5.3.3. Sko1

Sko1 is an ATF/CREB-related factor (Nehlin et al., 1992; Vincent and Struhl, 1992). Such factors possess a bZIP domain, i.e., a leucine zipper for dimerization, and a basic transcription activation domain. Sko1 inhibits transcription of several genes that are inducible by osmotic stress (Proft and Serrano, 1999; Garcia-Gimeno and Struhl, 2000) by recruiting the general corepressor complex Ssn6-Tup1. Release from Ssn6-Tup1 repression in response to osmotic stress requires direct phosphorylation of Sko1 by the Hog1 MAPK (Proft et al., 2001). Interestingly, Hog1 phosphorylation switches Sko1 activity from a repressing to an activating state, which involves recruiting of SWI/SNF and SAGA complexes (Proft and Struhl, 2002). Recent genome-wide studies have

demonstrated that yeast cells contain approximately 40 Sko1 target promoters in vivo (Proft et al., 2005). Sko1 binds to a number of promoters for genes directly involved in defense functions that relieve osmotic stress. In addition, Sko1 binds to the promoters of genes encoding transcription factors, including Msn2 among others. Lastly, Sko1 targets *PTP3*, one of the phosphatases that negatively regulates Hog1 kinase activity, and it is required for osmotic induction of *PTP3* expression.

1.5.3.4. Msn2/Msn4

Msn2 and Msn4 are Cys_2His_2 zinc finger proteins that activate the expression of a number of stress-inducible genes (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). The *MSN2* gene was selected as a multicopy suppressor in a temperature-sensitive Snf1 protein kinase mutant of *Saccharomyces cerevisiae*. Msn2 is a Cys2His2 zinc finger protein related to the yeast Mig1 repressor and to mammalian early growth response and Wilms' tumor zinc finger proteins. Deletion of *MSN2* caused no phenotype. Then, a second similar zinc finger gene, *MSN4*, was isolated. Deletion of both genes caused phenotypic defects related to carbon utilization.

Although often considered to be functionally redundant in part because they activate gene expression through a common binding site, they are differentially regulated and may play distinct roles under different environmental conditions (Garreau et al., 2000; Gasch and Werner-Washburne, 2002).

Msn2/Msn4 are considered the main response element against several stresses, such as glucose starvation, nitrogen starvation, heat shock or osmotic shock (Estruch, 2000). Thus, Msn2/Msn4 activate a set of genes of general protection to several stresses.

This general protective function has been recently related with aging: a mild stress that activate Msn2/Msn4 make yeast to live longer, the lifespan is extended (Medvedik et al., 2007). The molecular mechanism involved in this delay in aging effect is an essential part of the work.

1.5.4 Regulation of Msn2/Msn4 activity.

Msn2/Msn4 activity is regulated basically by two mechanisms: the subcellular localization and the nuclear degradation.

1.5.4.1. Msn2/Msn4 localization.

Msn2/Msn4 activity is regulated by the subcellular localization of the transcription factors, residing in the cytosol under standard growth conditions and translocating to the nucleus under stressful conditions.

This translocation is controlled by their phosphorylation state (Gorner et al., 1998). The phosphorylation is regulated by several signalling pathways. These include the target of rapamycin (TOR) pathway (Beck and Hall, 1999), the protein kinase C pathway (Heinisch et al., 1999; Nierras and Warner, 1999), the protein kinase A pathway (Gorner et al., 1998), and the HOG pathway (Rep et al., 2000). But the specific pattern of phosphorylations by the different pathways remains unclear.

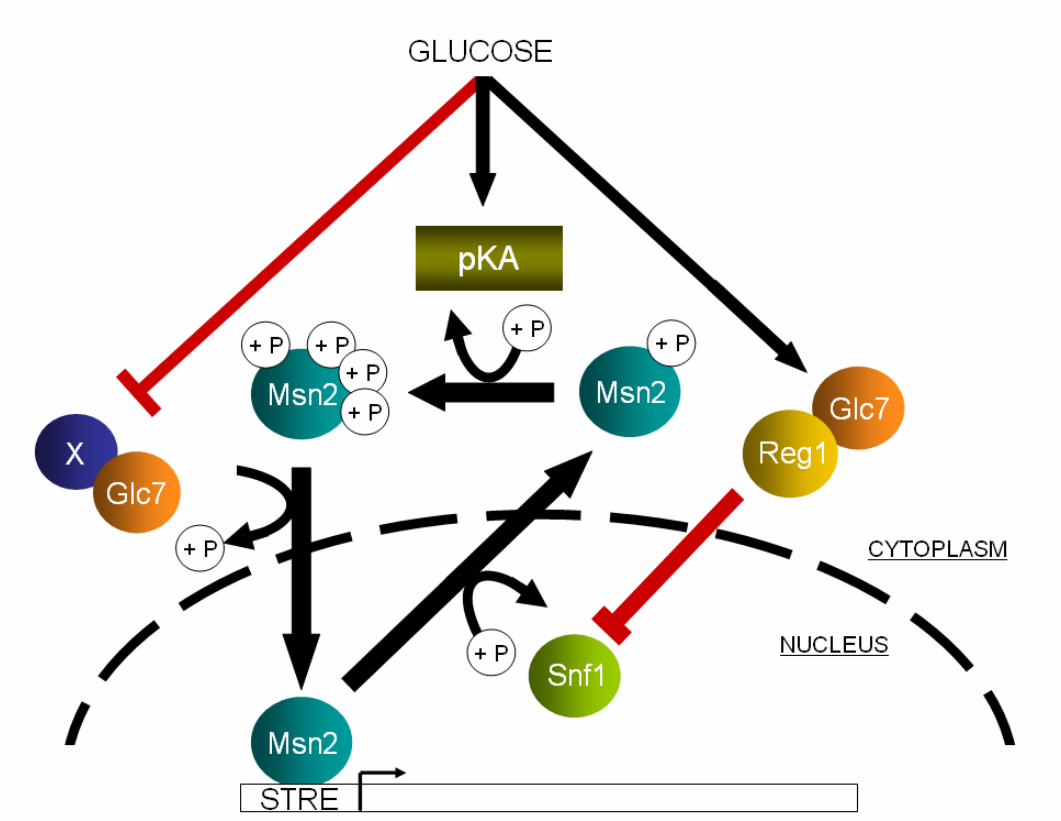

Figure 6. A diagram for the regulation of Msn2 upon acute glucose depletion. Msn2 is phosphorylated and inactivated by PKA during growth on glucose. Glucose depletion results in Msn2 dephosphorylation by PP1-Glc7 and nuclear accumulation. Activation of STRE-driven genes causes the rapid activation of Snf1 kinase. It phosphorylates Msn2 again, promoting nuclear entry of Msn2 (De Wever et al, 2005).

It is known that Msn2 is phosphorylated and inactivated by PKA during growth on glucose. Glucose depletion results in Msn2 dephosphorylation by PP1-Glc7 associated with unknown regulatory subunit(s). Then Msn2 enters into the nucleus, accumulate there and activate gene expression. It also causes the rapid activation of the Snf1 kinase. Cells adapt to the loss of glucose as the rising level of Snf1 kinase promotes nuclear entry of Msn2 (De Wever et al., 2005).

Hog1 activation localizes Msn2 to the nucleus, but a direct phosphorylation of Msn2 by Hog1 is not described (F. Posas, not published).

1.5.4.2. Msn2/Msn4 degradation.

Once in the nucleus, Msn2/Msn4 bind to their consensus site, called STRE element. It consists on a core sequence CCCCT in either orientation and it is usually found in two or more copies in front of Msn2/Msn4 target genes (Treger et al., 1998; Moskvina et al., 1998).

During Msn2 nuclear localization, Msn2 protein levels drop significantly. Moreover, Msn2 levels are also reduced under chronic stress or low protein kinase A (PKA) activity, both conditions that cause a predominant nuclear localization of Msn2 (Durchschlag et al., 2004). Similar effects were found in the *msn5*Δ mutant, cells that block Msn2 nuclear export. A mutant form with alanine substitutions in the PKA phosphorylation sites in Msn2, results in a similar effect (Durchschlag et al., 2004).

The decrease in Msn2 protein levels is caused by the proteosome (Durchschlag et al., 2004). In addition, Msn2 is efficiently ubiquitinated in vitro by SCF^{CDC4}. Therefore, Msn2 should be ubiquitinated by SCF^{CDC4} to be degradated by the proteosome to regulate Msn2 activity (Chi et al., 2001).

1.6. UBIQUITINATION

1.6.1. Ubiquitination: definition and mechanisms.

Ubiquitin is a highly-conserved small regulatory protein that is ubiquitously expressed in eukaryotes. Ubiquitination (or ubiquitination) refers to

the post-translational modification of a protein by the covalent attachment (via an isopeptide bond) of one or more ubiquitin monomers (Pickart et al., 2004).

The most prominent function of ubiquitin is labeling proteins for proteasomal degradation. It happens when ubiquitin molecules are added on to previously-conjugated ubiquitin molecules to form a polyubiquitin chain. If the chain is longer than 4 ubiquitin molecules, the tagged protein is rapidly degraded by the 26S-proteasome into small peptides, usually 3-24 amino acid residues in length (Ciechanover et al., 2000; Thrower et al. 2000). Ubiquitin moieties are cleaved off the protein by deubiquitinating enzymes and are recycled for further use. Besides degradation function, ubiquitination also controls the functionality and intracellular localization of a wide variety of proteins (Muratani et al., 2003; Lipford et al., 2003).

The process of marking a protein with ubiquitin (ubiquitination or ubiquitination) consists of a series of phases (Figure 7). The first one is the activation of ubiquitin. Ubiquitin is activated in a two-step reaction by an E1 ubiquitin-activating enzyme in a process requiring ATP as an energy source. The initial step involves production of a ubiquitin-adenylate intermediate. The second step transfers ubiquitin to the E1 active site cysteine residue, with release of AMP. This step results in a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1 cysteine sulphydryl group (Chi et al., 2001).

Then, there is a transfer of ubiquitin from the E1 to the active site cysteine of a ubiquitin-conjugating enzyme (E2) via a trans(thio)esterification reaction (Chi et al., 2001; Pickart et al., 2004).

The last phase of the ubiquitination cascade requires the activity of one of the hundreds of E3 ubiquitin-protein ligases (often termed simply ubiquitin ligase). E3 enzymes possess one of two domains: the HECT (Homologous to the E6-AP Carboxyl Terminus) domain; or the RING (Really Interesting New Gene) domain (or the closely- related U-box domain) (Deshaies, 1999; Jackson et al., 2000).

Once the E2, E3 and ubiquitin complex is formed, ubiquitin transfer can occur directly from E2, catalysed by RING domain, or via an E3 enzyme, via its HECT domain (Seol et al., 1999).

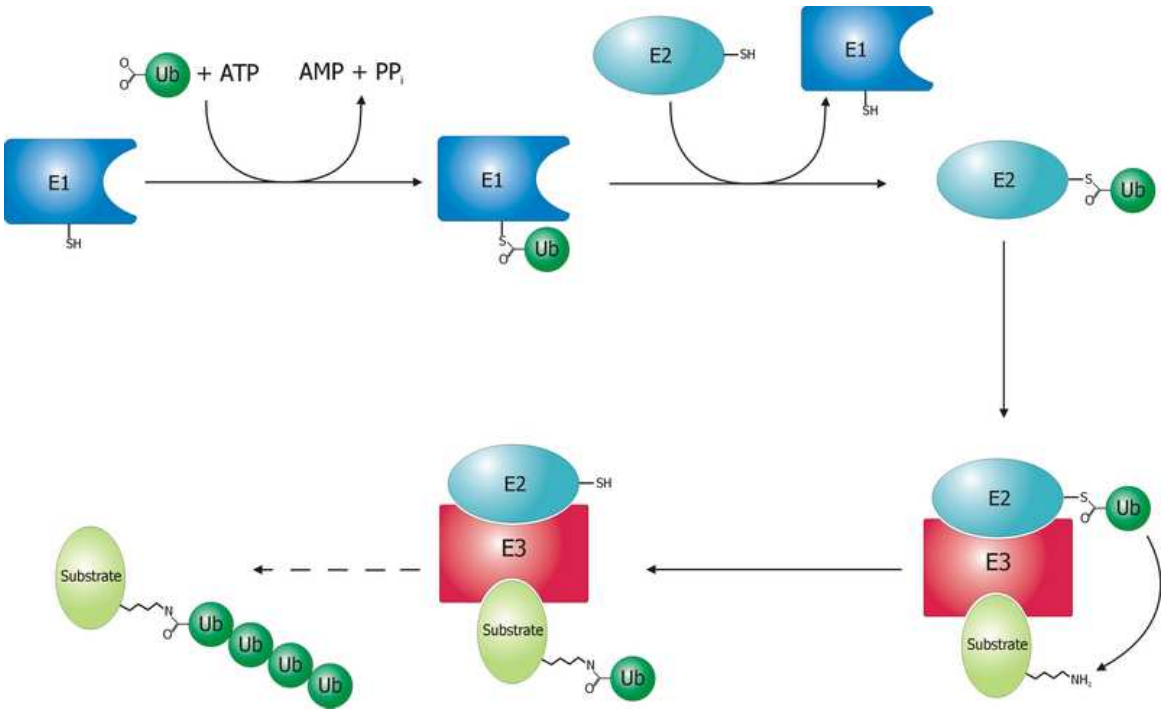

Figure 7. Schematic diagram of the ubiquitination system. The ubiquitin molecule is bound to an E1 ubiquitin-activating enzyme. Then, activated ubiquitin is transferred to the E2 ubiquitin-conjugating enzyme. E3 ubiquitin-protein ligase interacts with both E2 and substrate. Then, E2 is able to transfer the ubiquitin to the substrate. A substrate can be ubiquitinated several times to turn into a polyubiquitinated protein. © Roger B. Dodd

1.6.2. The SCF complex.

Many key proteins of cell are targeted for degradation by a recently described family of E3 ubiquitin protein ligases termed Skp1-Cdc53-F-box protein (SCF) complexes (Hershko et al., 1998). SCF complexes physically link substrate proteins to the E2 ubiquitin-conjugating enzyme Cdc34, which catalyses substrate ubiquitination, leading to subsequent degradation by the 26S proteasome. SCF complexes contain a variable subunit called an F-box and an invariant core complex composed of the subunits Cdc34, Skp1 and Cdc53 (Willems et al., 1999).

F-box proteins are the substrate-recognition components of SCF ubiquitin-protein ligases (Bai et al., 1996). F-box protein binds the SCF constant catalytic core by means of the F-box motif interacting with Skp1. The substrates are bound through the F-box variable protein–protein interaction domains (Bai et al.,

1996). The large number of F-box is thought to allow ubiquitination of diverse substrates. The most important F-box proteins are Cdc4, Grr1 and Met4 (Willems et al., 1999) (Figure 8).

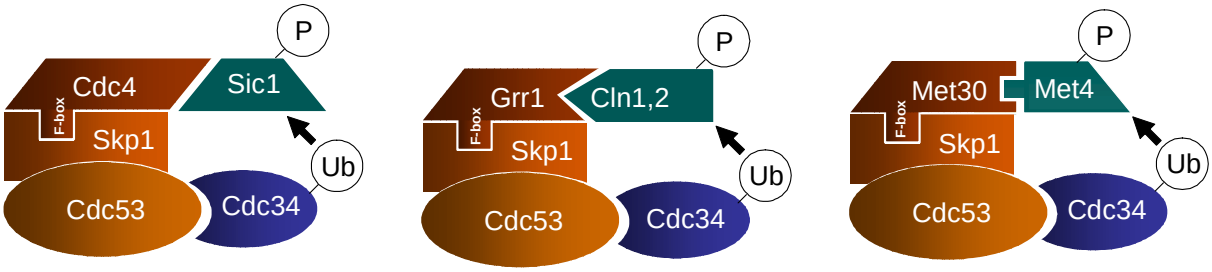

Figure 8. Schematic diagram of the different SCF – E2 ubiquitin ligase complexes. SCF complex has a constant part (formed by Skp1 and Cdc53) and a variable part (formed by the F-box protein). To recognize different substrates, there are several F-boxes, such as Cdc4, Grr1 or Met30.

SCF^{CDC4} is the prototype of the SCF family of ubiquitin ligases. SCF^{CDC4} was initially defined by *in vitro* reconstitution (Feldman et al 1997, Skowyra et al 1997). *CDC4* was first identified in budding yeast as a byproduct of a screen for cell-division-cycle defective (cdc) mutations (Patton et al., 1998). It is now known that the basis for the *CDC4*-mutant phenotype is the inability to ubiquitinate and target the Cdk inhibitor Sic1 for destruction, thereby blocking the G1–S transition (Schwob et al., 1994).

There are two general classes of protein ubiquitin ligase mechanisms to target a protein for destruction: APC/C and SCF (Reed et al., 2003). Whereas substrate regulation by APC/C activity is intrinsic to the ligase itself (Visintin et al., 1997), regulation of SCF activity is usually mediated by substrate phosphorylation (Skowyra et al., 1997). Every substrate ubiquitinated by SCF^{CDC4} has to be previously phosphoryated. For instance, in vitro studies showed that Srb10 is the kinase responsible to phosphorylate Msn2 (Chi et al., 2001).

1.6.3. Functions of ubiquitination

Many proteins have been described as substrates of SCF by a variety of criteria, including stabilization or accumulation in mutants on SCF pathway,

stabilization upon expression of dominant-negative F box proteins, and reconstitution of SCF-dependent ubiquitination with either purified proteins or in a crude system (Willems et al., 2004). These substrates represent a broad spectrum of proteins that participate in a variety of cellular functions, including regulation of CDK activity (by regulation of the Sic1 Cdc28-Clb5 inhibition, see Figure 9), activation of transcription, signal transduction, assembly of kinetochores and DNA replication (Reed et al., 2003).

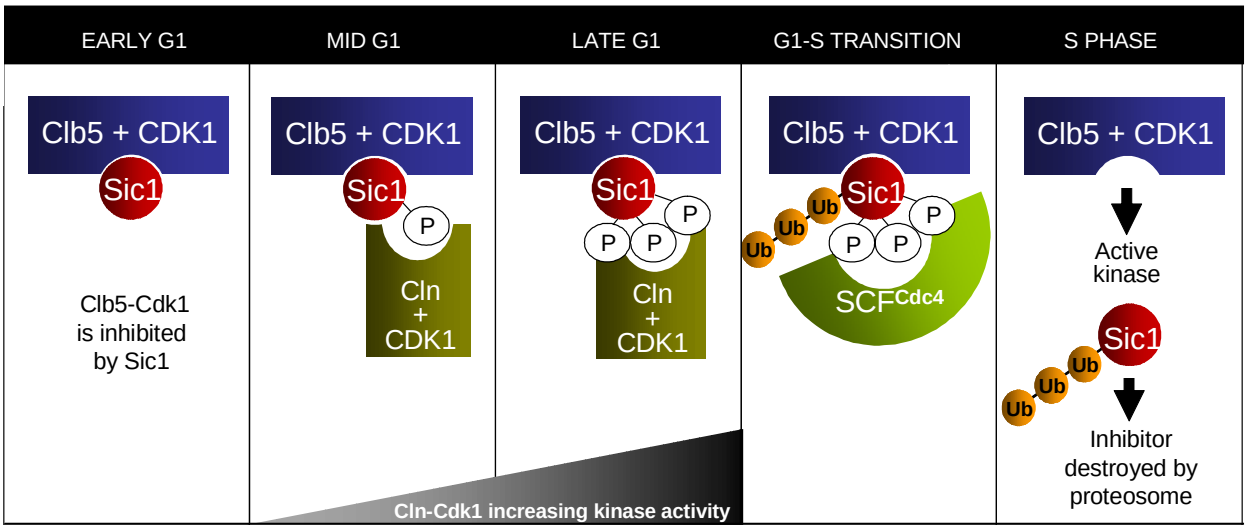

Figure 9. Sic1 evolution during the several cell cycle phases in *S. cerevisiae*. At early G1, Sic1 inhibits Clb5 + Cdk1 activity. When G1 progress, Cln1/Cln2 + Cdk1 complex phosphorylates Sic1 until Sic1 is recognized by SCF-Cdc4. Then, Sic1 is polyubiquitinated and destroyed. It releases Clb5/Cdk1 activity, promoting the transition to S-phase.

1.6.3.1. Gene expression regulation by ubiquitination.

Two models for understanding the role of posttranslational (phosphorylation and ubiquitination) modifications and proteasome-mediated degradation of activators in regulating their activity are shown: the "timer" model (Conaway et al., 2002; Gonzalez et al., 2002) and the "black widow" model (Muratani and Tansei, 2003).

The "timer" model assumes that activator monoubiquitination is a necessary licensing event for high-level gene expression and that extension of this modification into a K48-linked polyubiquitin chain would occur subsequently (Conaway et al., 2002; Gonzalez et al., 2002). At least four ubiquitin molecules

must be added to a protein to allow it to be recognized as a proteasome substrate (Thrower et al., 2000). Therefore, the activator would have the time required to go from one to four or more ubiquitins in which to drive gene expression, after which it would be destroyed by the proteasome.

It would be the "predictive" proteosome role: when gene expression has to stop, the transcription factor is destroyed. If there is no degradation, the polyubiquitinated form of the activator would simply build up over time, and gene expression would continue unabated. Here, the proteosome would have an inhibitory role in gene expression.

However, recent publication (Lipford et al., 2005) showed that, in Gcn4 dependent genes, proteosome inhibition lead to gene expression inhibition. Proteosome seems to have an activator role, at least for that activating factor. To explain this data, the "black widow" model was proposed.

The "black widow" model suggests that activator polyubiquitination and subsequent destruction is a necessary consequence of "mating" with the polymerase holoenzyme (Muratani and Tansei, 2003). When the activator is polyubiquitinated, it loses activity and, unless cleared by the proteasome, "gums up the works." Why a polyubiquitinated activator would be less active than the monoubiquitinated form is unknown, but the important point is that, rather than being essential for activation per se, proteasome-mediated degradation may be required to eliminate an "exhausted" form of the activator that would otherwise act as a competitive inhibitor of fresh activator. Without proteosome degradation, transcription remains blocked.

It would seem impossible to reconcile all the apparently disparate data described above into a new model to explain the regulation of a transcription activator through its ubiquitination and degradation. The "second-generation timer" model attempts to do it (Kodadek et al., 2006).

It focuses on the monoubiquitinated (and probably phosphorylated) activator as the intermediate and acknowledges the fact that monoubiquitination and subsequent extension into a polyubiquitin chain are two different chemical events. These events can occur at different rates, involving the recently discovered chain extenders (referred to as E4 ligases). Furthermore, deubiquitinases (DUBs) are invoked as a potential mechanism to antagonize chain growth. Finally, it assumes that the polyubiquitinated activator is poorly

active for reasons unknown (Kodadek et al., 2006). The model proposes that, if chain extension is efficient, the active period for the licensed monoubiquitinated activator would be short and proteasome activity would be critical to remove the inactive, polyubiquitinated molecule from the system and open the promoter to "fresh" transcription factor. However, if chain extension were slow due to poor activity of an E4 ligase or potent antagonism of extension by a DUB, the licensed activator could function for a considerable period of time, the activator-promoter complex would be long lived, and proteasome activity would be less important. Given that different E3 and E4 ligases and DUBs presumably work on different transcriptional activators, it seems reasonable to assume that the relative rates of these events can vary widely for different activators (Figure 10).

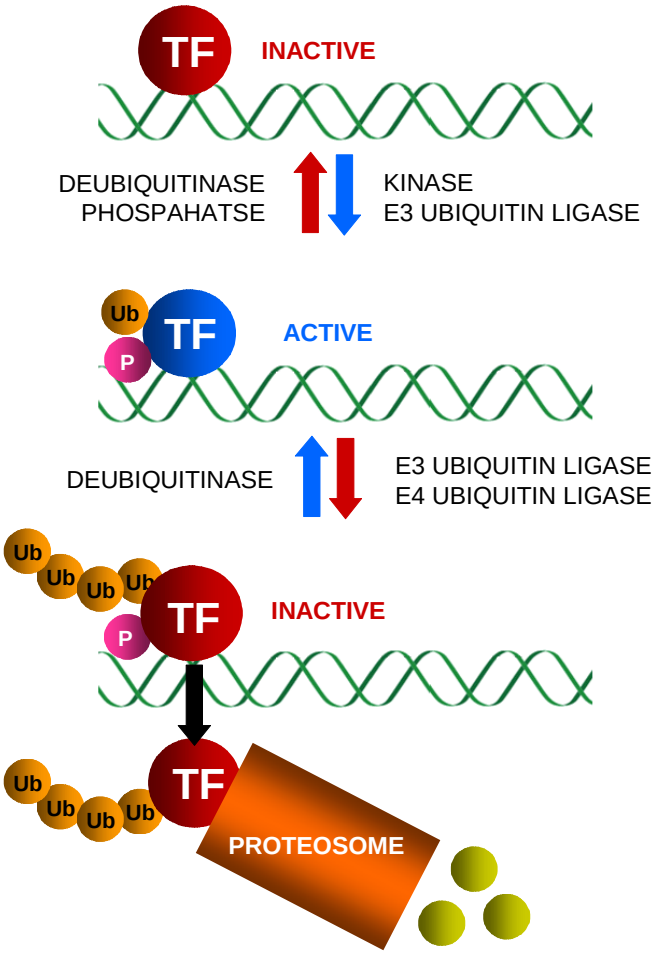

Figure 10. Scheme of the "Second-generation timer" model. Ubiquitination is a dynamic phenomenon, with different rates and proteins involved. If the transcription has to remain active for a longer period of time, the activator must be kept monoubiquitinated.

Msn2 is ubiquitinated by the SCF^{CDC4} (Chi et al, 2001). Interestingly, if Msn2 and Msn4 activity at the promoters was regulated by SCF^{CDC4}/proteosome, mutations in these two complexes should result in an increased Msn2/Msn4 gene expression. This has been addressed in this thesis.

1.7. REGULATION OF LIFESPAN.

Lifespan refers to the typical length of time that any particular organism can be expected to live. In yeast, we can disinguish between two types of lifespan: chronological lifespan and replicative lifespan (Maskell et al., 2003).

Chronological lifespan may be defined as the result of accumulation of irreversible damage to intracellular components during extended stationary phase, compromising cellular integrity and leading to death and autolysis (Maclean et al., 2001). In contrast, replicative lifespan relates to the number of divisions an individual cell has undertaken before entering a non-replicative state termed senescence, leading to cell death and autolysis (Harris et al., 2001).

In the budding yeast *Saccharomyces cerevisiae*, replicative lifespan is measured by the number of divisions that a mother cell undergoes before senescing (Medvedik et al., 2007). During yeast division, homologous recombination between ribosomal DNA (rDNA) repeats produces extrachromosomal rDNA circles, called ERCs (Sinclair et al., 1997). These ERCs are toxic for the cell, through an unknown mechanism. After around 40 divisions, these ERCs reach lethal levels that kill mother cells (Figure 11).

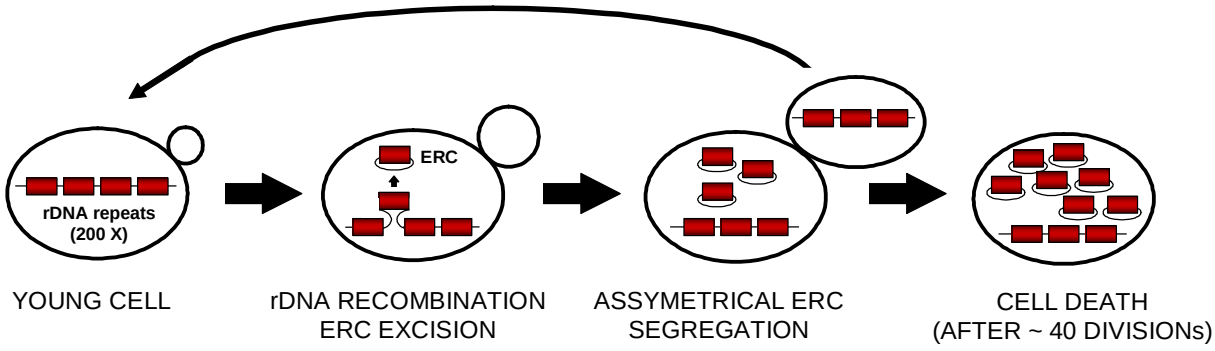

Figure 11. Process of ERC formation during replicative lifespan. In each division, rDNAs recombinate forming extrachromosomal rDNA circles (ERC). ERC accumulation drives to yeast death after 40 divisions.

The aging process induced by ERC production can be delayed. This phenomenon is called lifespan extension (Lin et al., 2000).

1.7.1. Lifespan extension

The restriction of energy intake or calories (called Calorie Restriction or simply CR) has been shown to extend the maximum lifespan of almost every species on which it has been tested, including rats, yeast, fruit flies, and nematodes (Cerami, 1985; Masoro, 1989; Lin et al., 2000; Clancy et al., 2001; Lin et al., 1997). In rodents, a roughly 30% maximum lifespan extension is seen with a roughly 40% restriction of calories from what would be consumed by freely-feeding animals (Masoro, 1989).

It has been discovered that lifespan also can be extended in a variety of species by a direct inhibition of TOR signaling, including *S. cerevisiae, C. elegans,* and *D. melanogaster* (Kaeberlein et al., 2005; Vellai et al., 2003; Kapahi et al., 2004). TOR (target of rapamycin) is a nutrient-responsive phosphatidylinositol-kinase-related kinase that regulates protein synthesis and cell growth, and is inhibited by rapamycin (Wullschleger et al., 2006). The molecular mechanism that extends lifespan extension throguh CR was described few years ago and is the same mechanism for TOR.

1.7.2 Molecular mechanism of lifespan extension: Sir2.

CR has been demonstrated to extend lifespan by boosting the activity of a large family of NAD-dependent deacetylase enzymes, named the sirtuins (Guarente, 2000; Howitz et al., 2003; Wood et al, 2004). These proteins are conserved from prokaryotes to eukaryotes. In yeast, the main member of this family is Sir2 (Kaeberlein et al, 1999; Guarente, 2000).

The main function of Sir2 is to deacetylate histones in an NAD-dependent manner: the loci regulated by Sir2 are hypoacetylated, and deletion of Sir2 results in a dramatic increase in the acetylation of the histones at these sites (Fritze et al., 1997; Guarente, 2000).

Deacetylation of histones is a known mechanism for causing chromatin condensation and transcriptional silencing; silencing gene expression occurs on the telomeres, silent mating-type loci and rDNA (Bitterman et al. 2003). It is

important to distinguish that Sir2 acts in two different complexes depending on the region silenced. On telomeres or silent mating-type loci, Sir2 acts together with Sir3 and Sir4, forming the SIR complex whereas, to silence the rDNA region, Sir2 acts with the RENT complex (REgulator of Nucleolar silencing and Telophase exit) that is composed by Sir2, Cdc14 and Net1 (Figure 12) (Strahl-Bolsinger et al., 1997; Huang et al., 2003).

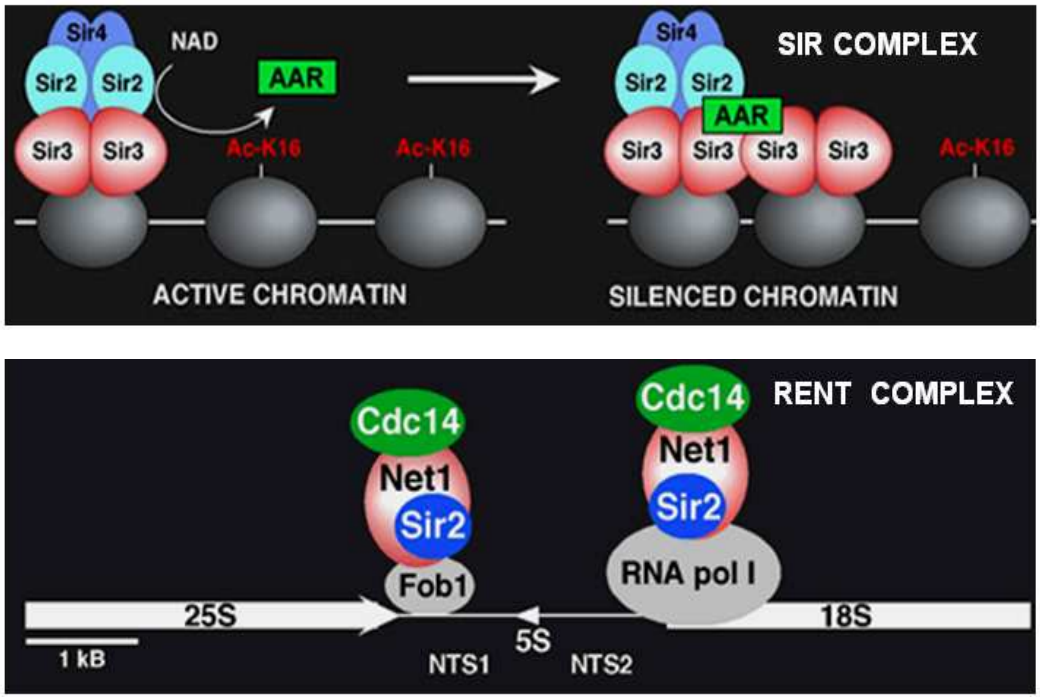

Figure 12. Elements involved on the two Sir2 complexes. SIR complex (Sir2 + Sir3 + Sir4) silences mating loci and telomeres whereas the RENT complex (Sir2 + Cdc14 + Net1) only is able to silence the rDNA region. © Danesh Moazed

The antiaging effect of Sir2 is exerted on the rDNA region (Bitterman et al. 2003). There, the homologous recombination between ribosomal DNA (rDNA) repeats results in the formation of extrachromosomal rDNA circles (ERCs) that accumulate to toxic levels in mother cells. ERC accumulation induces aging in yeast (Sinclair et al., 1997). Sir2 silencing suppresses the formation of this toxic repetitive ribosomal DNA (rDNA) circles.

Because Sir2 protein levels do not increase in response to calorie restriction (Anderson et al., 2002), lifespan extension must be due to an

increase in Sir2 activity. Through upregulation of Sir2 activity, yeast increases rDNA silencing and extends its lifespan.

1.7.3 Sir2 activity regulation: Pnc1.

Two models for how CR stimulates Sir2 activity were proposed: by the increase either of NAD^+ or the NAD^+:NADH ratio (Lin et al., 2000) or the depletion of the nicotinamide (NAM), a product of the Sir2 reaction that inhibits Sir2 *in vitro* and *in vivo* (Landry et al., 2000; Bitterman et al., 2002). Manipulating genetically NAD^+ metabolic pathways, it is possible to increase intracellular NAD^+ levels and affect Sir2 activity (Anderson et al., 2002). However, under aerobic conditions, the steady-state levels of NAD^+ do not fluctuate greatly upon CR (Anderson et al., 2003a). Moreover, NAD^+ fluctuations during CR negatively correlate with Sir2 activity (Anderson et al., 2003a). For this reason, the levels of NAD^+ are unlikely to regulate Sir2 activity.

On the other hand, cells grown in medium containing 5 mM of NAM showed a dramatically reduction in lifespan upon caloric restriction. In addition, when cells grow without glucose, NAM levels decrease and Sir2 is more active (Bitterman et al., 2002). Thus, NAM is the regulator of lifespan extension by Sir2 activity.

The cellular levels of NAM are regulated by a protein called Pnc1, a nicotinamidase that converts nicotinamide to nicotinic acid in the NAD^+ pathway (Anderson et al., 2003b; Gallo et a., 2004). *PNC1* expression is regulated by environmental stimuli that extend lifespan, such as heat, osmotic stress, low amino acids, and CR (Medvedik et al., 2007). The *pnc1*Δ strain did not exhibit an extension of lifespan under either of these conditions, demonstrating that Pnc1 is necessary for lifespan extension by calorie restriction and on response to low-intensity stress (Anderson et al., 2003b; Gallo et a., 2004; Medvedik et al., 2007). Under non-stressing conditions (2% glucose, 30ºC), a strain with additional copies of *PNC1* lived 70% longer than the wild-type and some cells lived for more than 70 divisions, which is the longest reported lifespan extension in this organism (Anderson et al., 2003b).

Therefore, Sir2 activity regulation is likely to go through *PNC1* expression (Figure 13). A polemic point was that, in absence of Sir2, some yeast strains were able to continue extending lifespan under CR (Kaeberlein et al., 2004). Thus, it could exist another mechanism in addition to Sir2. It has been discovered that another NAD-dependent deacetilase, called Hst2, is able to make the same function as Sir2 (Lamming et al., 2005). Thus, when Sir2 is deleted, depending on the strain, Hst2 replaces Sir2.

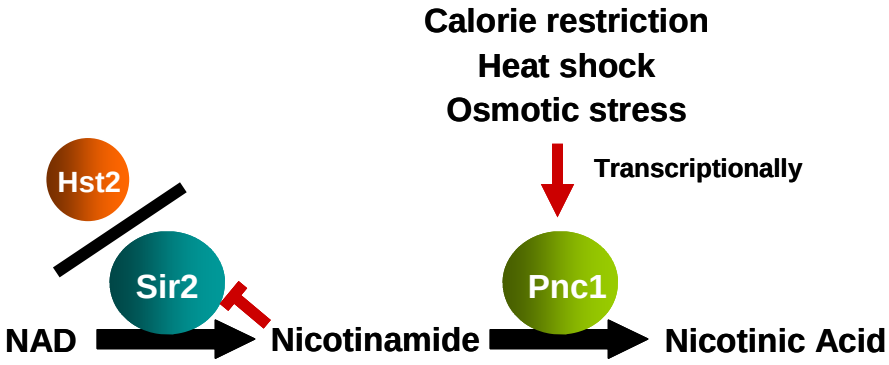

Figure 13. Schematic diagram of the regulation of Sir2 activity. Sir2 activity is inhibited by nicotinamide. To increase Sir2 activity, cell expresses *PNC1* which degrades nicotinamide.

1.7.4 Lifespan extension, Pnc1 and Msn2/Msn4: a tentative model.

It has been published recently that lifespan extension by CR is completely *MSN2/MSN4*-dependent (Medvedik et al., 2007). Sites for the stress-responsive zinc-finger transcription factors Msn2 and Msn4 have been identified in the *PNC1* promoter (Ghislan et al., 2002). In addition, Msn2/Msn4 relocalize from the cytoplasm to the nucleus during CR (Medvedik et al., 2007). *PNC1* expression is completely dependent on Msn2/Msn4 regulation in all the stresses described that induce *PNC1,* such as CR, aminoacid starvation, and osmotic stress (Medvedik et al., 2007).

Therefore, a tentative model for lifespan extension is that CR induces *PNC1* through Msn2/Msn4 activation. Msn2/Msn4 activation by CR is mediated through inhibition of Tor1 and PKA pathways, that leads to Msn2/Msn4 nuclear

localization. Then, Pnc1 promotes NAM degradation and enhances Sir2 activity, increasing rDNA silencing. The increased rDNA silencing diminishes ERC formation and increases yeast longevity (Figure 14).

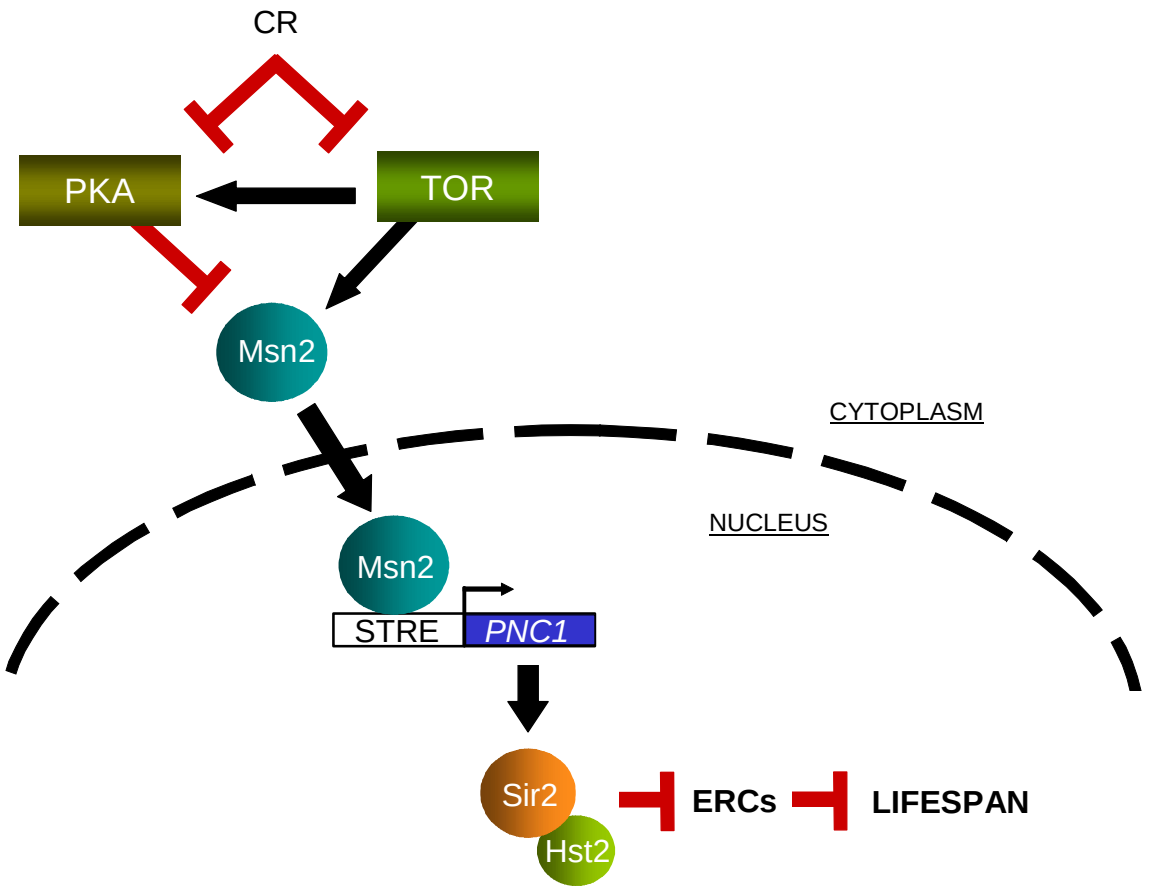

Figure 14. Schematic diagram for the Msn2/Msn4-mediated lifespan extension. Under caloric restriction, PKA or Tor pathways are inhibited and Msn2/Msn4 localize into the nucleus. There, Msn2/Msn4 induce *PNC1*. Pnc1 degradates nicotinamide, enhancing Sir2 (and Hst2) activity which leads to rDNA silencing and extended lifespan.

1.8. APOPTOSIS

1.8.1 Apoptosis in higher eukaryotes

Apoptosis is a form of programmed cell death (PCD) used by multicellular organisms to eliminate excess, damaged or harmful cells. This process of cell suicide is crucial for developmental morphogenesis, tissue homeostasis and defense against pathogens (Jacobson et al., 1997; Vaux et al., 1999).

Apoptosis involves a series of biochemical events leading to a characteristic cell morphology and death. In more specific terms, apoptotic events lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Kerr et al., 1972).

In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis, in general, confers advantages during an organism's life cycle.

1.8.1.1. Apoptotic mechanisms.

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (*extrinsic inducers*) or intracellularly (*intrinsic inducers*).

Extracellular signals may include hormones, growth factors, nitric oxide or cytokines, and therefore must either cross the plasma membrane or transduce to effect a response. However, the main extracellular activators are FAS and TNFα. They bind to their receptors, transmit the signal to the cytoplasm and activate the apoptotic cascade (Schmitz et al., 2000).

Intracellular apoptotic signalling is a response initiated by a cell in response to stress, and may ultimately result in cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, and hypoxia are all factors that can lead to the release of intracellular apoptotic signals by a damaged cell (Danial et al., 2004).

The intracellular apoptotic signals are always acting directly over the mitochondria. From there, apoptotic signals are released. On the other hand, extracellular signals can activate apoptosis both dependently (as an intracellular effector) or independently from the mitochondria.

When apoptosis is caused through the mitochondria, mitochondria are affected in different ways: forming membrane pores or increasing the permeability of the mitochondrial membrane, allowing apoptotic effectors to leak out (Dejean, L.M et al., 2006).

There are three main pro-apoptotic effectors released from mitochondria into the cytosol: Cytochrome C, SMACs and AIFs. Cytochrome C is released from mitochondria due to formation of a channel, called MAC, in the outer

mitochondrial membrane . Once cytochrome c is released binds with Apaf-1, ATP and pro-caspase-9 to create a protein complex known as apoptosome. The apoptosome activates the apoptotic effectors caspase-3, 6 and 9. These caspases are cysteine proteases which carry out the degradation of many proteins in the cell (Dejean, L.M et al., 2006).

MAC is subject to regulation by various proteins, such as those encoded by the mammalian Bcl-2 family of anti-apoptopic genes. Bcl-2 family proteins are able to promote or inhibit apoptosis by direct action on MAC. Concretely, Bcl-2, Bcl-XL, Bcl-w and Mcl-1 inhibit MAC formation whereas Bax, Bak and Bok promote MAC formation (Dejean, L.M et al., 2006).

SMACs (second mitochondria-derived activator of caspases) bind to inhibitor of apoptosis proteins (IAPs) and deactivate them, preventing the IAPs from arresting the apoptotic process and therefore allowing apoptosis to proceed. IAP also normally suppresses the activity of caspases. Therefore, the caspase activivity is activated by both citochrome C and SMACs (Fesik et al., 2001).

Finally, AIF (Apoptosis-inducing factor) is the last pro-apoptotic factor released from the mitochondria. It is a protein that is normally located in the intermembrane space of mitochondria. When AIF is released, migrates into the nucleus, binds to DNA, and triggers the destruction of the DNA and cell death (Candé et al., 2002).

1.8.1.2. Apoptosis execution: hallmarks and phases

Although many pathways and signals lead to apoptosis, there is only one mechanism that actually causes the death of the cell in this process: after the appropriate stimulus has been received by the cell, the cell will undergo the organized degradation of cellular organelles by activated proteolytic caspases (Fesik et al., 2001).

A cell undergoing apoptosis shows a characteristic morphology that can be observed with a microscope. First, there is cell shrinkage and rounding due to the breakdown of the proteinaceous cytoskeleton by caspases. The cytoplasm appears dense, the organelles appear tightly packed, and the cell membrane shows irregular buds known as blebs.

Chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis. In

addition, the nuclear envelope becomes discontinuous and the DNA inside it is fragmented in a process referred to as karyorrhexis. The nucleus breaks into several discrete chromatin bodies or nucleosomal units due to the degradation of DNA. Finally, cell breaks apart into several vesicles called apoptotic bodies, which are then phagocytosed (Hacker et al., 2000).

1.8.1.3. ROS and apoptosis.

Reactive oxygen species (ROS) include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons.

The main ROS productor mechanism in cell is electron transport chain and oxidative phosphorylation, the mitochondrial processes by which ATP is produced from acetyl-CoA. This process involves the transport of protons across the inner mitochondrial membrane by means of the electron transport chain. In the electron transport chain, electrons are transferred through a series of proteins via oxidation-reduction reactions to an oxygen molecule to produce water (Bouveris and Cadenas, 1982). However, in about 0.1-2% of the transferred electrons within the chain, especially in the Ubiquinone complex, oxygen is prematurely and incompletely reduced to give the superoxide radical ($\cdot O_2^-$). This superoxide produces hidroxil radical (·OH), the most dangerous ROS (Cadenas and Davies, 2000).

ROS levels are normally low, product of the normal metabolism of oxygen and have important roles in cell signaling. However, during times of environmental stress ROS levels can increase dramatically. This seems to be because an uncoupling of the respiratory chain. It makes that electrons remain longer in the ubiquinone complex, and, superoxide rate production increase (Cadenas and Davies, 2000).

Superoxides, and especially hidroxil radicals, initiate lipid peroxidation with the result that the fatty acid side chains of membrane lipids are altered and results to membrane damage (Halliwell and Gutteridge, 1984).

Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases and catalases. But in stress situations, if too much damage is accumulated to the mitochondrial

membrane, cell undergoes apoptosis or programmed cell death due to a proapoptotic proteins release (Huang et al., 2000).

1.8.2 Apoptosis in yeast

There are recent studies that support that apoptosis might also occur in unicellular organisms. In the yeast *Saccharomyces cerevisiae*, it has been detected cell death with typical markers of apoptosis such as DNA fragmentation and chromatin condensation (Madeo et al., 1997). Apoptosis has been observed in aged cells (Laun et al., 2001; Herker et al., 2004), after heterologous expression of human pro-apoptotic genes, such as Bax (Ligr et al., 1998), after mild treatment with stress agents, after exposure to low doses of hydrogen peroxide (Madeo et al.,1999), acetic acid (Ludovico et al., 2001), or α–mating-type pheromone (Severin and Hyman, 2002). In many cases, affected cells produce reactive oxygen species (ROS), which are responsible for apoptotic cell death. Exposure to low doses of H_2O_2 or accumulation of ROS by depletion of glutathione induces apoptosis in wild-type yeast cells, indicating that, as in metazoans, ROS are a key regulator of yeast apoptosis (Madeo et al., 1999).

In yeast, there are proteins relative of caspases called metacaspases and paracaspases (Uren et al., 2000). Their homology to human caspases is not restricted to the primary sequence, but extends to the secondary structure as well. Metacaspases encompass a member in *S. cerevisiae*, Yor197w, also called *MCA1,* which is cleaved and displays a caspase-like proteolytic activity which is activated during the apoptotic process. Disruption of Yor197w abrogates hydrogen peroxide-induced apoptosis, whereas overexpression of Yor197w increases hydrogen peroxide-induced caspase-like activity and apoptosis.

Recently, other orthologs of key regulators such as the apoptosis inducing factor, Aif1, (Wissing et al., 2004) have been observed in yeast. In addition, evidence has been provided for Cytochrome C associated mitochondrial involvement in yeast apoptosis. Ludovico et al. (2002) observed in yeast cells undergoing apoptosis induced by acetic acid, cytochrome c release and mitochondrial dysfunction.

1.8.3 Apoptosis and high osmolarity in yeast.

In mammalian cells, hyperosmotic stress induces apoptosis and is involved in several pathological states such as ischaemia, septic shock and diabetic coma (Wright and Rees, 1998; Galvez et al., 2001). Moreover, during hyperosmotic shock-induced cell death, several proteins are cleaved and activated via a caspase-dependent mechanism which suggests the involvement of ROS production in the induction of this process (Chan et al., 1999)

In *S. cerevisiae*, cell death induced by hyperosmotic stress is not a lytic process and is temperature, aeration and growth phase dependent, indicating that cells death maybe linked to a metabolic process. It seems an apoptotic process as results in chromatin condensation, DNA strand breaks, ROS production and preservation of plasma membrane integrity. In addition, deletion of *MCA1* reduced cell death in response to hyperosmotic stress (Ribeiro et al., 2006).

Although metacaspase Mca1 and Cytochrome C are key factors in the yeast apoptotic process, it should be stressed that their absence only reduces but not abolishes the apoptotic death indicating the existence of alternative pathways.

OBJECTIVES

"Combatrem als mars i oceans, a les platges , als camps i als carrerss ; defensarem la nosrta illa a qualsevol preu, ; mai no ens rendirem »

Winston Churchill

Research in our group is focused on understanding the mechanisms by which the stress-activated kinase Hog1 regulates protective responses to osmostress.

A sustained activation of Hog1 is detrimental for cell growth. However, the molecular mechanisms of this cell growth inhibition remained unknown. We observed that mutants in the SCF^{CDC4} complex were able to survive under continuous Hog1 activation. Therefore, we aimed to investigate the mechanisms by which SCF^{CDC4} mutation prevents the inhibition of cell growth induced by Hog1.

The specific objectives of this PhD project were as follows:

(1) To characterize the molecular mechanism by which SCF^{CDC4} prevents inhibition of cell growth under sustained Hog1 activation.
(2) To identify the molecular basis by which Hog1 inhibits cell growth.

MATERIALS AND METHODS

"Només l'amo de la casa sap per on goteja la teulada »

Proverbi del Txad

MATERIALS.

TABLE 1. Yeast strains. YAL strains have been generated during this work.

Strain	Genotype	Source
W303-1a	MATa *ade2—1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1 -100*	Standard wild-type strain
CMY1035	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc4-1*	A gift from Mike Tyers.
KY441	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc34-2*	A gift from Mike Tyers.
KY442	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc53-1*	A gift from Mike Tyers.
KY553	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 skp1-11*	A gift from Mike Tyers.
YPC24O	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 sln1*::*URA* p*GAL1-PTP2*	This lab.
YPC242	*MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc4-1 sln1*::*URA* p*GAL1-PTP2*	This lab.
YAL62	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 msn2*::NAT *msn4*::*KANMX4*	This study.
YAL63	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc4-1 msn2*::*NAT msn4*::*KANMX4*	This study.
YAL84	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 pnc1*::*KANMX4*	This study.

Strain	Genotype	Source
YAL86	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc4-1 pnc1*::*KANMX4*	This study.
YAL80	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 sir2*::*KANMX4*	This study.
YAL81	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc4-1 sir2*::*KANMX4*	This study.
YAL68	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 hda2*::*KANMX4*	This study.
YAL69	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc4-1 hda2*::*KANMX4*	This study.
YAL70	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 hst1*::*KANMX4*	This study.
YAL71	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc4-1 hst1*::*KANMX4*	This study.
YAL82	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 sir4*::*KANMX4*	This study.
YAL83	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cdc4-1 sir4*::*KANMX4*	This study.
YAL51	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 hog1-as*	This study.

TABLE 2. Plasmid DNAs.

Construction	Description	Source
pYES-*PBS2*^{DD}	*GAL1* promoter - *PBS2*^{DD} in pYES2 (URA3)	A gift from Haruo Saito.
p*GAL1-PTP2*	*GAL1* promoter - *PTP2* in pCH1122 (URA3)	A gift from Haruo Saito.
Hog1-GFP	*HOG1* promoter - *HOG1* - *GFP* in pRS416 (URA3)	This lab.
pY*MSN2*-HA	*MSN2* promoter - *MSN2* – *3xHA* in YCPlac111	A gift from Christoph Schüller.
p*ADH1MSN2*-HA	*ADH1* promoter - *MSN2* – *3xHA* in YCPlac111	A gift from Christoph Schüller.

TABLE 3. Oligonucleotides.

Oligonucleotides	Sequence	Used for
CTT1-7	GCCGATCCAGAAGGGTTATT	ChIP analysis (*CTT1* promoter)
CTT1-8	TGAGAAGCGTATGGGTGATG	ChIP analysis (*CTT1* promoter)
GRE2-1	ATTGGCCCTCACCTCTTTTG	ChIP analysis (*GRE2* promoter)
GRE2-2	CCCGTTAGCACCTGAAACGA	ChIP analysis (*GRE2* promoter)

Oligonucleotides	Sequence	Used for
TEL-2a	AGTGCAAGCGTAACAAAGCC	ChIP analysis (Telomeric region)
TEL-2b	GCCTCACTGGTTTTTACCCT	ChIP analysis (Telomeric region)
5CTT1	ATACTCAAGACCAGACGGCC	Northern blot analysis (*CTT1* ORF)
3CTT1	TTAATTGGCACTTGCAATGG	Northern blot analysis (*CTT1* ORF)
5ALD2	ATGCCTACCTTGTATACTG	Northern blot analysis (*ALD2* ORF)
3ALD2	TTAGTTGTCCAAAGAGAG	Northern blot analysis (*ALD2* ORF)
5GRE2	ATGTCAGTTTTCGTTTCAGGTG	Northern blot analysis (*GRE2* ORF)
3GRE2	TGCCCTCAAATTTTAAAATTTG	Northern blot analysis (*GRE2* ORF)
5ACT1	ATGGATTCTGGTATGTTCTAG	Northern blot analysis (*ACT1* ORF)
3ACT1	TTAGAAACACTTGTGGTGAAC	Northern blot analysis (*ACT1* ORF)

METHODS.

Growth in solid media.

Yeast cultures were grown to early log phase (OD_{660} = 0.6-1.0). Then 5 μl of culture diluted at OD_{660} = 0.05 were spotted onto plates containing specific media. Cell growth was scored after 3 days at 25°C.

Propidium iodide cell permeability assay

Yeast cultures were grown to early log phase (OD_{660} = 0.6-1.0) in minimal medium containing raffinose. Then, cells were washed with minimal medium and diluted to OD_{660} = 0.3 in minimal medium containing galactose. 300 μl of yeast culture (OD_{660} = 0.3-0.8) were added to the same volume of a solution of PI (4 μg/ml propidium iodide in 50 mM sodium citrate) at indicated times and incubated in the dark for 15 min at 4°C. Samples were diluted 10 times in 50 mM sodium citrate and PI fluorescence was measured in a FACScan flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ) in the FL3 channel. Each analysis was done on 10000 cells. Every 4 hours the cultures were diluted again to a OD_{660} = 0.3 in minimal medium containing galactose.

Immunoblot analysis of Hog1 and phosphorylated Hog1.

Yeast cultures were grown to early log phase (OD_{660}= 0.6-1.0). When necessary, cells were either subjected to stress (0.4 M NaCl, indicated times) or untreated. 1 ml of culture per sample was collected by 1 min of centrifugation at 13200 rpm. Supernatant was removed. Samples were resuspended in 100 μl of SDS loading buffer, immediately boiled for 4 min, and resolved by SDS-10% polyacrylamide gel electrophoresis. Threonine-Tyrosine-phosphorylated Hog1 was detected with Phospho-p38 MAPK (Thr180/Tyr182) rabbit monoclonal antibody (Cell Signalling Technology Inc, Danvers, MA) at a dilution of 1:5000. Total Hog1 was detected with Hog1 goat polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at a dilution of 1:10000. Immunoblots were developed by using horseradish peroxidase-conjugated secondary antibodies against rabbit (Amersham, GE Healthcare Life Sciences, Piscataway, NJ) or goat (Santa Cruz Biotechnology Inc, Santa Cruz, CA) both at a dilution of 1:25000

and an Amersham ECL kit (GE Healthcare Life Sciences, Piscataway, NJ). Probes were removed from membranes by incubation at 65°C for 30 min in stripping buffer (60 mM Tris-HCl pH = 6.8, SDS 2%, 100 mM β-mercaptoetanol) prior to incubation of the same membrane with another antibody.

Fluorescence microscopy.

Yeast cultures were grown to early log phase (OD_{660}=0.6-1.0). When necessary, cells were either subjected to stress (0.4 M NaCl, indicated times) or untreated. GFP was detected in unfixed cells with a Nikon (Garden City, NY) Eclipse E600 epifluorescence microscope equipped with Hamamatsu C4742-98 camera (Hamamatsu Photonics, Shizuoka, Japan). Images were taken at 100x magnification and converted to Photoshop version 7.0 format (Adobe Systems, Mountain View, CA).

Growth curves.

Yeast cultures were inoculated in 96-we|l plates at OD=0.001 in YPD medium (control cells) and YPD + indicated concentrations of NaCl. Cell growth was scored after 16 h at 25°C by measuring OD_{660} with a Microplate reader Model 680 (Bio-Rad, Hercules, CA).

Northern blot analysis.

Yeast cultures were grown to early log phase (OD_{660} = 0.6-1.0). When necessary, cells were either subjected to stress (0.4 M NaCl, indicated times) or untreated. 15 ml of culture per sample was collected by 3 min of centrifugation at 13200 rpm. Supernatant was removed. Samples were resuspended in 400 μl of AE buffer (50 mM sodium acetate pH = 5.3, 10 mM EDTA pH = 8.0) plus 40 μl of SDS 10% and 300 μl of phenol solution (SIGMA-Aldrich, St. Louis, MO). Inmediately, samples were heated for 5 minutes at 65°C. Then, samples were put on ice for 1 min, and 300 μl of chloroform were added. After 2 min. of centrifugation at 13200 rpm, the aquose phase was recovered. Then, 400 μl of chloroform were added to the aquose phase. After 2 min. of centrifugation at 13200 rpm, the aquose phase was recovered again. Then, 40 μl of sodium acetate 3 M and 1 ml of ethanol were added to the aquose phase. The RNA was

extracted by precipitation for 2 h at -20°C and a centrifugation for 20 min at 13200 rpm. The pellet was washed with ethanol 70%, and centrifuged 5 min at 13200 rpm, and resuspended in 50 µl of H_20 DEPC. 20 µg of total RNA per sample were run in an 1% agarose gel by electrophoresis. RNAs were transferred to a nylon membrane (Roche, Basel, Switzerland) by a Vacuum blotter model 785 (Bio-Rad, Hercules, CA). Total RNA and expression of specific genes were probed by using radiolabeled PCR fragments containing the entire ORF of *CTT1* (1.7 kbp), *ALD2* (1.5 kbp), *GRE2* (1.1 kpb) and *ACT1* (1.4 kbp). Signals were quantified by using a storage phosphor screen, a Typhoon 8600 phosphorimager and the ImageQuant software (Amersham, GE Healthcare Life Sciences, Piscataway, NJ).

Chromatin immunoprecipitation.

Yeast cultures were grown to early log phase (OD_{660}=0.5-0.8) before aliquots of the culture were exposed to osmotic stress treatment (0.4 MNaCl) for the time specified in the figure legends. To crosslink, yeast cells were treated with 1% formaldehyde for 20 min at room temperature. Reaction was quenched by the addition of 125 mM glycine for 15 min at room temperature, and cells were washed with TBS 1X (20 mM Tris-HCl pH 7.5 and 150 mM NaCl) and frozen at -20ºC. Then, pellets were resuspended in 300 µl of lysis buffer (50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1mM PMSF, 10 µg/ml aprotinin, 1µg/ml leupeptin, 1µg/ml pepstatin and 1 mM benzamidine) and 300 µl of Ø=0.40-0.60 mm glass beads (Sartorius AG, Goettingen, Germany) were added. Cells were broken by vortexing for 13 min at 4ºC in a Vortex-GENIE2 (Scientific Industries, Bohemia, NY). Extracts were transferred to a new 1.5 ml microcentrifugue tube, sonicated on ice with a Branson Digital Sonifier Model S-250D (Branson Ultrasonic Corporation, Danbury, CT) and harvested by spinning at 3000 rpm for 2 min at 4ºC. At this time, 10 µl of extracts were taken as whole cell extract (WCE) samples. 50 µl of DynaBeads Pan mouse IgG (Invitrogen Dynal AS, Oslo, Norway), previously blocked with PBS/BSA 5mg/ml and incubated with 4 µg of anti-HA antibody overnight at 4ºC, were added to samples. Then, samples were incubated for 2 h while rotating at 4ºC, and washed with lysis buffer (twice), lysis buffer plus 360 mM NaCl (twice), washing buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deocycholate and 1 mM EDTA) (twice), and

once with TE 1X (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). Samples were eluted with elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% SDS) and crosslinkings were reversed by placing eppendorfs at 65ºC overnight. After that, proteins were digested with Proteinase K (15 μl of 7 mg/ml) and Glycogen (6 μl of 20 mg/ml) for 2 h, and eliminated using phenol:chloroform extraction. DNA was precipitated with 4 μl NaCl 5M/100 μl sample plus one volume of isopropanol for 1 h at -20ºC, harvested and resuspended in TE 1X. Quantitative PCR analysis of stress genes and constitutively expressed genes with the primers showed above (see table 3).

Immunoblot analysis of Msn2 degradation and Pnc1 accumulation.

Yeast cultures were grown to early log phase (OD_{660}= 0.6-1.0). When necessary, cells were either subjected to stress (0.4 M NaCl, indicated times) or untreated. 1 ml of culture per sample was collected and mixed with 300 μl of TCA 85%. Cells were collected by 1 min of centrifugation at 13200 rpm. and supernatant was removed. Then, cells were resuspended in 100 μl of TCA loading buffer (20% SB 5X, 20% Tris 1.5 M pH = 8.8) and 300 μl of glass beads were added. Cells were broken by 30 s at potency = 5 in the FastPrep-24 (MP Biomedicals Inc, lrvine, CA). Samples were boiled 10 minutes at 100°C, and 10 μl of supernatant were run into SDS-10% polyacrylamide gel. Msn2-HA was detected with anti-HA mouse monoclonal antibody (12CA5). Pnc1 was detected with anti-Pnc1 rabbit polyclonal antibody (gift of Dr. D. Sinclair, Boston, MA) at a dilution of 1:5000. Irnmunoblots were developed as described above, as well as the probes removal.

Determination of SubG1 population by FACS.

Yeast cultures were grown to early log phase (OD_{660} = 0.6-1.0) in minimal medium containing raffinose. Then, cells were washed with minimal medium and diluted to OD_{660} = 0.3 in minimal medium containing galactose. 300 μl of yeast culture (OD_{660} = 0.3-0.8) were fixed with 1 ml of 70% etanol for 1 hour. Then, cells were centrifuged, supernatant was removed and cells were incubated in 1 ml of RNAase solution (0.1 mg/ml RNAase in 50 mM sodium citrate) at 37°C for 16 hours. Then, the same volume of a PI solution (4 μg/ml propidium iodide in 50 mM sodium citrate) was added and cells were incubated in the dark for 15

min at 4ºC. Samples were diluted 10 times in 50 mM so dium citrate and PI fluorescence was measured in a FACScan flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ) in the FL3 channel. Each analysis was done on 10000 cells. Every 4 hours the cultures were diluted again to a OD_{660} = 0.3 in minimal medium containing galactose.

TUNEL assay adapted to flow cytometry.

In the standard protocol developed by Roche (In Situ Cell Death Detection Kit, Roche, Basel, Switzerland), the cells are first fixed with paraformaldehyde and then permeabilised with a TritonX-100 solution before incubation with terminal transferase and fluorescein-dUTP. Carré et al. observed that these steps could be skipped and that incubation with 70% ethanol was sufficient to ensure cell membrane permeability to both enzyme and substrate. They set up a shorter protocol described below that ensures a maximal labelling of fragmented DNA (see below).

Yeast cultures were grown to early log phase (OD_{660} = 0.6-1.0) in minimal medium containing raffinose. Then, cells were washed with minimal medium and diluted to OD_{660} = 0.3 in minimal medium containing galactose. 2 ml of yeast culture (OD_{660} = 0.3-0.8) were centrifuged (1000 g, 2 min), and resuspended into 2 ml of Tris-HCl solution (0,02 M Tris + 0,01 M $CaCl_2$; pH=7.4). This step was repeated three times. Then, cells were resuspended and fixed with 1 ml of 70% etanol for 1 hour. Cells were centrifuged (1000 g, 2 min) and resuspended into 1 ml PBS solution (0,002 M NaH_2PO_4 + 0,02 M Na_2HPO_4 + 0,154 M NaCl; pH adjusted to 7.4 by HCl). This step was repeated five times. Finally, the pellet was resuspended into 50 µl of TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche, Basel, Switzerland) and incubated for 16 h at 37°C. Samples were diluted 10 times in 50 mM sodium citrate and TUNEL fluorescence was measured in a FACScan flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ) in the FL1 channel. Each analysis was done on 10000 cells.

Tetrazolium overlay assay.

Yeast cultures were grown to early log phase (OD_{660} = 0.6-1.0). Then 5 µl of culture were spotted onto plates containing specific media. After 16 h. of

incubation at 25°C, an overlay was added to the plates. The composition of the overlay medium was 1% glucose and 1.5% agar in 0.02 M phosphate buffer pH=7, and 0.01% 2,3,5-triphenyl tetrazolium chloride solution (SIGMA-Aldrich, St. Louis, MO). After cells were embedded with the overlay solution, plates were incubated for 16 h at 25°C in the dark.

RESULTS

"L'ànima de l'home es forja a l'adversitat"

Lucí Anneu Sèneca

4.1 Inhibition of cell growth by sustained Hog1 activation is prevented in SCF^{CDC4} mutants.

The Hog1 pathway can be sustainedly activated by mutations that inactivate the Sln1 osmosensor, by expression of constitutively active MAP3K mutants or a constitutively active MAPKK mutant (*PBS2^{DD}*) (Maeda et al., 1995; Posas et al., 1997).

The sustained Hog1 activation is detrimental to cell growth (Maeda et al., 1994). For instance, cells with the deletion of *SLN1* are not able to grow. Inhibition of cell growth by sustained Hog1 activation is also observed when cells carrying a hyperactive allele of the PBS2 MAPKK (*PBS2^{DD}*) under the control of the *GAL1* promoter are grown in the presence of galactose.

We took advantage of the systems described above to induce a sustained Hog1 activation to analyze inhibition of cell growth by Hog1. *sln1*Δ cells carrying a plasmid with the phosphatase *PTP2* under the control of the *GAL1* promoter were grown on glucose or galactose plates at 25ºC for 3 days. As we expected, *sln1*Δ only grew on galactose, where *PTP2* overexpression prevented Hog1 activation (Figure 1b). On the other hand, cells carrying a plasmid with *PBS2^{DD}* under the control of the *GAL1* promoter were grown on glucose or galactose plates at 25ºC for 3 days. As we expected, cells grown on galactose, where *PBS2^{DD}* was expressed, were not able to grow (Figure 1a).

Inhibition of cell growth caused by inactivation of Sln1 can be prevented by mutations on downstream components of the Hog1 pathway (i.e. *SSK1, SSK2, PBS2* and *HOG1*) (Wurgler-Murphy et al., 1997). However, mutations downstream of Hog1 that could suppress inhibition of cell growth due to sustained activation of Hog1 have not been identified. Only mutations at *RCK2* or *HOT1* have some residual preventive effect (Teige et al., 2001).

While carrying out studies on cell cycle control by the Hog1 MAPK, we realized that mutations on *CDC4*, the E3 ligase of the SCF^{CDC4} complex (see introduction), suppressed the growth defect caused by *PBS2^{DD}* overexpression. Thus, we tested whether mutations on the different components of the SCF complex prevented inhibition of cell growth upon sustained Hog1 activation. For this, thermosensitive mutants *cdc34-2*, *skp1-11*, *cdc53-1* and *cdc4-1* carrying a plasmid with *PBS2^{DD}* under the control of the *GAL1* promoter were grown on glucose or galactose plates at 25ºC for 3 days. As we expected, wild-type cells carrying *PBS2^{DD}* were not able to grow onto galactose, whereas the several

mutants in SCF displayed an increase in cell survival (Figure 1a).

The preventive effect of *CDC4* mutant was verified achieving sustained Hog1 activation by *sln1*Δ. *sln1*Δ p*GAL1-PTP2* cells carrying or not a mutation in *CDC4* were grown on glucose or galactose plates at 25ºC for 3 days. As shown in Figure 1b, *sln1*Δ cell growth was highly compromised on glucose plates. However, *sln1*Δ *cdc4-1* strain grew on glucose almost to the same extent as on galactose (Figure 1b). Taken together, $PBS2^{DD}$ and *sln1*Δ results clearly support that SCF^{CDC4} mutations prevent inhibition of cell growth by sustained Hog1 activation.

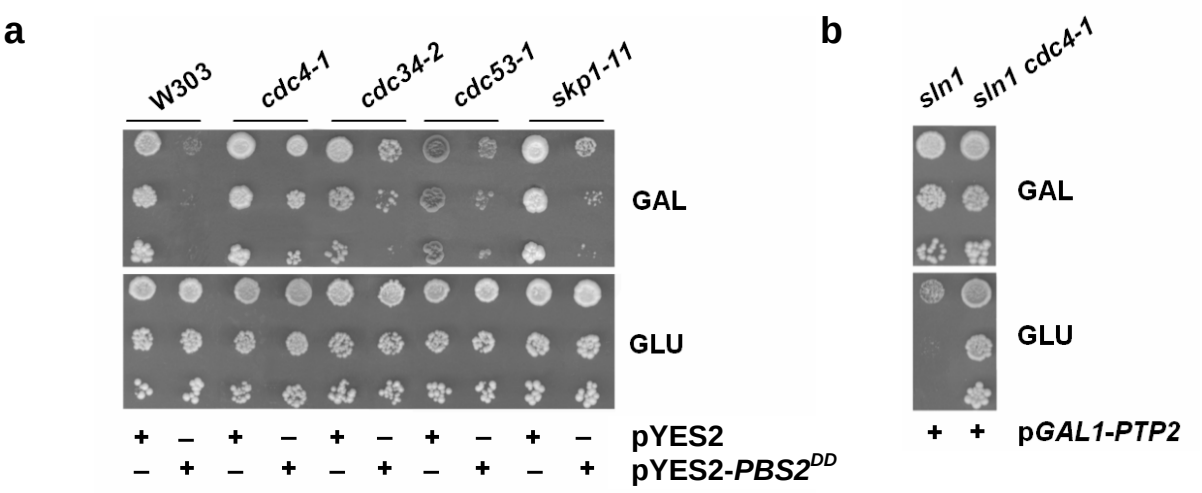

Figure 1. Inhibition of cell growth by sustained Hog1 activation is prevented by SCF^{CDC4} and thermosensitive mutants.

(a) Mutations on SCF^{CDC4} complex prevent inhibition of cell growth due to $PBS2^{DD}$ expression. The wild-type (W303) and indicated mutant strains carrying pYES or pYES-$PBS2^{DD}$ were spotted on plates with glucose or galactose. Growth was scored after 3 days at 25ºC.

(b) Mutation on *cdc4-1* prevents inhibition of cell growth due to *sln1*Δ. The *sln1*Δ and *sln1*Δ *cdc4-1* strains carrying p*GAL1-PTP2* were spotted on plates with glucose or galactose. Growth was scored after 3 days at 25ºC.

4.2. SCF^{CDC4} mutation prevents inhibition of cell growth by sustained Hog1 due to a decrease in cell death.

A sustained Hog1 activation leads to an inhibition of cell growth that classically has been related to a lethal effect of Hog1 activation (Maeda et al., 1994). However, no data about whether sustained Hog1 activation inhibits cell growth by inducing cell death has been reported.

To test whether inhibition of cell growth caused by sustained Hog1 was caused by an increase in cell death, we performed viability assays using FACS analysis of propidium iodide (PI) cell permeability. As shown in Figure 2, wild-type cells carrying a plasmid expressing *PBS2^{DD}* under the control of the *GAL1* promoter displayed a 45% of dead cells after 24 h of growth in galactose, whereas wild-type cells carrying an empty vector displayed only 1% of dead cells after 24 h of growth in galactose. Therefore, sustained Hog1 activation increases cell death. We also performed viability assays using FACS analysis of propidium iodide (PI) cell permeability in cdc4-1 mutant cells. As shown in Figure 2a, *cdc4-1* cells carrying a plasmid expressing *PBS2^{DD}* under the control of the *GAL1* promoter displayed 30% of dead cells after 24 h of growth in galactose. Thus, SCF^{CDC4} mutation reduces cell death by sustained Hog1 activation.

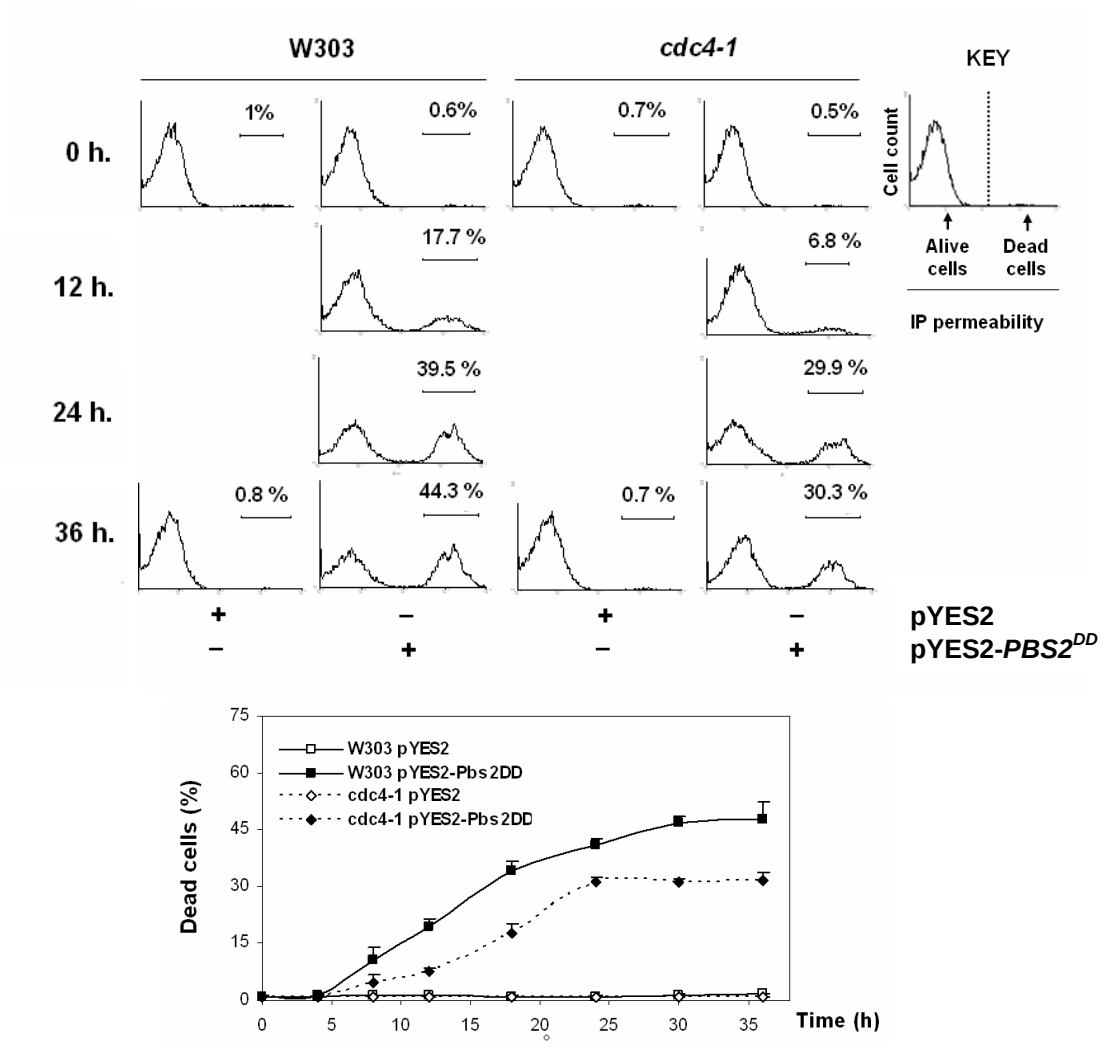

Figure 2. Sustained Hog1 activation increases cell death. Wild-type and indicated mutant strains were grown in liquid media with galactose as carbon source. Propidium iodide (PI) cell permeability assay was measured by FACS at the indicated times.

Taken together, our data support that inhibition of cell growth by sustained Hog1 activation is caused by an increase in mortality rate and that SCF^{CDC4} mutant preventive is explained by a decrease in mortality rate caused by sustained Hog1 activation.

4.3. SCF^{CDC4} mutants show an improved cell survival to osmostress.

The preventive effect of SCF^{CDC4} mutations over lethality by sustained Hog1 activation prompted us to look for differences in the activation of the Hog1 MAPK pathway between a wild-type and a *cdc4-1* mutant.

To analyze the Hog1 phosphorylation, we performed Western Blot analysis with antibodies against Hog1 and phosphorylated Hog1. Cells from wild-type and *cdc4-1* yeast strains stressed at 0.4 M NaCl were collected and analysed by PAGE. As shown in Figure 3a, Hog1 phosphorylation levels were not altered in a *cdc4-1* mutant.

Since signalling is not altered in a *cdc4-1* mutant, we then tested the nuclear import and export of Hog1. Fluorescence microscopy was used to measure GFP-Hog1 nuclear import and export at 0.4 M NaCl in a wild-type and a *cdc4-1* mutant. As shown in Figure 3b, GFP-Hog1 nuclear import and export were not altered in a *cdc4-1* mutant. Taken together, Western blot and GFP-Hog1 data suggest that Hog1 signalling and localization are not the cause of the preventive effect of SCF^{CDC4} mutations on the lethality caused by sustained Hog1 activation.

To characterize in more detail the effect of the Hog1 pathway in SCF^{CDC4} mutants, we performed a comprehensive growth analysis by dots of a full set of SCF^{CDC4}mutants at several osmolarities. As shown in Figure 3c, *cdc4-1*, *cdc34-2* and *cdc53-1* mutations did not reduce cell growth at high osmolarities. Actually, *cdc4-1*, *cdc34-2* and *cdc53-1* mutants had an increased cell survival to hyperosmotic shock. To confirm the *cdc4-1* and *cdc34-2* osmoresistance observed on plates, we performed growth assays in liquid media analyzing sensitivity to increasing NaCl concentrations. As shown in Figure 3d, *cdc4-1* and *cdc34-2* showed increased cell viability when grown in salt. Therefore, although Hog1 signalling and localization seems to be not affected, SCF^{CDC4} mutants display a certain osmoresistance.

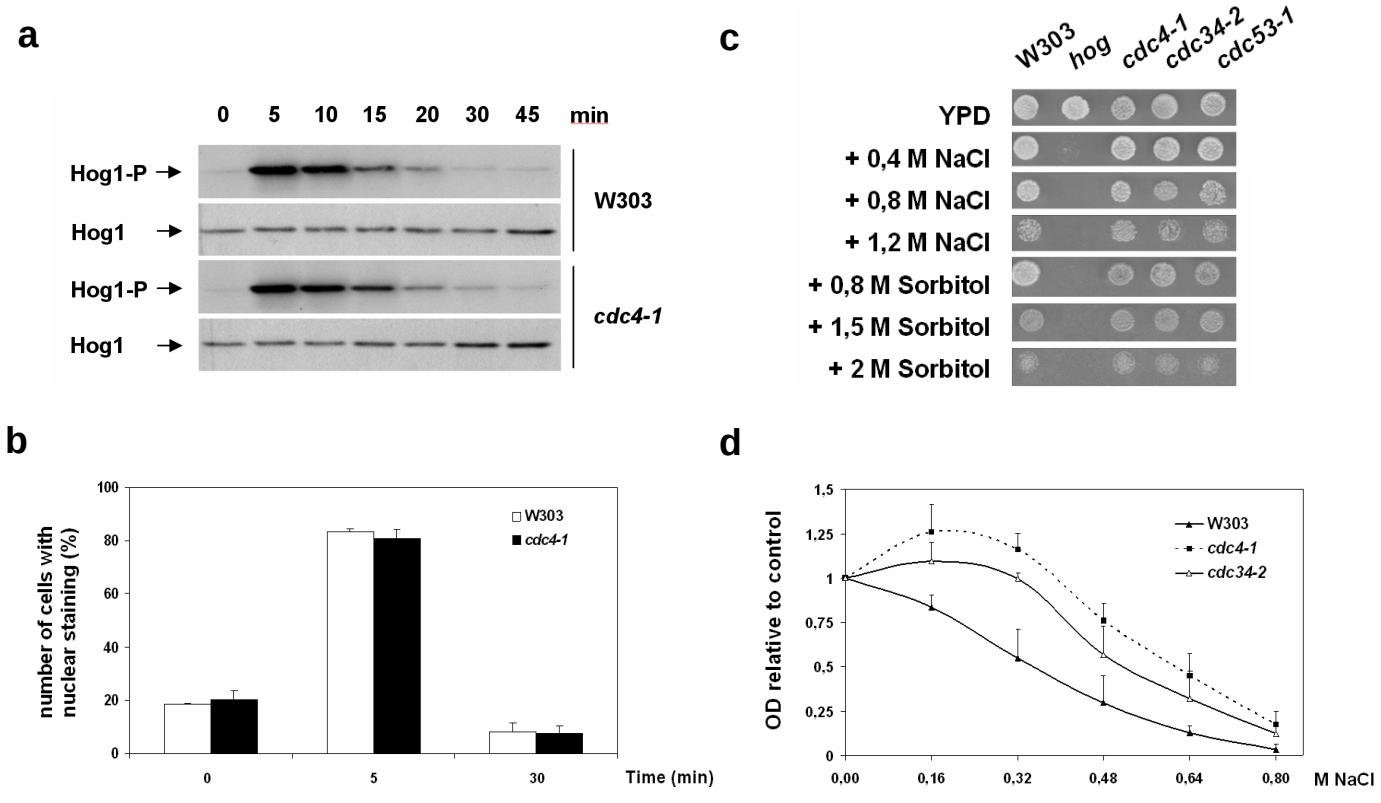

Figure 3. Hog1 signalling and localization are not altered in SCF^{CDC4} mutants. Unexpectedly, SCF^{CDC4} mutants are osmoresistant.

(a) Hog1 phosphorylation is not affected in SCF^{CDC4} mutants. Wild-type and *cdc4-1* strains were subjected to an osmostress (0.4 M NaCl) at the indicated times. Hog1 phosphorylation was followed by immunoblotting using specific antibodies against phospho-p38 MAPK (Cell Signalling) in whole cell extracts. Total Hog1 was followed using specific antibodies against Hog1 (Santa Cruz).

(b) Hog1 nuclear import/export is not affected in SCF^{CDC4} mutants. Wild-type and *cdc4-1* cells carrying pRS416-GFP-Hog1 were grown in minimal medium and subjected to an osmostress (0.4 M NaCl) at the indicated times. GFP proteins were visualized by direct fluorescence. Results shown are from three independent assays.

(c) SCF^{CDC4} mutations render cells osmoresistant. Wild-type (W303) and indicated mutant strains were spotted on YPD plates with or without several NaCl and sorbitol concentrations. Growth was scored after 3 days at 25ºC.

(d) SCF^{CDC4} mutations render cells osmoresistant. Wild-type (W303) and indicated mutant strains were grown overnight in YPD liquid media with or without several NaCl concentrations. The OD was monitored by spectrophotometry. Results shown are from three independent assays.

4.4 *SCF*^{CDC4} mutant shows increased Msn2 and Msn4 dependent gene expression.

We were then interested in finding the mechanism responsible for the preventive effect of SCF^{CDC4} mutations on Hog1 lethality. The increase on cell

survival on high osmolarity in SCF-Cdc4 mutants suggested that SCF^{CDC4} was regulating a downstream effector of Hog1.

Recently, it has been reported that Msn2, a transcription factor under the control of the Hog1 MAPK is ubiquitinated in vitro by SCF^{CDC4} (Chi et al., 2001). On the other hand, it has been also reported that SCF^{CDC4} mutation increases gene expression (Conaway et al., 2002; Gonzalez et al., 2002). To test whether Msn2 dependent gene expression was altered in a SCF^{CDC4} mutant, we performed a Northern Blot analysis comparing expression of several stress-responsive genes in a wild-type and SCF^{CDC4} mutant strains. As shown in Figure 4a, mutations in SCF^{CDC4} resulted in cells with an increase in Msn2 and Msn4 dependent gene expression (i.e. *CTT1* and *ALD2*) in response to mild osmostress conditions (0.4 M NaCl), but not in other osmoresponsive genes such as *GRE2,* that depends on the Sko1 transcription factor, and without showing an increase on general gene expression (i.e. *ACT1*).

To verify that Msn2 accumulation is responsible of increased Msn2 and Msn4 dependent gene expression observed in SCF^{CDC4} mutants, we performed ChIP analysis of Msn2 on wild-type and *cdc4-1* strains carrying a plasmid with Msn2-HA under the *ADH1* promoter. We tested whether binding of Msn2 to promoters increased in SCF^{CDC4} mutants. Chromatin from a wild-type and a *cdc4-1* yeast strains was immunoprecipitated with antibodies against HA and analyzed by PCR. As shown in Figure 4b, binding of Msn2 to the *CTT1* promoter increased in a *cdc4-1* strain, whereas binding to the *GRE2* promoter control, was not detected (data not shown). The results observed by PCR were quantified by Real Time PCR. ChIP analysis showed that binding of Msn2 to its promoters increases upon osmostress in SCF^{CDC4} mutants. Thus, our data suggest that the increased recruitment of RNA polymerase II to promoters in SCF^{CDC4} mutants is due to an increased binding of Msn2.

To test that Msn2 enlarged recruitment to its promoters in a *cdc4-1* mutant is due to a delayed ubiquitination and, therefore, inefficient degradation, we performed Western Blot analysis to quantify Msn2 protein levels. TCA cell extracts from a wild-type and a *cdc4-1* yeast strains carrying a monocopy plasmid with epitope tagged *MSN2-HA* under the *ADH1* promoter were analyzed

by Western Blot using antibodies against HA. As shown in Figure 4c, Msn2 levels decreased slower in a *cdc4-1* strain than in a wild-type strain. These data support that Msn2 degradation is delayed in an SCF^{CDC4} mutant, and this is consistent with the observation that the presence of Msn2 to osmoresponsive promoters in a *cdc4-1* strain.

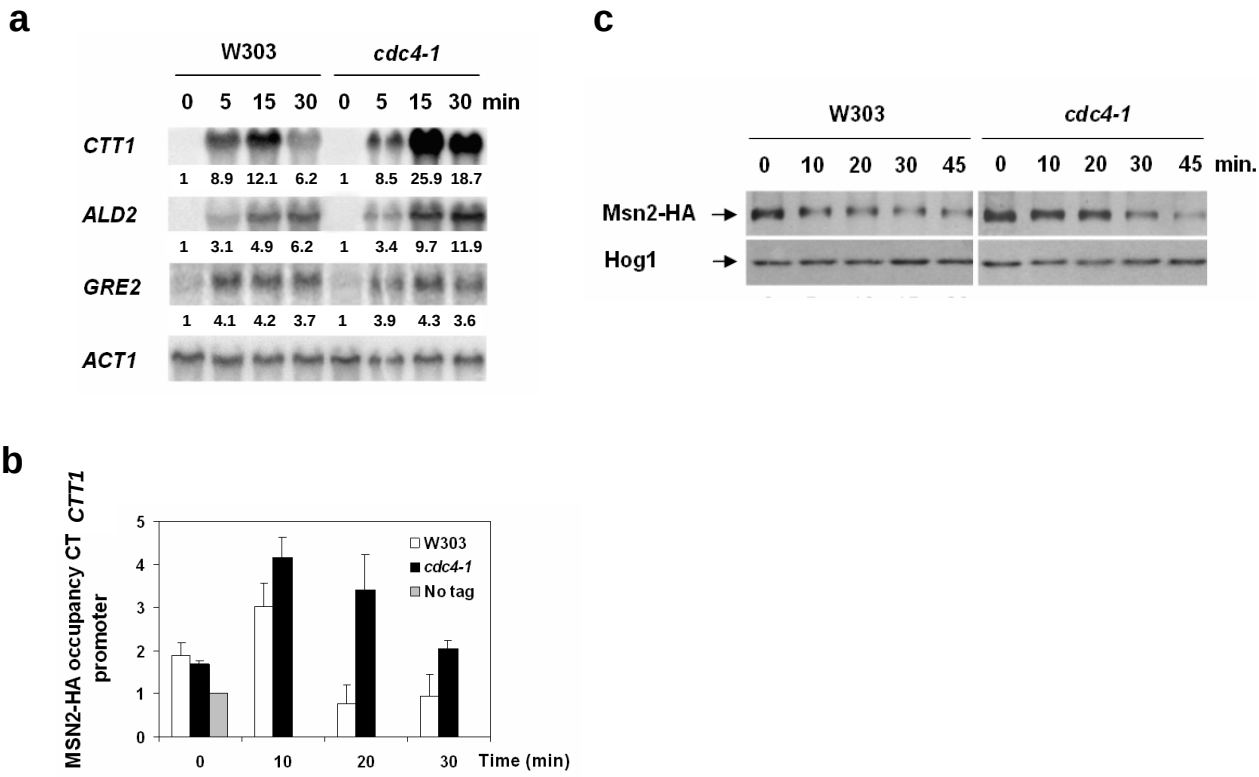

Figure 4. *cdc4-1* mutation increases Msn2 dependent gene expression.

(a) Mutation in the SCF^{CDC4} complex increases Msn2 and Msn4 dependent gene expression under mild osmostress. Wild-type and the indicated mutant strains were grown to mid-log phase in rich medium and treated with 0.4 M NaCl for the indicated times. Total RNA was assayed by Northern blotting for *CTT1*, *ALD2*, *GRE2* and *ACT1* (as a loading control).

(b) Msn2 recruitment at osmoresponsive promoters increases in a SCF^{CDC4} mutant. Wild-type and *cdc4-1* cells carrying a monocopy plasmid with *MSN2*-HA under the *ADH1* promoter were grown to mid-log phase and treated with 0.4 M NaCl for the indicated times. Proteins were immunoprecipitated with anti-HA antibody. Binding to *CTT1* promoters was determined by Real Time PCR.

(c) Msn2 degradation is delayed in a *cdc4-1* mutant upon osmotic stress. Wild-type and *cdc4-1* cells carrying a monocopy plasmid with *MSN2*-HA under the *ADH1* promoter were grown to mid-log phase and treated with 0.4 M NaCl for the indicated times.The presence of Msn2 was analysed using monoclonal antibodies against HA. Hog1 was monitored as a loading control using anti-Hog1 antibody.

4.5 Prevention of Hog1 induced lethality by SCF^{CDC4} mutations depends on Msn2 and Msn4 transcription factors.

To confirm that Msn2 actually was responsible for recovery from sustained Hog1 activation, it should be expected that deletion of *MSN2/MSN4* genes inhibited the preventive effect of *cdc4-1* on cell lethality upon sustained Hog1 activation. *cdc4-1* and *cdc4-1 msn2*Δ*msn4*Δ cells carrying a plasmid expressing $PBS2^{DD}$ under the control of the *GAL1* promoter were grown on glucose or galactose plates at 25ºC for 3 days. As shown in Figure 5a, deletion of *MSN2/MSN4* genes in *cdc4-1* cells abolished cell growth upon $PBS2^{DD}$ overexpression in *cdc4-1* mutant. Therefore, Msn2 and Msn4 transcription factors are essential for cell growth mediated by SCF^{CDC4} mutations to sustained Hog1 activation.

We then tested whether overexpression of *MSN2* in a wild-type strain was able to mimic the preventive effect of SCF^{CDC4} mutations to sustained Hog1 activation. Wild-type and *cdc4-1* cells carrying a monocopy plasmid expressing *MSN2* under the *ADH1* promoter together with a plasmid with $PBS2^{DD}$ under the control of the *GAL1* promoter were grown on glucose or galactose plates at 25ºC for 3 days. As shown in Figure 5b, cells with $PBS2^{DD}$ cells overexpressing *MSN2* had an increased cell survival when grown on galactose.

Taken together, our data supports that the preventive effect of SCF^{CDC4} mutations over lethality by sustained Hog1 activation depends on the presence of Msn2 and Msn4 transcription factors.

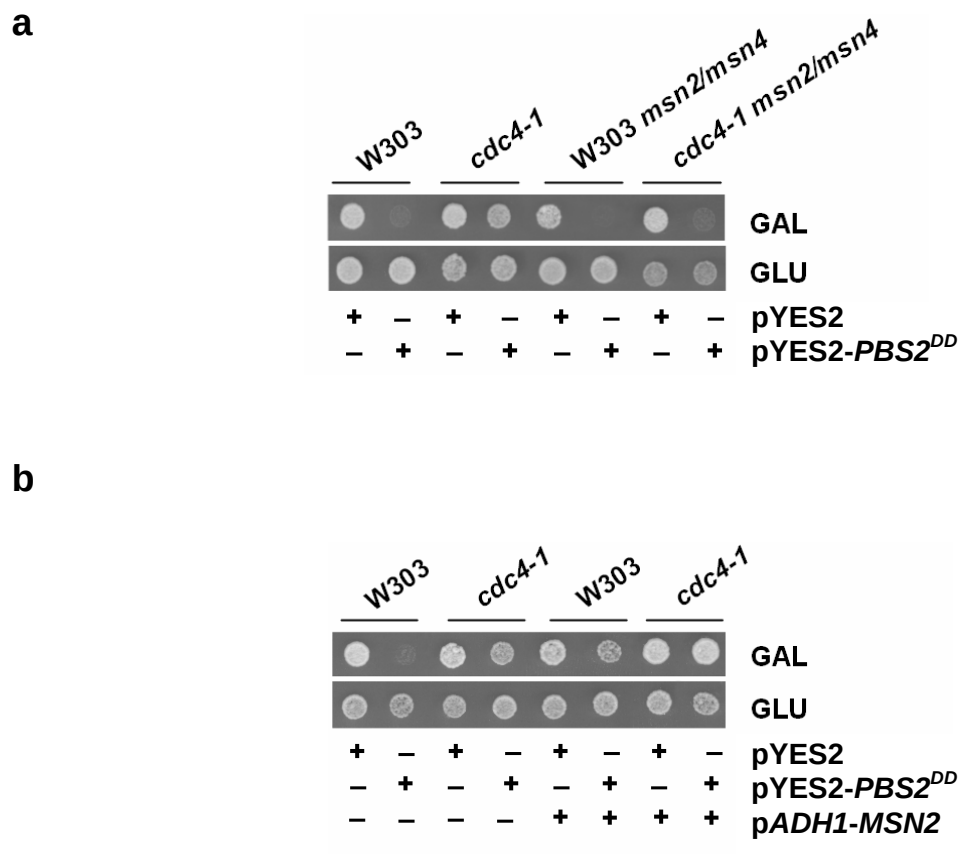

Figure 5. Suppresion of the lethality caused by sustained Hog1 activation in SCF^{CDC4} mutants depends on Msn2 and Msn4 transcription factors.
(a) *msn2*Δ and *msn4*Δ double deletion abolishes cell growth on a *cdc4-1* mutant upon sustained Hog1 activation. Wild-type (W303) and the indicated mutant strains carrying pYES or pYES-$PBS2^{DD}$ were spotted on plates with glucose or galactose. Growth was scored after 3 days at 25ºC.
(b) *MSN2* overexpression increases cell survival upon $PBS2^{DD}$ overexpression. Wild-type (W303) and the indicated mutant strains carrying p*ADH1-MSN2* and pYES or pYES-$PBS2^{DD}$ were spotted on plates with glucose or galactose. Growth was scored after 3 days at 25ºC.

4.6 Prevention of Hog1 induced lethality by SCF^{CDC4} mutations depends on *PNC1, a* Msn2 and Msn4 target gene.

It has recently been reported that an element downstream of Msn2 and Msn4 is the gene *PNC1* (Medvedik et al., 2007). Increased expression of the *PNC1* gene, which encodes a nicotinamidase, extends yeast lifespan upon caloric restriction and other mild stresses, including heat stress and osmotic shock. The relationship between *PNC1* gene expression levels and extended

lifespan prompted us to analyze the levels of Pnc1 protein under sustained Hog1 activation in a wild-type and a *cdc4-1* mutant strains. As shown in Figure 6a, Pnc1 protein levels increased upon Hog1 activation. Furthermore, Pnc1 protein levels increased further in the *cdc4-1* mutant compared to wild-type.

Then, we analysed *PNC1* involvement in the preventive effect of SCF^{CDC4} mutations on lethality by sustained Hog1 activation. *cdc4-1 pnc1*Δ carrying a plasmid expressing $PBS2^{DD}$ under the control of the *GAL1* promoter were grown on glucose or galactose plates at 25ºC for 3 days. As shown in Figure 6b, deletion of *PNC1* in *cdc4-1* cells carrying $PBS2^{DD}$ abolished cell growth on galactose caused by *cdc4-1*. Thus, *PNC1* is essential for the increased survival of SCF^{CDC4} mutants to sustained Hog1 activation.

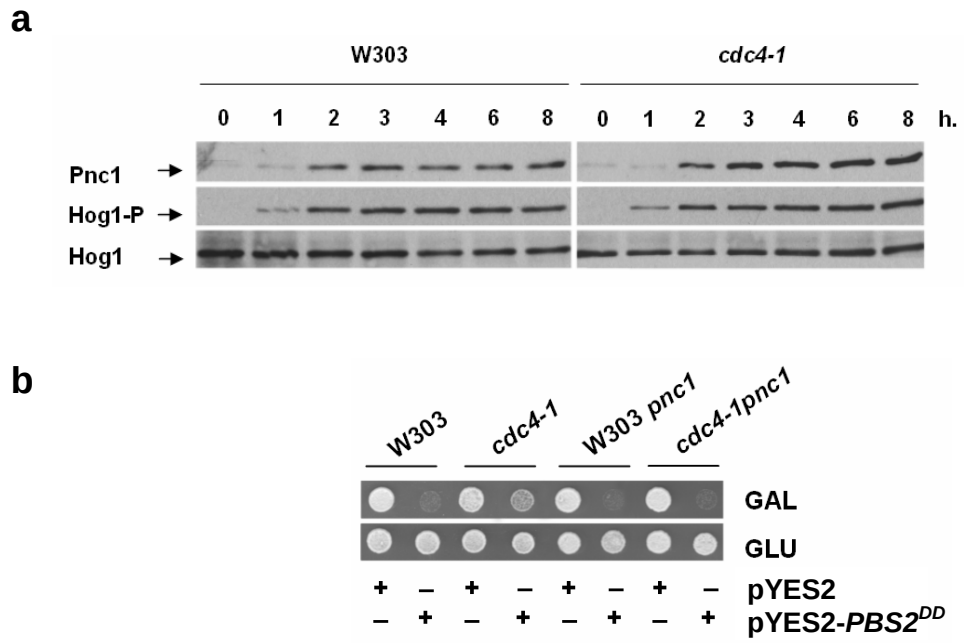

Figure 6. Suppression of the lethality caused by sustained Hog1 activation in SCF^{CDC4} mutants depends on *PNC1* gene expression.

(a) Hog1 activation leads to *PNC1* induction that is enhanced in a cdc4-1 mutant. Wild-type and *cdc4-1* strains were grown to mid-log phase in minimal medium with raffinose, and shifted to minimal medium with galactose, and then cells were collected at indicated times. The presence of Pnc1 was analysed using polyclonal antibodies against Pnc1, a generous gift of Dr. D. Sinclair. Hog1 phosphorylation was followed by immunoblotting using specific antibodies against phospho-p38 MAPK (Cell Signalling) in whole cell extracts. Total Hog1 was monitored as a loading control using specific antibodies against Hog1 (Santa Cruz).

(b) *PNC1* deletion abolished prevention of $Pbs2^{DD}$ lethality by *cdc4-1* mutation. Wild-type (W303) and the indicated mutant strains carrying pYES or pYES-$PBS2^{DD}$ were spotted on plates with glucose or galactose. Growth was scored after 3 days at 25ºC.

4.7 *cdc4-1* suppression of Hog1 induced lethality depends on *SIR2*, a NAD^+ dependent deacetilase activated by *PNC1.*

In the lifespan extension pathway, Pnc1 acts upstream of Sir2 (see introduction). If the role of *PNC1* was to activate the lifespan extension pathway to prevent cell death by sustained Hog1 activation, it could be that its immediate target *SIR2* could be mediating cell growth in *cdc4-1* mutants. Thus, we tested whether a *cdc4-1 sir2*Δ carrying pYES-$PBS2^{DD}$ strain was able to grow on galactose. For this, *cdc4-1 sir2*Δ carrying a plasmid with $PBS2^{DD}$ under the control of the *GAL1* promoter were grown on glucose or galactose plates at 25ºC for 3 days. As shown in Figure 7a, *cdc4-1 sir2*Δ cells carrying $PBS2^{DD}$ were not able to grow on galactose. Therefore, Sir2 is also essential for cell survival to sustained Hog1 activation.

To establish the specificity of Sir2 deacetilase activity in cell survival to sustained Hog1 activation, we tested whether the absence of other deacetilases, in a *cdc4-1* strain compromised cell growth upon $PBS2^{DD}$ overexpression. As shown in Figure 7a, deletion of *HDA2* or *HST1* did not compromise cell growth of a *cdc4-1* strain expressing $PBS2^{DD}$. This indicates that Sir2 deacetilase activity has an specific role in the preventive effect of SCF^{CDC4} mutations over lethality by sustained Hog1 activation.

The requirement of *SIR2* and *PNC1* to mediate cell survival in a *cdc4-1* upon sustained Hog1 activation suggested but not fully confirmed the involvement of the lifespan extension pathway. To discard the involvement of the SIR complex rather than an specific role for Sir2, we tested whether a *cdc4-1 sir4*Δ pYES-$PBS2^{DD}$ strain (see introduction) was able to grow on galactose. As shown in Figure 7a, in contrast to *sir2*Δ, deletion of *sir4*Δ in cdc4-*1* cells carrying $PBS2^{DD}$ allowed the growth on galactose to the same extent as *cdc4-1*. Therefore, the SIR complex is not involved in the preventive effect of SCF^{CDC4} mutations over lethality by sustained Hog1 activation, but rather is a specific effect of *SIR2.*

To support our genetic evidence linking *SIR2* to cell survival in the *cdc4-1* mutants, we tested whether resveratrol, a drug that induces Sir2 activity (see introduction), was able to improve cell survival to sustained Hog1 activation in a wild-type strain. As shown in Figure 7b, wild-type cells carrying pYES-$PBS2^{DD}$

were able to grow on galactose plates when resveratrol was added. The increase in cell survival due to resveratrol treatment depends on the presence of *SIR2* because it is not observed in a *sir2*Δ strain. Correspondingly, resveratrol could not promote growth in a *cdc4-1* cells that carried a *sir2*Δ mutation. Therefore, direct activation of Sir2 is able to prevent lethality by sustained Hog1 activation, mimicking the effect of *cdc4-1*. Taken together, our data support that the preventive effect of SCF^{CDC4} mutations on lethality by sustained Hog1 activation depends on the activation of the lifespan extension pathway.

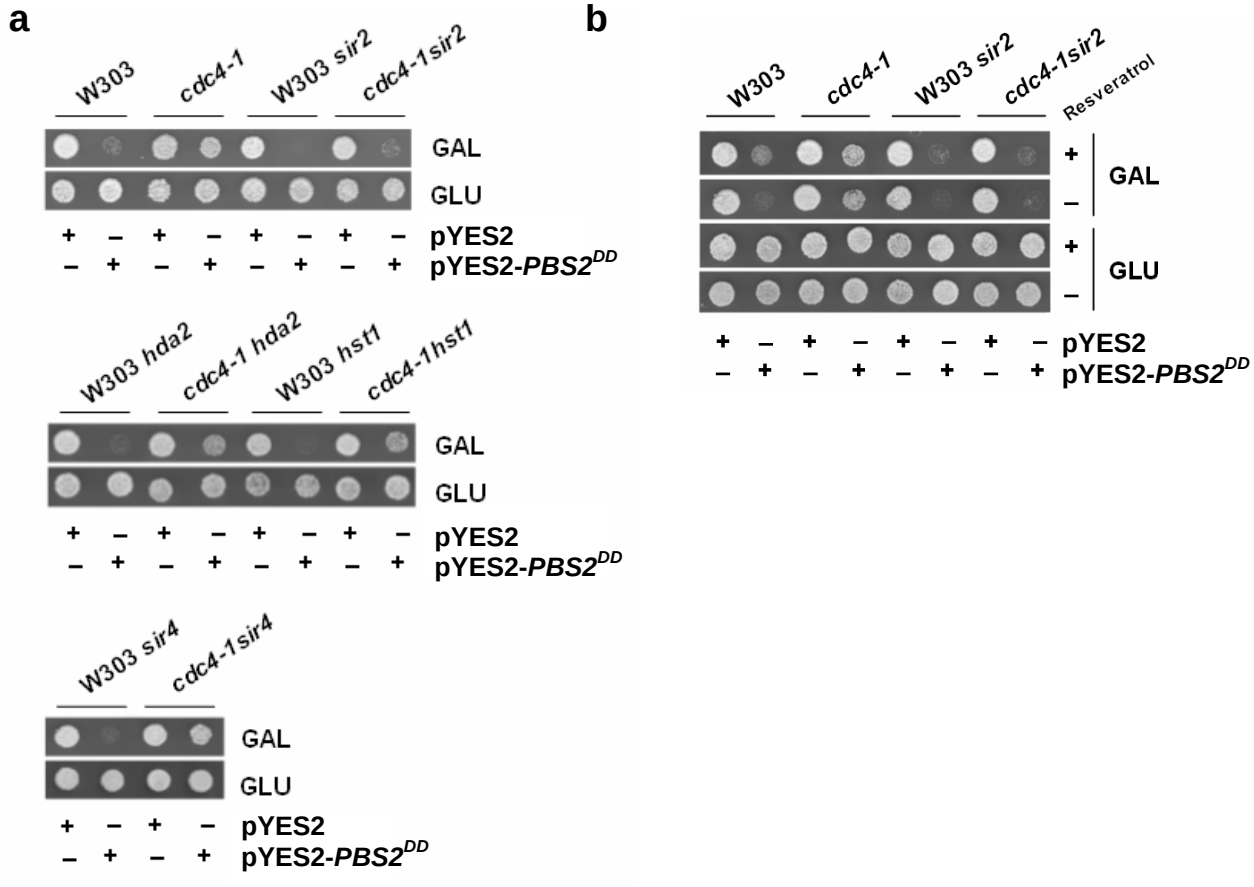

Figure 7. The lifespan extension pathway suppressed the cell death caused by sustained Hog1 activation.

(a) *SIR2* deletion abolishes prevention of $Pbs2^{DD}$ lethality in a SCF^{CDC4} mutant. Wild-type (W303) and the indicated mutant strains carrying pYES or pYES-$PBS2^{DD}$ were spotted on plates with glucose or galactose. Growth was scored after 3 days at 25ºC.

(b) Resveratrol prevents $Pbs2^{DD}$ lethality in a *SIR2* dependent manner. Wild-type (W303) and the indicated mutant strains carrying pYES or pYES-$PBS2^{DD}$ were spotted on plates with glucose or galactose containing resveratrol (5 μM). Growth was scored after 3 days at 25ºC.

4.8 SCF^{CDC4} mutation prevents Hog1-induced cell death by reducing apoptosis.

Recently, it has been proposed that yeast extended lifespan by delaying apoptosis (Hamann et al., 2008). Hence, we asked whether the cell-death preventing effect of the lifespan extension pathway upon sustained Hog1 activation was due to an inhibition of cell death by apoptosis. To address this question, wild-type and *cdc4-1* mutant strains carrying a plasmid with $PBS2^{DD}$ under the control of the *GAL1* promoter were grown on galactose liquid media and analysed for apoptotic hallmarks. Phenotypic apoptotic markers included the appearance of SubG1 population of cells on flow cytometry sorting and DNA breakage followed by TUNEL assay.

When DNA content is analysed by flow cytometry, a population of cells in SubG1 indicates DNA degradation that appears when cells die. To detect a SubG1 population formation, wild-type and *cdc4-1* mutant cells carrying pYES-$PBS2^{DD}$ were grown to log-phase in raffinose, and 2% galactose was added for 24 hours to express $PBS2^{DD}$. Then, cells were processed for detection of SubG1 peak by FACS (see Materials and methods). As shown in Figure 8a, wild-type carrying pYES-$PBS2^{DD}$ showed a SubG1 peak that includes 17.5% of the population, whereas *cdc4-1* carrying pYES-$PBS2^{DD}$ cells showed a SubG1 peak that includes only 9% of the population.

To detect yeast cells with DNA single-strand breaks, we employed the TUNEL assay (see Materials and methods). The TUNEL assay detects DNA with free 3' ends generated by chromosome fragmentation by attaching labelled nucleotides with a terminal deoxinucleotydil transferase. Wild-type and *cdc4-1* mutant cells carrying pYES-$PBS2^{DD}$ were grown to log-phase in raffinose, and 2% galactose was added for 24 hours to express $PBS2^{DD}$ as before. Cells were processed by TUNEL assay and detected by FACS. As shown in Figure 8b, wild-type carrying pYES-$PBS2^{DD}$ cells grown on galactose for 24 hours showed a 15.2% of TUNEL-positive cells (fluorescence increased in the FLH1 filter), whereas *cdc4-1* cells showed only 8% of TUNEL-positive cells.

Therefore, our data suggest that cell death by sustained Hog1 activation is due to an induced apoptosis. Moreover, *cdc4-1* displays reduced cell death under sustained Hog1 activation.

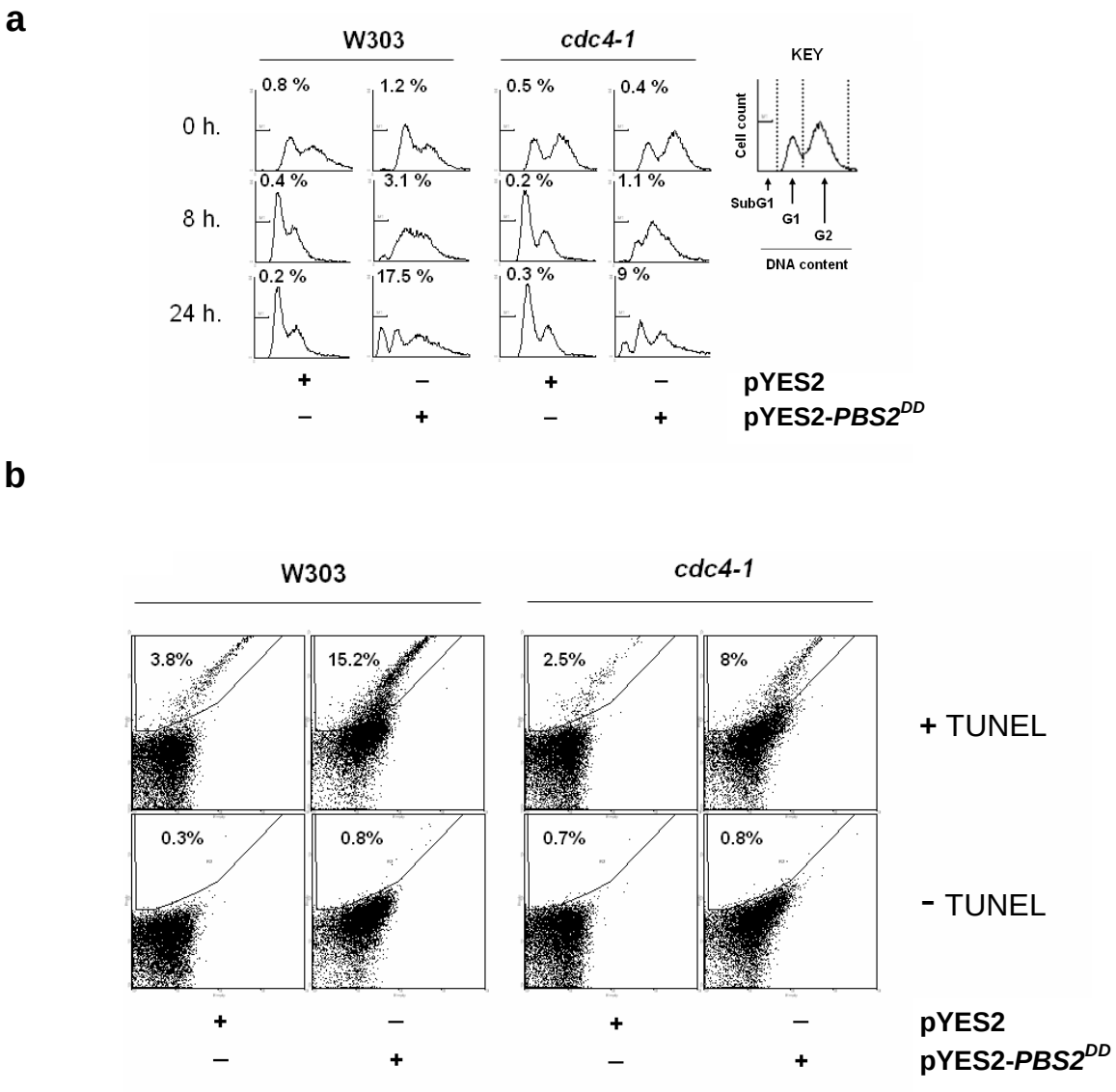

Figure 8. *cdc4-1* mutation prevents apoptosis induced by sustained Hog1 activation.

(a) SubG1 population is reduced in *cdc4-1* mutant cells. Wild-type and *cdc4-1* mutant cells carrying pYES-*PBS2*^{DD} were grown to log-phase in raffinose and 2% galactose was added for 24 hours to express *PBS2*^{DD} and analysed for SubG1 population by FACS. Data shown are representative of 3 independent experiments with similar results.

(b) Cells containing DNA single strand breaks are reduced in *cdc4-1* mutant cells. Wild-type and *cdc4-1* mutant cells carrying pYES-*PBS2*^{DD} were grown to log-phase in raffinose and 2% galactose was added for 24 hours to express *PBS2*^{DD}, processed for the TUNEL assay and analysed for presence of cells with single-strand DNA breaks by FACS. Data shown are representative of 3 independent experiments with similar results.

4.9 Cell death caused by sustained Hog1 activation might be caused by increased ROS levels.

The fact that ROS formation can trigger apoptosis under several stimuli prompted us to analyze whether ROS could mediate cell death upon sustained Hog1 activation. We tested whether in the absence of O_2, an environment on which where cells are unable to accumulate ROS, wild-type cells did not suffer cell death upon sustained Hog1 activation. For this, wild-type and *cdc4-1* cells carrying a plasmid with *PBS2*^{DD} under the control of the *GAL1* promoter were grown on glucose or galactose plates at 25ºC for 3 days under anaerobiosis by placing them inside an hermetic jar with a Mikrobiology Anaerocult A (Merck, Darmstadt, Germany). As shown in Figure 9a, in plates incubated under anaerobiosis, wild-type cells carrying pYES-*PBS2*^{DD} were able to grow on galactose as efficiently as wild-type cells carrying pYES empty vector. This suggests that inhibition of ROS formation is able to prevent cell death under sustained Hog1 activation. It still exists the possibility that an inhibition of respiration by anaerobiosis could also be involved in preventing cell death.

Thus, to confirm that a decrease in ROS levels and not inhibition of respiration was mediating cell growth in SCF^{CDC4} mutant cells, we tested whether cell viability of *cdc4-1* mutant cells under sustained Hog1 activation was affected by antimycin A treatment. Antimycin A binds to the Qi site of Complex III, inhibiting the oxidation of ubiquinol in the electron transport chain, ultimately preventing the formation of ATP but promoting the formation of large quantities of ROS. For this, wild-type and *cdc4-1* cells carrying a plasmid with *PBS2*^{DD} under the control of the *GAL1* promoter were grown on glucose or galactose plates treated with antimycin or not at 25ºC for 3 days. As shown in Figure 9b, when cells were treated with antimycin, neither wild-type nor *cdc4-1* mutant cells expressing *PBS2*^{DD} were not able to grow on galactose plates. Therefore, inhibition of the respiratory chain does not prevent cell death under sustained Hog1 activation. Moreover, ROS formation induced by antimycin treatment avoids the preventive effect the *cdc4-1* mutation.

To discard that an increase in ROS levels and not the inhibition of ATP formation is avoiding *cdc4-1* preventive effect, we tested whether cell viability of *cdc4-1* mutant cells under sustained Hog1 activation was affected by dinitrophenol treatment. Dinitrophenol uncouples mitochondrial ATPasse ultimately preventing the formation of ATP without promoting the formation of

ROS. For this, wild-type and *cdc4-1* cells carrying a plasmid with *PBS2*^{DD} under the control of the *GAL1* promoter were grown on glucose or galactose plates treated with dinitrophenol or not at 25ºC for 3 days. As shown in Figure 9c, when cells were treated with antimycin, *cdc4-1* mutant cells carrying pYES-*PBS2*^{DD} were able to grow on galactose plates. Therefore, an inhibition of ATP formation has no effect on the suppression of cell death under sustained Hog1 activation in the *cdc4-1* mutant. Taken together, our data suggest that inhibition of ROS formation is critical to prevent cell death under sustained Hog1 activation.

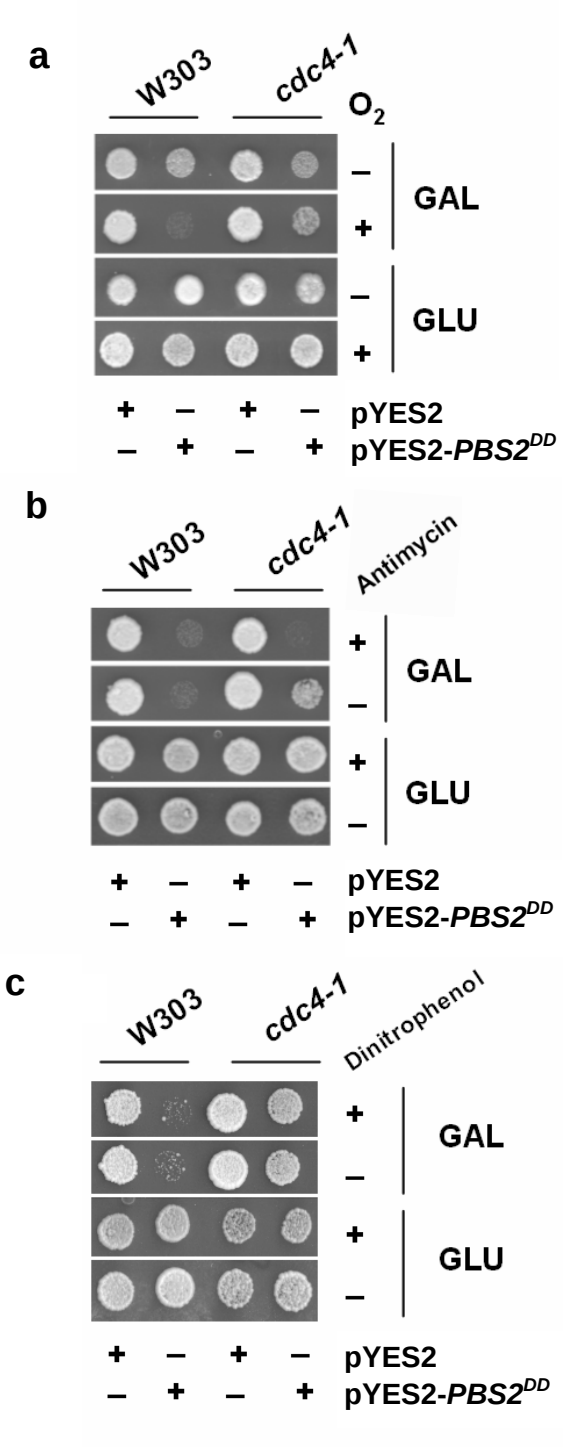

Figure 9. Inhibition of ROS formation prevents cell death under sustained Hog1 activation.

(a) Anaerobiosis prevents cell death under sustained Hog1 activation. Wild-type (W303) and *cdc4-1* mutant strains carrying pYES or pYES-*PBS2*^{DD} were spotted on plates with glucose or galactose. Growth was scored after 3 days at 25ºC.

(b) Antimycin does not prevent cell death under sustained Hog1 activation and avoids the preventive effect of SCF^{CDC4} mutant. Wild-type (W303) and *cdc4-1* mutant strains carrying pYES or pYES-*PBS2*^{DD} were spotted on plates with glucose or galactose treated or not with antimycin (2.5 μg/ml). Growth was scored after 3 days at 25ºC.

(c) Inhibition of ATP formation by dinitrophenol treatment does not affect cell death under sustained Hog1 activation. Wild-type (W303) and *cdc4-1* mutant strains carrying pYES or pYES-*PBS2*^{DD} were spotted on plates with glucose or galactose treated or not with dinitrophenol (25 μg/ml). Growth was scored after 3 days at 25ºC.

4.10 Inhibition of mitochondrial respiration by Hog1 as a mechanism of apoptosis induction.

It is kown that a mechanism to increase ROS levels and to induce apoptosis is to inhibit mitochondrial respiration (Pelicano et al., 2003). Thus, we tested whether mitochondrial respiration was affected in high osmolarity by Hog1 activity. We performed a triphenyl tetrazolium overlay analysis with wild-type or ATP analogue sensitive Hog1 (*hog1as)* mutant cells grown in plates with or without salt (0.8 M NaCl). This assay allows to monitor the activity of mitochondrial respiration (Kobayashi et al., 1974). As shown in Figure 10, tethrazolium chloride was not reduced to a red color when cells were grown in high osmolarity in contrast to cells grown in the absence of osmostress. We then tested whether inhibition of the Hog1 MAPK prevented inhibition of mitochondrial function. As shown in Figure 10, when the specific inhibitor for *hog1as* called NMPP1 was added, cells were able to reduce tethrazolium chloride even in the presence of salt. Therefore, Hog1 activity is sufficient to inhibit the mitochondrial respiration in response to osmotic stress. This could be the mechanism that leads to ROS formation and induces apoptosis under sustained Hog1 activation.

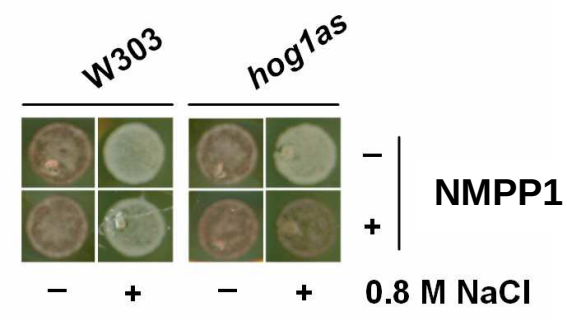

Figure 10. In response to high osmolarity, mitochondrial respiration is inhibited. The wild-type and *hog1as* mutant strains were spotted on YPD plates with or without NaCl and in the presence or absence of a *hog1-as* inhibitor called NMPP1 (5 μM). Cells grew overnight at 25ºC, and then, tetrazolium chloride was added as an overlay. Pictures were taken 12 h. after the addition of tetrazolium chloride.

DISCUSSION

"En el punt mig és on trobarem la virtut"

Arístòtil

Mutations on the SCF^{CDC4} complex prevent inhibition of cell growth caused by sustained Hog1 activation.

Modulation of Hog1 activation is essential to respond to enviromental changes without compromising cell growth. However, sustained Hog1 activation inhibits cell growth. No mutations downstream of Hog1 have been identified that could suppress inhibition of cell growth due to sustained activation of Hog1. Only mutations in *RCK2* or *HOT1* have some residual preventive effect. Here, we have found that mutations in the SCF^{CDC4} complex are able to prevent inhibition of cell growth caused by sustained Hog1 activation.

SCF^{CDC4} is a multisubunit protein complex involved in the polyubiquitination of several proteins, mainly cell cycle regulators, such as Sic1, and transcription factors, such as Gcn4 or Msn2. At present, SCF^{CDC4} complex is essential for cell viability. For this reason, we employed SCF^{CDC4} thermosensitive mutants. Notably, the reversion of Hog1 lethality by SCF^{CDC4} mutants was observed at permissive tempearature (25ºC). Therefore, SCF^{CDC4} thermosensitive mutants are not completely functional at 25ºC.

Inhibition of cell growth by sustained Hog1 activation was described at early 90's since Hog1 pathway was described (Maeda et al., 1994). However, the mechanism by which sustained Hog1 activation inhibited cell growth remained completely unknown. Here, we have found that sustained Hog1 activation leads to a high degree of cell death. Therefore, inhibition of cell growth under sustained Hog1 activation is due to an increase in cell mortality.

Interestingly, we have found that SCF^{CDC4} mutation showed a reduced percentage of dead cells when compared to wild-type. Thus, the preventive effect of SCF^{CDC4} mutation is a consequence of the reduction in cell mortality under sustained Hog1 activation.

***cdc4-1* mutation does not affect Hog1 signalling nor localization but leads to cells with increased osmoresistance.**

No relationship between SCF^{CDC4} and the Hog1 pathway had been described before. Here we have found that mutations in SCF^{CDC4} interfere somehow with the HOG pathway response. An initial hypotesis was that SCF^{CDC4}

mutations could inhibit signaling on the Hog1 MAPK pathway to increase cell survival upon sustained Hog1 activation. However, we observed that SCF^{CDC4} mutant cells have proper Hog1 signalling and localization of the MAPK when cells are exposed to osmostess. However, it is worth noting that SCF^{CDC4} mutants were more osmoresistant than wild-type cells which suggested that the HOG responses were somehow hyperactive in these mutant strains.

The osmoresistance observed in SCF^{CDC4} mutants prompted us to look for a mechanism induced by the Hog1 MAPK that prevented cell death, and that, in addition, was inhibited by SCF^{CDC4}. We have found that lifespan extension pathway is an excellent candidate: a) Lifespan extension pathway is activated by osmotic stress; b) Lifespan extension pathway is involved in prevention of cell death; c) This pathway is activated by Msn2 and Msn4 dependent gene expression, two transcription factors under the control of Hog1; d) SCF^{CDC4} inhibits gene expression by ubiquitination of several transcription factors; e) Msn2 is ubiquitinated by SCF^{CDC4} in vitro. Taken together these five observations, we proposed the following hypothesis: in a SCF^{CDC4} mutant, cell death by sustained Hog1 activation was prevented by a lifespan extension pathway hyperactivation due to an increased Msn2 and Msn4 gene expression.

Msn2 and Msn4 dependent gene expression increases in a SCF^{CDC4} mutant strain.

Initially, we analyzed gene expression in a *cdc4-1* mutant strain compared to a wild-type strain to verify the hypothesis stated above. We have found by Northern Blot analyses that Msn2 and Msn4 dependent gene expression is clearly increased in a *cdc4-1* mutant strain. Correspondingly, ChIP analyses have shown that an increase in Msn2 and Msn4 dependent gene expression correlates to an enhanced Msn2 binding to stress-response promoters in the *cdc4-1* mutant.

Recently, it has been suggested that a transcription factor remains bound to its promoter until the transcription factor is polyubiquitinated (Kodadek et al., 2006). Moreover, a delay in polyubiquitination would result in an increase in gene expression. Therefore, enhanced Msn2 promoter binding in the SCF^{CDC4} mutant could be due to a delay in Msn2 polyubiquitination. We have not been

able to detect Msn2 ubiquitinated forms to confirm the delay in ubiquitination in the SCF^{CDC4} mutant strain (data not shown). However, we have observed by Western Blot analysis of Msn2 levels a reduced degradation of Msn2 upon stress in the *cdc4-1* mutant that suggests a delayed Msn2 polyubiquitination.

Thus, we can conclude that, in the *cdc4-1* mutant, Msn2 is degraded slower, suggesting a delayed polyubiquitination. Then, Msn2 accumulation results in an enhanced Msn2 promoter binding and an increased Msn2 and Msn4 dependent gene expression. Therefore, SCF^{CDC4} polyubiqutylation has an inhibitory role in Msn2 and Msn4 dependent gene expression.

Msn2/Msn4 as a model for gene regulation by destruction of a transcriptional activator.

The inhibitory role of SCF^{CDC4} in Msn2 and Msn4 dependent gene expression is a notable discovery since there is a certain amount of contention about degradation of transcription activators and gene expression regulation. It is not fully understood why for some transcription factors, such as Gcn4, ubiquitination and proteosomal degradation are necessary to allow for proper gene expression, whereas other activators, such as Gal4, do not depend on degradation (Lipford et al., 2005).

A third model has arose to explain these observations. This is called the "second generation timer" model and it is based on one main concept: a transcription factor is active only when it remains monoubiquitinated. When a transcription factor is polyubiquitinated, then it becomes inactive and it needs to be degraded to allow a new active protein be bound to the promoter (Kodadek et al., 2006).

To understand the mechanism underlying the different transcription factors, the "second generation timer" model suggests that they might have different polyubiquitination kinetics. For instance, if a transcription factor is polyubiquitinated very slowly, it is never inactive. Therefore, it does not need to be removed and gene expression is not affected by mutations that reduce degradation or polyubiquitination (i.e. Gal4). In contrast, if a transcription factor is ubiquitinated rapidly, then it is inactivated after a few transcription rounds. The non-functional factor has then to be degraded to restart transcription, or transcription would be blocked (i.e. Gcn4).

A transcription factor whose activity was inhibited when proteosomal degradation was inhibited but enhanced when polyubiquitination was inhibited would confirm the "second generation timer". However, this transcription factor has not yet been found. Gal4 dependent gene expression is not affected by inhibition of degradation whereas Gcn4 dependent gene expression is blocked by inhibition of proteosomal degradation (Lipford et al., 2005). Unexpectedly, a decrease of Gcn4 polyubiquitination completely blocks gene expression instead of increasing it (Lipford et al., 2005). Classically, it has been thought that inhibition of degradation should cause protein accumulation and gene expression enhancement. But we know that probably an inhibiton of degradation will cause a block in gene expression.

Our first observations about Msn2 and Msn4 would suppot the "second generation timer" model to explain how the cell regulates transcription factor activity through protein polyubiquitination and degradation. We have described an enhancement of Msn2 and Msn4 dependent gene expression due to a mutation on a polyubiquitination complex that leads to slower Msn2 degradation. In the future, we will test whether an inhibition of degradation inhibits Msn2 and Msn4 dependent gene expression to confirm, or not, the "second generation timer".

Msn2 and Msn4 accumulation prevents cell death upon sustained Hog1 activation.

The increased Msn2 and Msn4 dependent gene expression observed in mutants on SCF^{CDC4} cells suggested that Msn2 and Msn4 should be critical to suppress Hog1 sustained activation.

The dramatical reduction of SCF^{CDC4} mutation preventive effect by deletion of *MSN2* and *MSN4* suggested that both transcription factors were involved in the increased cell survival under sustained Hog1 activation. *MSN2* overexpression assays confirmed that an Msn2 accumulation increases resistance to cell death upon sustained Hog1 activation.

Therefore, our data suggest that Msn2 and Msn4 accumulation is able to prevent cell death upon sustained Hog1 activation by increasing Msn2 and Msn4 dependent gene expression.

PNC1 overexpression in SCF^{CDC4} mutants prevents cell death upon sustained Hog1 activation.

Activation of *PNC1* gene expression by Msn2 and Msn4 transcription factors is a critical step on the lifespan extension pathway. An increased *PNC1* expression prevents cell death. We have found that, indeed, *PNC1* expression is induced upon Hog1 activation and the induction is stonger in a SCF^{CDC4} mutant compared to wild-type. Moreover, the dramatic reduction of the preventive effect of the SCF^{CDC4} mutation observed by deletion of *PNC1* confirmed that *PNC1* is involved in the increased cell survival upon sustained Hog1 activation.

Sir2 hyperactivation prevents cell death upon sustained Hog1 activation.

PNC1 overexpression leads an extension in the yeast lifespan due to a reduction of nicotinamide, a natural inhibitor of the NAD^+ dependent deacetilase Sir2 (Anderson et al., 2003b). Hyperactivation of Sir2 leads to rDNA silencing, the mechanism that finally extends lifespan (Bitterman et al., 2003). Therefore, Sir2 effect over rDNA is the key element of lifespan extension pathway.

Here we have shown that, as for *PNC1,* deletion of *SIR2* results in a dramatic decrease of SCF^{CDC4} mutant preventive effect on Hog1 induced cell death. The deletion of other deacetilases did not reduce the preventive effect of the SCF^{CDC4} mutation on Hog1 induced cell death, and thus, Sir2 deacetilase activity is specifically required. In addition, we have shown that, as in the lifespan extension pathway, suppression of silencing in HMLs and telomeres is not important.

Resveratrol, a compound that induces Sir2 activity, also increases cell survival under sustained Hog1 activation, which confirms the relevance of Sir2 hyperactivation in preventing cell death under sustained Hog1 activation. Interestingly, resveratrol does not reach the increase in cell survival of a *cdc4-1* strain. This is due to resveratrol is a chemical inducer of Sir2 activity that enhances Sir2 affinity for its substrates as a maximum of two fold.

Taken together, the results from the genetic and chemical analyses of the lifespan extension pathway elements - Msn2/Msn4, Pnc1 and Sir2 - strongly indicates that the lifespan extension pathway is involved in prevention of cell death under sustained Hog1 activation by the *cdc4-1* mutation.

Continuous Hog1 activation leads to apoptosis.

The mechanism underlying the lifespan extension pathway to delay replicative aging remains unknown. However, recent reports have suggested that lifespan could be limited by an apoptotic process (Hamann et al., 2008). For instance, yeast metacaspase deletion slightly increases lifespan (Madeo et al., 2002). Therefore, an activation of the lifespan extension pathway by, for instance, caloric restriction, could delay apoptosis.

The fact that the lifespan extension pathway is required to decrease cell death under sustained Hog1 activation suggested that sustained Hog1 activation could induce apoptosis. Detection of a SubG1 population by FACS, that represents cells that have lost part of their DNA usually indicates apoptosis. Here we have shown by FACS analysis that sustained Hog1 activation induces the appearance of a SubG1 population up to 17% of the total population after 24 h of Hog1 induction. TUNEL analysis confirmed that indeed sustained Hog1 activation leads to a nuclear single-strand DNA breakage, a typical apoptotic pattern, that appears at 15% of the population after 24 h of Hog1 induction. It is worth noting that both methods, SubG1 and TUNEL analyses, showed that around 15% of the population underwent apoptosis after 24 h of Hog1 induction.

The observation that sustained Hog1 activation induces apoptosis is notable, since it is the first time that apoptosis induced by the direct activation of a MAPK is described in yeast. In mammalian cells, the requirement of some MAPKs, as p38 or JNK, to induce apoptosis under stress conditions have been described (De Zutter et al., 2001; Oskouian et al., 2006).

SCF^{CDC4} mutation prevents apoptosis induced by sustained Hog1 activation.

SubG1 and TUNEL analyses showed that *cdc4-1* mutation reduces the percentage of apoptotic cells to half. We have previously demonstrated that SCF^{CDC4} mutation prevents cell death due to a hyperactivation of the lifespan extension pathway. Therefore, taken together our data support the hypothesis that the lifespan extension pathway prevents apoptosis.

The effects of the lifespan extension pathway in aging prevention has also been observed in higher eukaryotes, such as *C.elegans*, *D. melanogaster*, and

mouse (Cerami, 1985; Masoro, 1989; Lin et al., 2000; Clancy et al., 2001; Lin et al., 1997). Surprisingly, the lifespan extension pathway activation also showed protective effects in other degenerative pathologies, such as diabetes or Alzheimer (Milne et al., 2007). Our results could bring some light in the protective effect of lifespan extension pathway in stop aging and degenerative pathologies.

It has been recently suggested that Alzheimer or diabetes could be mediated by inflammatory response that leads to apoptosis of affected cells (Bordone et al., 2007). Classically, activation of MAPKs, and specially p38 (the human homolog of Hog1), has been related to inflammatory processes (Han et al., 2001). We have observed that activation of the lifespan extension pathway prevents apoptosis induced by sustained MAPK activation. Therefore, we propose the following mechanism for the role of the lifespan extension pathway in the preventive effect on degenerative pathologies. In human cells, activation of the lifespan extension pathway could reduce Alzheimer or diabetes progression by inhibiting apoptosis induced by sustained MAPK activation that is caused by inflammatory stimuli. Aging prevention could be explained by the same mechanism. Lifespan extension pathway would be delaying aging by preventing loss of old cells by apoptosis.

Cell death caused by sustained Hog1 activation is caused by ROS formation.

An increase in reactive oxygen species (ROS) is the most frequent cause that induces apoptosis (Perrone et al., 2007). Here we have shown that in anaerobiosis, a condition where cell is not able to produce ROS, the lethal effect of sustained Hog1 activation was dramatically reduced This data suggests that ROS formation is essential for Hog1 induced cell death.

However the preventive effect of anaerobiosis over Hog1 induced cell death could be caused by a depletion of ATP produced in the mitochondrial respiratory chain. To test whether respiration or ROS formation were inducing cell death by sustained Hog1 activation, we employed two different drugs. We treated cells with antimycin, a drug that blocks respiration and increases ROS production, and with dinitrophenol, a drug that blocks ATP production by mitochondrial respiration without increasing ROS levels (Zuin et al., 2008). Both drugs were unable to prevent cell death induced by Hog1, supporting the

hypothesis that ROS production and neither respiration nor ATP production are necessary for Hog1 induced cell death.

On the other hand, the fact that antimycin, a drug that increases ROS production, abolishes preventive effect of SCF^{CDC4} mutation suggests that the protective mechanism of SCF^{CDC4} mutation could be based on a reduction of ROS formation.

Hog1 inhibits mitochondrial respiration.

Apoptosis induced by an increase on ROS production is usually linked to altered mitochondrial respiratory chain, the major source of ROS (Pelicano et al., 2003). Strong inhibition or activation of the mitochondrial respiration induce ROS formation. Here we have shown that, under osmotic stress, Hog1 activitation inhibits respiration. Therefore, we propose the following model: a continuos inhibition of mitochondrial respiration by sustained Hog1 activation triggers an increase in ROS production that induces apoptosis. Currently, we are working on the detection of increases in ROS production under sustaind Hog1 activation.

Biological economy or the sense of lifespan extension pathway regulation.

Under low stress conditions, such as moderated caloric restriction, moderated aminoacid deprivation, mild heat shock or mild hyperosmotic shock, cells stop proliferation, inhibit respiration and activates autophagy (Sohal et al., 1996). These responses promote cells enter in a "energy-saving" mode, to accumulate energy resources enough for facing the aggression and increase cell survival.

However, under high stress conditions, such as strong caloric restriction, strong aminoacid deprivation, severe heat shock or severe hyperosmotic shock, despite of activating the same "energy-saving" mode, an important percentage of the yeast population enters into apoptosis (Ribeiro et al., 2006).

In this work we have shown that a mutation in SCF^{CDC4} complex which activates the lifespan extension pathway, prevents apoptotic cell death by sustained Hog1 activation. Why yeast cells have not a physiological higher

activation of lifespan extension pathway to avoid apoptotic cell death under high stress?

The answer might be that yeast cells should take some advantage of entering into apoptosis. However, in a unicellular organism, such as yeast, it is difficult to find an advantage for apoptosis. Because of this apparent lack of sense, the discovery of apoptotic markers and mechanims in yeast has been refused by many investigators for years. Recent reports have suggested that apoptosis has been developed in unicellular organisms to improve the energy employement to increase the survival rate of the population (Madeo et al., 2002).

Under low stress conditions, inhibition of metabolism of the cells is sufficient to provide energy to face the stress. However, under severe stresses, such as strong caloric deprivation, metabolism inhibiton is not sufficient. A part of the population should die to increase the resources available for the rest of the population. The lifespan extension pathway might be tuned to prevent cell death only under mild stresses, which could be bypassed by inhibition of metabolism.

The next question is how the cell is able to sense the strength of the stress. The main agent involved in apoptosis regulation should be ROS. Under low stress conditions, inhibition of mitochondrial respiration increases moderately ROS formation. However, lifespan extension pathway somehow should be able of decreasing mild ROS accumulation. Therefore, cell death stimulus is not enough to induce cell death. However, under severe stresses, inhibition of mitochondrial respiration should be stronger and longer. Lifespan extension pathway effect is not enough to repress apoptotic stimulus by ROS accumulation, and cell enter into apoptosis.

Data recently published reinforces this model. Low caloric restriction (0,5% glucose) extends lifespan in a 30% in a Sir2 dependent manner, whereas stronger caloric restriction (0,05% glucose) have no effect in lifespan (Easlon et al., 2007). Thus, we propose that lifespan extensión pathway decreases cell oxidation levels only under mild stresses.

We have suggested a model to explain why lifespan extension pathway has been developed in the cell. Lifespan extension pathway would be developed to inhibit ROS formation under a low stress induced-metabolic stop. This inhibitory effect over ROS formation should be the responsible of the anti-aging

effect, since it has been demonstrated a clear relationship between cell oxidation and lifespan (Kaeberlein et al., 2007). It is worth noting that lifespan extension when cell proliferation is stopped is also a mechanism to preserve population size.

CONCLUSIONS

"La ciència és un arbre d'arrels amargues però de fruits molt dolços"

Diògenes Laerci

The following conclusions can be drawn from the results of the work presented in this PhD thesis:

- ✓ SCF^{CDC4} mutants prevents inhibition of cell growth under sustained Hog1 activation.
- ✓ The SCF^{CDC4} complex has an inhibitory role over the Msn2 and Msn4 dependent gene expression.
- ✓ Prevention of Hog1 lethality in SCF^{CDC4} mutation is due to a hyperactivation of the lifespan extension pathway, that includes *PNC1* and the *SIR2* deacetylase.
- ✓ Sustained activation of Hog1 leads to apoptosis.
- ✓ Prevention of Hog1 lethality by SCF^{CDC4} mutation is due to a reduced Hog1-induced apoptosis.
- ✓ Mitochondrial respiration is inhibited under a hyperosmotic shock. It could be the mechanism through Hog1 induces apoptosis.

SUPPLEMENTARY ARTICLES

"Un no és necí per seure's a un formiguer ; és necí si no s'aixeca"

Proverbi xinès

ARTICLE 1.

During the PhD. period spent in Dr. Posas' laboratory, we were aimed at studying the role of Hog1 in the control of cell responses under osmotic shock. Besides the results concerning the role of Hog1 in cell death, I have also contributed to elucidating other processes driven by the MAPK.

Specifically, I spent the two first years of PhD. thesis looking for new interactions between Hog1 and downstream targets. To unravel new unknown functions of Hog1, we set up several proteomical techniques, specially the TAP purification protocol coupled to mass espectometry. The TAP protocol was a very innovative idea, but this technique only works for stable complexes, such as RNA polymerase II, SAGA, etc. In the case of Hog1, we obtained very low amounts of interacting proteins. To increase the faint bands obtained in the initial trials, we worked with larger volumes of cultures (until 16 l). Thanks to this modification, we began to observe bands strong enough to be identified by mass espectometry.

However, the problem was then about the sensitivity of the mass spectrometry facility. It was not sensible enough to identify the purified bands. We needed a previous Liquid Chromatography (LC) coupled to the Ion Trap Mass Espectometer to improve the sensibility. We began a collaboration with Gustav Ammerer's group at the BioZentrum in Viena. They were able to identify the purified bands, and new lines of work are being opened in the laboratory with these results.

The methods employed to detect new interactions of Hog1 were used in another project. We employed the TAP protocol coupled to photocrosslinking assays to identify which subunit of the RNA polymerase II interacted with the MAPK Hog1. This was my contribution to the article explained below.

The Stress-Activated Hog1 Kinase is a Selective Transcriptional Elongation Factor for Genes Responding to Osmotic Stress.

Markus Proft, Glòria Mas, Eulàlia de Nadal, Alexandre Vendrell, Núria Noriega, Kevin Struhl and Francesc Posas.

Molecular Cell, Vol 22 pp.241- 250, 21st July 2006.

In response to hyperosmolarity, the MAPK Hog1 coordinates the transcription programme required for cell adaptation. Hog1 tightly binds

chromatin in response to osmostress and elicits gene expression by mechanisms which were not completely understood. New insights into the mechanisms used by Hog1 to activate gene expression, other than simple modification of activators, were published previously. Specifically, it was published that Hog1 is directly bound to the chromatin (Alepuz et al, 2004). This binding is completely necessary to perform RNA polymerase II recruitment. This evidence prompted us to think that probably RNA polymerase II and Hog1 were interacting directly.

To answer this question, we planned a proteomics approach. We set up the TAP protocol to purify the RNA polymerase II complex. Then, we used a photocrosslinking assay to identify which subunit of RNA polymerase II complex was directly interacting with Hog1. The photocrosslinking assay is based in the SULFO-SBED (Pierce), a commercial molecule designed to bind covalently proteins that are at distances smaller than 10 nm.

Thanks to the photocrosslinking assay, we found that Hog1 and the RNA polymerase II subunit, Rpb1 were interacting. It was the first evidence that demonstrates a direct interaction between the MAPK Hog1 and the RNA polymerase II.

The interaction between Hog1 and the RNA polymerase II could occur at the promoters, since ChIP analysis had found Hog1 at the promoters (Alpeuz et al., 2004). However, maybe the recruiter function of Hog1 at the promoter was also necessary at the ORF for a proper elongation. For this, ChIP and "ChiP on ChIP" analysis were performed. Both of them verified that Hog1 is also bound at the ORF.

The role of Hog1 in elongation was completely unknown. To test whether Hog1 was essential for a proper elongation, we uncoupled initiation from elongation and we designed a plasmid with an osmoresponsive gene, *STL1,* under the LexA promoter. In this plasmid *STL1* expression is initiated by LexA-Vp16 expression in a Hog1 independent manner. Therefore, changes in RNA levels were due to an effect of Hog1 on elongation. Northern blot analysis showed an increase of *STL1* expression in a wild-type strain that was not observed in a *hog1*Δ strain. Correspondingly, ChIP analyses showed that RNA polymease II bound to the LexA promoter was not altered in a *hog1*Δ, whereas was highly reduced in the *STL1* ORF. Finally, Hog1 involvement in elongation

was confirmed by the coimmunoprecipitation of Hog1 with several elongation factors.

These results showed a new role for Hog1 in gene expression regulation by promoting elongation in osmoresponsive genes.

My personal contribution to this work (Proft et al., 2006) was focused on the photocrosslinking assay presented in Figure 1A. In addition, I also followed closely the whole work.

The Stress-Activated Hog1 Kinase is a Selective Transcriptional Elongation Factor for Genes Responding to Osmotic Stress

Markus Proft, Glòria Mas, Eulàlia de Nadal, Alexandre Vendrell, Núria Noriega, Kevin Struhl and Francesc Posas

Molecular Cell, Vol 22 pp.241- 250, 21st July 2006.

Proft M, Mas G, de Nadal E, Vendrell A, Noriega N, Struhl K, Posas F.
The stress-activated Hog1 kinase is a selective transcriptional elongation factor for genes responding to osmotic stress.
Mol Cell. 2006 Jul 21;23(2):241-50.

ARTICLE 2.

During the first two years of PhD spent in seeting up proteomical techniques, we were also involved in a European Project called QUASI. The QUASI project was a multidisciplinary project with the goal to obtain a coherent and detailed picture of the dynamic operation of a model signalling transduction network such as the Hog1 MAP kinase cascade module. MAP kinase pathways are currently being explored as drug targets since they are implicated in human diseases such as cancer and inflammatory disorders. A better understanding of the dynamic operation of these pathways offers new opportunities for drug discovery and for efficient individualised treatment based on the genetic setup of the patient (pharmacogenomics). To achieve the goals of QUASI, quantitative data of high definition on signal transduction activation and deactivation was obtained using frontline experimental approaches encompassing global gene expression, proteomics, bioimaging and chemical genetics.

Specifically, we were involved in the development of the chemical genetics approach. Initially, we created a collection of analog sensitive mutant alleles of *HOG1, PBS2, STE11, SSK2 and SSK22* (*hog1-as, pbs2-as, ste11-as, ssk2-as and ssk22-as* alleles) by replacing a conserved bulky residue with a glycine or alanine in the active site. Then, a complementary "bump" was created by attaching bulky substituents to ATP or to pyrazolo-pyrimidinel (PP1) derived inhibitors. The sensitization of the kinase's active site for a specific structurally modified labeled nucleotide analog provides a unique handle by which the direct substrates of any particular kinase can be traced in the presence of other protein kinases. Similarly, a cell-permeable, chemically modified "bumped" inhibitor in the presence of an analog sensitive mutant kinase is an extremely potent tool for dissecting the dynamic processes of any kinase of interest *in vivo.* The *as* collection was distributed to the QUASI partners to be employed in new projects.

Once the collection was done, we were involved in the second part of the chemical genetics approach. In collaboration with Professor Morten Grötli's laboratory, we developed a new selective orthogonal inhibitor that is able to inhibit *as* kinases faster than previous inhibitors. Specifically, we tested whether the new inhibitor could inhibit the Hog1 effect on cell cycle and gene expression faster than inhibitors previously described. This was my contribution to the article explained below.

Design, synthesis and characterization of a highly effective and fast acting orthogonal inhibitor for analog-sensitive (as) kinases

Michael Klein, Montse Morillas, Alexandre Vendrell, Lars Brive, Francesc Posas and Morten Grötli.

Submitted to **Angewandte Chemie**.

Signal transduction pathways are the cellular information routes with which cells monitor their surrounding as well as their own state and adjust to environmental changes or hormonal stimuli. Signalling encompasses the processes with which cells sense changes, generate intracellular signals, transduce the signal and ultimately mount a response. In doing so, signal transduction pathways orchestrate cellular metabolism, establish stress tolerance, control growth, proliferation and development and determine morphogenesis. Initially, the understanding of cellular signal transduction has been restricted to the wiring schemes of signaling pathways. However, it is also necessary to understand the dynamics of these pathways.

To study real-time cellular function of a kinase, highly selective cell permeable inhibitors of kinases have appeared (Bishop et al., 2000). This selective inhibitors are based on the modification of a kinase inhibitor to eliminate its binding affinity for its native target and subsequent mutation of a protein to allow it to recognize the orthogonal inhibitor.

However, the inhibitors reported are not fast enough to study signaling events that occurs within minutes, since a longer pre-incubation time with the inhibitor is needed. We have designed, synthesised and evaluated a selective ortogonal inhibitor that could inhibit *as* kinases faster than previous before.

Specifically, we have tested the ability of the new orthogonal inhibitor to decrease the *hog1-as* kinase effect on gene expression and cell cycle upon osmotic stress. We have shown that the new inhibitor acts faster than the old inhibitor on the Hog1 dependent gene expresión induction as well as in the cycle arrest induced by Hog1.

These results confirmed that the new inhibitor acts faster than the inhibitor described before.

My personal contribution to this work (Klein et al., submitted) was focused on the construction of the plasmids and yeast strains, as well as the performance of the β-galactosidase activity assays. In addition, I also followed closely the whole work.

The whole article is not included in this thesis because the compound described in the article is being submitted to a patent process.

REFERENCES

"Qui controla el passat, controla el futur"

George Orwell

Alepuz,P.M., Jovanovic,A., Reiser,V., and Ammerer,G. (2001). Stress-induced map kinase Hog1 is part of transcription activation complexes. Mol. Cell *7*, 767-777.

Alepuz,P.M., de Nadal,E., Zapater,M., Ammerer,G., and Posas,F. (2003). Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II. EMBO J *22*, 2433-2442.

Anderson, R.M., Bitterman, K.J., Wood, J.G., Medvedik, O., Cohen, H., Lin, S.S., Manchester, J.K., Gordon, J.I. and Sinclair, D.A. Manipulation of a nuclear NAD+ salvage pathway delays aging without altering steady-state NAD+ levels. J Biol Chem. (2002). 21:18881-90.

Anderson, R.M., Latorre-Esteves, M., Neves, A.R., Lavu, S., Medvedik, O., Taylor, C., Howitz, K.T., Santos, H. and Sinclair, D.A. (2003a) Yeast life-span extension by calorie restriction is independent of NAD fluctuation. *Science*. 302:2124-6.

Anderson RM, Bitterman KJ, Wood JG, Medvedik O, Sinclair DA (2003b) Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. *Nature* 423: 181–185.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W. and Elledge, S.J. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell*. (1996). 2:263-74.

Beck, T., Hall, M.N. (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature*. 402:689-92.

Bilsland-Marchesan,E., Arino,J., Saito,H., Sunnerhagen,P., and Posas,F. (2000). Rck2 kinase is a substrate for the osmotic stress-activated mitogen-activated protein kinase Hog1. *Mol. Cell Biol.* 20, 3887-3895.

Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M., and Sinclair, D.A. (2002). Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast Sir2 and human SirT1. *J. Biol. Chem*. 277:45099–45107.

Bitterman, K.J., Medvedik, O. and Sinclair, D.A. (2003) Longevity regulation in Saccharomyces cerevisiae: Linking metabolism, genome stability, and heterochromatin. *Microbiol Mol Biol Rev* 67: 376–399.

Blomberg,A. and Adler,L. (1992). Physiology of osmotolerance in fungi. *Adv. Microb. Physiol*. *33:145-212.*, 145-212.

Bordone, L. and Guarente, L. (2007). Sirtuins and beta-cell function. *Diabetes Obes Metab.* 2:23-7.

Bouveris A. and Cadenas E. (1982) Production of superoxide radicals and hydrogen peroxide in mitochondria*. L.W. Oberely Ed. CRC press*,.15–30.

Brewster,J.L., de Valoir,T., Dwyer,N.D., Winter,E., and Gustin,M.C. (1993). An osmosensing signal transduction pathway in yeast. *Science 259*, 1760-1763.

Brown,A.D. (1976). Microbial water stress. *Bacteriol. Rev*. *40*, 803-846.

Cadenas, E. and Davies, K.J. (2000). Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic. Biol. Med.* 29 222–230.

Candé, C., Cohen, I., Daugas, E., Ravagnan, L., Larochette, N., Zamzami, N. and Kroemer, G. (2002). Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. *Biochimie*. 84:215-22

Causton,H.C., Ren,B., Koh,S.S., Harbison,C.T., Kanin,E., Jennings,I.E., Lee,T.I., True,H.L., Lander,E.S., and Young,R.A. (2001). Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell.* 12, 323-337.

Cerami, A. (1985). Hypothesis: Glucose as a mediator of aging. *J. Am. Geriatr. Soc.* 33, 626-634.

Chan, W.H., Yu, J.S. and Yang, S.D. (1999) PAK2 is cleaved and activated during hyperosmotic shock-induced apoptosis via a caspase-dependent mechanism: evidence for the involvement of oxidative stress. *J Cell Physiol.* 178:397-408.

Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A. and Deshaies, R.J. (2001) Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes Dev.* 9:1078-92.

Cid,V.J., Shulewitz,M.J., McDonald,K.L., and Thorner,J. (2001). Dynamic localization of the Swe1 regulator Hsl7 during the Saccharomyces cerevisiae cell cycle. *Mol. Biol. Cell* 12, 1645-1669.

Ciechanover, A., Orian, A. and Schwartz, A.L. (2000) Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays*. 5:442-51. Review.

Clancy, D. J., Gems, D., Harshman, L. G., Oldham, L. G., Stocker, S., Hafen, E., Leevers, S. J. and Partridge, L. (2001). Extension of life-span by loss of CHICO, a Drosophila receptor substrate protein. *Science* 292:104-106.

Clotet,J., Escote,X., Adrover,M.A., Yaakov,G., Gari,E., Aldea,M., de Nadal,E., and Posas,F. (2006). Phosphorylation of Hsl1 by Hog1 leads to a G2 arrest essential for cell survival at high osmolarity. *EMBO J*. 25, 2338-2346.

Conaway, R.C., Brower, C.S. and Conaway, J.W. (2002) Emerging roles of ubiquitin in transcription regulation. *Science*. 296:1254-8. Review.
de Nadal,E., Alepuz,P.M., and Posas,F. (2002). Dealing with osmostress through MAP kinase activation. *EMBO Rep 3*, 735-740.

Danial, N.N. and Korsmeyer, S.J. (2004). Cell death: critical control points. *Cell*. 116:205-19.

De Nadal,E., Casadome,L., and Posas,F. (2003). Targeting the MEF2-like transcription factor Smp1 by the stress-activated Hog1 mitogen-activated protein kinase. *Mol. Cell Biol. 23*, 229-237.

De Wever, V., Reiter, W., Ballarini, A., Ammerer, G., and Brocard, C. (2005). A dual role for PP1 in shaping the Msn2-dependent transcriptional response to glucose starvation. *EMBO J.* 23:4115-23.

De Zutter, G.S. and Davis, R.J. Pro-apoptotic gene expression mediated by the p38 mitogen-activated protein kinase signal transduction pathway (2001). Proc Natl Acad Sci U S A. 98:6168-73.

Dejean, L.M., Martinez-Caballero, S. and Kinnally, K.W. (2006). Is MAC the knife that cuts cytochrome c from mitochondria during apoptosis? *Cell Death Differ.* 13:1387-95.

Deshaies, R.J. (1999) SCF and cullin/RING H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.* 15: 435–467.

Dihazi,H., Kessler,R., and Eschrich,K. (2004). High osmolarity glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress. *J. Biol. Chem. 279*, 23961-23968.

Dove,S.K., Cooke,F.T., Douglas,M.R., Sayers,L.G., Parker,P.J., and Michell,R.H. (1997). Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. *Nature*. *390*, 187-192.

Durchschlag, E., Reiter, W., Ammerer, G. and Schüller, C. Nuclear localization destabilizes the stress-regulated transcription factor Msn2 (2004). J Biol Chem. 53:55425-32.

Easlon, E., Tsang, F., Dilova, I., Wang, C., Lu, S.P., Skinner, C., Lin and S.J. (2007). The dihydrolipoamide acetyltransferase is a novel metabolic longevity factor and is required for calorie restriction-mediated life span extension. *J Biol Chem.* 282:6161-71.

Escote,X., Zapater,M., Clotet,J., and Posas,F. (2004). Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1. Nat. Cell Biol. *6*, 997-1002.
Estruch, F. (2000). Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiol Rev.* 24:469-86. Review.

Feldman, R.M., Correll, C.C., Kaplan, K.B. and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1. *Cell*. 2:221-30.

Fesik, S.W. and Shi, Y. (2001). Structural biology. Controlling the caspases. *Science*. 294:1477-8

Ferrigno,P., Posas,F., Koepp,D., Saito,H., and Silver,P.A. (1998). Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. *EMBO J* 17, 5606-5614.

Fritze, CE; Verschueren, K; Strich, R; Easton Esposito, R. Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. *EMBO J.* 1997;16:6495–6509.

Gallo CM, Smith DL Jr, Smith JS (2004) Nicotinamide clearance by Pnc1 directly regulates Sir2-mediated silencing and longevity. *Mol Cell Biol* 24: 1301–1312.

Garreau,H., Hasan,R.N., Renault,G., Estruch,F., Boy-Marcotte,E., and Jacquet,M. (2000). Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in Saccharomyces cerevisiae. *Microbiology*. 146, 2113-2120.

Galcheva-Gargova,Z., Derijard,B., Wu,I.H., and Davis,R.J. (1994). An osmosensing signal transduction pathway in mammalian cells. *Science 265*, 806-808.

Galvez, A., Morales, M.P., Eltit, J.M., Ocaranza, P., Carrasco, L., Campos, X., Sapag-Hagar, M., Díaz-Araya, G. and Lavandero, S. (2001) A rapid and strong apoptotic process is triggered by hyperosmotic stress in cultured rat cardiac myocytes. *Cell Tissue Res.* 304:279-85.

Garcia-Gimeno,M.A. and Struhl,K. (2000). Aca1 and Aca2, ATF/CREB activators in Saccharomyces cerevisiae, are important for carbon source utilization but not the response to stress. *Mol. Cell Biol.* 20, 4340-4349.

Gasch,A.P., Spellman,P.T., Kao,C.M., Carmel-Harel,O., Eisen,M.B., Storz,G., Botstein,D., and Brown,P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* 11, 4241-4257.

Gasch,A.P. and Werner-Washburne,M. (2002). The genomics of yeast responses to environmental stress and starvation. *Funct. Integr. Genomics. 2*, 181-192.

Gonzalez, F., Delahodde, A., Kodadek, T. and Johnston, S.A. (2002) Recruitment of a 19S proteasome subcomplex to an activated promoter. *Science*. 296:548-50.

Görner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., Schüller, C. (1998) Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev.* 12:586-97.

Guarente, L. (2000). Sir2 links chromatin silencing, metabolism, and aging. *Genes Dev.* 14:1021–1026.

Ghislain, M., Talla, E. and Francois, J.M. (2002). Identification and functional analysis of the Saccharomyces cerevisiae nicotinamidase gene, PNC1. *Yeast* 19: 215–224.

Gustin,M.C., Albertyn,J., Alexander,M., and Davenport,K. (1998). MAP kinase pathways in the yeast Saccharomyces cerevisiae. *Microbiol. Mol. Biol. Rev* 62, 1264-1300.

Halliwell B. and Gutteridge J.M. (1984). Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. *Lancet* 1:1396–1397.

Hamann, A., Brust, D. and Osiewacz, H.D. (2008). Apoptosis pathways in fungal growth, development and ageing. Trends Microbiol. 16:276-83.

Han, Z., Boyle, D.L., Chang, L., Bennet, B., Karin, M., Yang, L., et al. (2001). c-Jun N-terminal kinase is required for metalloproteinase expresión and joint destruction in inflammatory arthritis. *J. Clin Invest.* 108: 73–81.

Harris, N., Maclean, M., Hatzianthis, K., Panaretou, B. and Piper, P.W. (2001). Increasing Saccharomyces cerevisiae stress resistance, through overactivation of the heat shock response resulting from defects in the Hsp90 chaperone, does not extend the replicative life-span but can be associated with slower chronological ageing of nondividing cells. *Mol. Genet. Genome* 265, 258-263.

Heinisch, J.J., Lorberg, A., Schmitz, H.P., Jacoby, J.J. (1999) The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in Saccharomyces cerevisiae. *Mol Microbiol.* 4:671-80. Review.

Herker, E., Jungwirth, H., Lehmann, K.A., Maldener, C., Fröhlich, K.U., Wissing, S., Büttner, S., Fehr, M., Sigrist, S. and Madeo, F. (2004) Chronological aging leads to apoptosis in yeast. *J Cell Biol.* 164:501-7.

Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem*. 67:425-79. Review.

Hohmann, S. and Mager, W.H. (1997). *Yeast Stress Responses.* R.G. Landes Company, Austin TX.

Hohmann,S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev* 66, 300-372.

Howitz, K.T., Bitterman, K.J., Cohen, H.Y., Lamming, D.W., Lavu, S., et al. (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* 425:191–196.

Huang, P., Feng, L., Oldham, E.A., Keating, M.J. and Plunkett, W. (2000) Superoxide dismutase as a target for the selective killing of cancer cells. *Nature*. 407:390-5.

Huang, J. and Moazed, D. (2003) Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. *Genes Dev* 17: 2162–2176.

Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J.D. (2000) The lore of the RINGs: Substrate recognition and catalysis by ubiquitin ligases. *Trends Cell Biol.* 10: 429–439.

Jacobson, M.D., Weil, M. and Raff, M.C. (1997). Programmed cell death in animal development. *Cell*. 88:347-54.

Jacoby,T., Flanagan,H., Faykin,A., Seto,A.G., Mattison,C., and Ota,I. (1997). Two protein-tyrosine phosphatases inactivate the osmotic stress response pathway in yeast by targeting the mitogen-activated protein kinase, Hog1. *J Biol. Chem. 272*, 17749-17755.

Jansen,G., Buhring,F., Hollenberg,C.P., and Ramezani,R.M. (2001). Mutations in the SAM domain of STE50 differentially influence the MAPK-mediated pathways for mating, filamentous growth and osmotolerance in Saccharomyces cerevisiae. *Mol. Genet. Genomics. 265*, 102-117.

Kaeberlein, M., McVey, M. and Guarente, L. (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. *Genes Dev* 13: 2570–2580.

Kaeberlein M, Kirkland KT, Fields S, Kennedy BK (2004) Sir2-independent lifespan extension by calorie restriction in yeast. *PLoS Biol* 2: e296.

Kaeberlein, M., Powers, R.W., Steffen, K.K., Westman, E.A., Hu, D., et al. (2005). Regulation of yeast replicative lifespan by TOR and Sch9 in response to nutrients. *Science.* 310:1193–1196.

Kaeberlein, M., Burtner, C.R., Kennedy, B.K. (2007). Recent developments in yeast aging. *PLoS Genet.*3:e84.

Kapahi, P., Zid, B.M., Harper, T., Koslover, D., Sapin, V., et al. (2004). Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway. *Curr Biol.* 14:885–890.

Karin, M. and Hunter, T. (1995). Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr Biol.* 5:747-57. Review.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. (1972). 26:239-57.

Kobayashi, G.S., Cheung, S.S., Schlessinger, D. and Medoff, G. (1974). Effects of rifamycin derivatives, alone and in combination with amphotericin B, against Histoplasma capsulatum. *Antimicrob. Agents. Chemother.* 5:16-8.

Kodadek, T., Sikder, D. and Nalley, K. (2006) Keeping transcriptional activators under control. *Cell*. 2:261-4. Review.

Krisak,L., Strich,R., Winters,R.S., Hall,J.P., Mallory,M.J., Kreitzer,D., Tuan,R.S., and Winter,E. (1994). SMK1, a developmentally regulated MAP kinase, is required for spore wall assembly in Saccharomyces cerevisiae. *Genes Dev. 8*, 2151-2161.

Kyriakis,J.M. and Avruch,J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev 81*, 807-869.

Lamming DW, Latorre-Esteves M, Medvedik O, Wong SN, Tsang FA, et al. (2005) HST2 mediates SIR2-independent life-span extension by calorie restriction. Science 309: 1861–1864.

Landry, J., Slama, J.T., and Sternglanz, R. (2000). Role of NAD(+) in the deacetylase activity of the Sir2-like proteins. *Biochem. Biophys. Res. Commun.* 278, 685–690.

Laun, P., Pichona, A., Madeo, F., Fuchs, J., Ellinger, A., Kohlwein, S., Dawes, I., Fröhlich, K.U. and Breitenbach, M. Aged mother cells of Saccharomyces cerevisiae show markers of oxidative stress and apoptosis. Mol Microbiol. 2001 Mar;39(5):1166-73.

Ligr, M., Madeo, F., Fröhlich, E., Hilt, W., Fröhlich, K.U. and Wolf, D.H. (1998). Mammalian Bax triggers apoptotic changes in yeast. *FEBS Lett.* 438:61-5.

Lin, K., Dorman, J. B., Rodon, A. and Kenyon, C. (1997). Daf-16, A HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science*. 278:1319-1322.

Lin, S.J., Defossez, P.A. and Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. *Science*. 289:2126-8.

Lipford, J. R. and Deshaies, R. J. (2003) Diverse roles for ubiquitin-dependent proteolysis in transcriptional activation. *Nature Cell Biol.* 5, 845–-850.

Lipford, J.R., Smith, G.T., Chi, Y. and Deshaies, R.J. (2005) A putative stimulatory role for activator turnover in gene expression. *Nature*. 438:113-6.

Ludovico, P., Sousa, M.J., Silva, M.T., Leão, C. and Côrte-Real, M. (2001). Saccharomyces cerevisiae commits to a programmed cell death process in response to acetic acid. *Microbiology*. 147:2409-15.

Ludovico, P., Rodrigues, F., Almeida, A., Silva, M.T., Barrientos, A. and Côrte-Real, M. (2002) Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in Saccharomyces cerevisiae. *Mol Biol Cell.* 13:2598-606.

Maclean, M., Harris, N. and Piper, P. (2001). Chronological lifespan of stationary phase yeast cells ; a model for investigating the factors that might in£uence the ageing of post mitotic tissues in higher organisms. *Yeast* 18, 499-509.

Madeo, F., Fröhlich, E. and Fröhlich, K.U. (1997) A yeast mutant showing diagnostic markers of early and late apoptosis. *J. Cell. Biol.* 139:729-34.

Madeo, F., Fröhlich, E., Ligr, M., Grey, M., Sigrist S.J., Wolf, D.H. and Fröhlich, K.U. (1999) Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biol.* 145:757-67.

Madeo, F., Engelhardt, S., Herker, E., Lehmann, N., Maldener, C., et al. (2002) Apoptosis in yeast: A new model system with applications in cell biology and medicine. *Curr. Genet.* 41: 208–216.

Maeda,T., Wurgler-Murphy,S.M., and Saito,H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* 369, 242-245.

Maeda,T., Takekawa,M., and Saito,H. (1995). Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science* 269, 554-558.

Marchler,G., Schuller,C., Adam,G., and Ruis,H. (1993). A Saccharomyces cerevisiae UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO J.* 12, 1997-2003.

Martinez-Pastor,M.T., Marchler,G., Schuller,C., Marchler-Bauer,A., Ruis,H., and Estruch,F. (1996). The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J *15*, 2227-2235.

Maskell, D.L., Kennedy, A.I., Hodgson, J.A. and Smart, K.A. (2003). Chronological and replicative lifespan of polyploid Saccharomyces cerevisiae (syn. S. pastorianus). *FEMS Yeast Res*. 2:201-9.

Masoro EJ. (1989). Overview of the effects of food restriction. *Prog Clin Biol Res*. 287:27-35. Review.

Mattison,C.P., Spencer,S.S., Kresge,K.A., Lee,J., and Ota,I.M. (1999). Differential regulation of the cell wall integrity mitogen-activated protein kinase pathway in budding yeast by the protein tyrosine phosphatases Ptp2 and Ptp3. *Mol. Cell Biol.* 19, 7651-7660.

McKinsey, T.A., Zhang, C.L., Olson, E.N. (2002) Signaling chromatin to make muscle. *Curr. Opin. Cell. Biol.* 14:763-72. Review.

Medvedik, O., Lamming, D.W., Kim, K.D., Sinclair, D.A. (2007) MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in Saccharomyces cerevisiae. PLoS Biol. 5:e261.

Milne, J.C., Lambert, P.D., Schenk, S., Carney, D.P., Smith, J.J., Gagne, D.J., Jin, L. et al. (2007). Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature.* 450:712-6.

Moskvina, E., Schüller, C., Maurer, C.T., Mager, W.H. and Ruis, H. (1998) A search in the genome of Saccharomyces cerevisiae for genes regulated via stress response elements. *Yeast*. 11:1041-50.

Muratani, M. and Tansey, W. P. (2003) How the ubiquitin–-proteasome system controls transcription. *Nature Rev. Mol. Cell Biol.* 4, 192–-201

Nehlin,J.O., Carlberg,M., and Ronne,H. (1992). Yeast SKO1 gene encodes a bZIP protein that binds to the CRE motif and acts as a repressor of transcription. Nucleic Acids Res. *20*, 5271-5278.

Nierras, C.R. and Warner, J.R. (1999) Protein kinase C enables the regulatory circuit that connects membrane synthesis to ribosome synthesis in Saccharomyces cerevisiae *J Biol Chem.* 19:13235-41.

Norbeck,J. and Blomberg,A. (2000). The level of cAMP-dependent protein kinase A activity strongly affects osmotolerance and osmo-instigated gene expression changes in Saccharomyces cerevisiae. *Yeast* 16, 121-137.

O'Rourke,S.M. and Herskowitz,I. (1998). The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in Saccharomyces cerevisiae. *Genes Dev. 12*, 2874-2886.

O'Rourke,S.M. and Herskowitz,I. (2004). Unique and redundant roles for HOG MAPK pathway components as revealed by whole-genome expression analysis. Mol. Biol. Cell. *15*, 532-542.

Oskouian, B., Sooriyakumaran, P., Borowsky, A.D., Crans, A., Dillard-Telm, L., Tam, Y.Y., Bandhuvula, P. and Saba, J.D. (2006). Sphingosine-1-phosphate lyase potentiates apoptosis via p53- and p38-dependent pathways and is down-regulated in colon cancer. Proc Natl Acad Sci U S A. 103:17384-9.

Patton, E.E., Willems, A.R. and Tyers, M. (1998). Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis, *Trends Genet.* 14:236–243.

Pelicano, H., Feng, L., Zhou, Y., Carew, J.S., Hileman, E.O., Plunkett, W., Keating, M.J. and Huang, P. (2003). Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. *J Biol Chem.* 278:37832-9.

Perrone, G.G., Tan, S.X. and Dawes, I.W. (2008). Reactive oxygen species and yeast apoptosis. *Biochim Biophys Acta.* 1783:1354-68.

Pickart, C. M. (2004). Back to the future with ubiquitin. *Cell* 116, 181-190.

Posas,F., Wurgler-Murphy,S.M., Maeda,T., Witten,E.A., Thai,T.C., and Saito,H. (1996). Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. *Cell* 86, 865-875.

Posas,F. and Saito,H. (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. Science *276*, 1702-1705.

Posas,F., Witten,E.A., and Saito,H. (1998). Requirement of STE50 for osmostress-induced activation of the STE11 mitogen-activated protein kinase kinase kinase in the high-osmolarity glycerol response pathway. *Mol. Cell Biol*. 18, 5788-5796.

Posas,F., Chambers,J.R., Heyman,J.A., Hoeffler,J.P., de Nadal,E., and Arino,J. (2000). The transcriptional response of yeast to saline stress. *J Biol. Chem.* 275, 17249-17255.

Proft,M. and Serrano,R. (1999). Repressors and upstream repressing sequences of the stress-regulated ENA1 gene in Saccharomyces cerevisiae: bZIP protein Sko1p confers HOG-dependent osmotic regulation. Mol. Cell Biol. *19*, 537-546.

Proft,M., Pascual-Ahuir,A., de Nadal,E., Arino,J., Serrano,R., and Posas,F. (2001). Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. EMBO J *20*, 1123-1133.

Proft,M. and Struhl,K. (2002). Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. Mol. Cell *9*, 1307-1317.

Proft,M. and Struhl,K. (2004). MAP kinase-mediated stress relief that precedes and regulates the timing of transcriptional induction. *Cell* 118, 351-361.

Proft,M., Gibbons,F.D., Copeland,M., Roth,F.P., and Struhl,K. (2005). Genomewide Identification of Sko1 Target Promoters Reveals a Regulatory Network That Operates in Response to Osmotic Stress in Saccharomyces cerevisiae. Eukaryot. Cell *4*, 1343-1352.

Raitt,D.C., Posas,F., and Saito,H. (2000). Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. *EMBO J* 19, 4623-4631.

Reed SI. (2003) Ratchets and clocks: the cell cycle, ubiquitination and protein turnover. *Nat Rev Mol Cell Biol.* 11:855-64. Review

Reiser,V., Ruis,H., and Ammerer,G. (1999). Kinase activity-dependent nuclear export opposes stress-induced nuclear accumulation and retention of Hog1 mitogen-activated protein kinase in the budding yeast Saccharomyces cerevisiae. Mol. Biol. Cell *10*, 1147-1161.

Reiser,V., Salah,S.M., and Ammerer,G. (2000). Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42. *Nat. Cell Biol. 2*, 620-627.

Rep,M., Albertyn,J., Thevelein,J.M., Prior,B.A., and Hohmann,S. (1999a). Different signalling pathways contribute to the control of GPD1 gene expression by osmotic stress in Saccharomyces cerevisiae. *Microbiology* 145 *(3)*, 715-727.

Rep,M., Reiser,V., Gartner,U., Thevelein,J.M., Hohmann,S., Ammerer,G., and Ruis,H. (1999b). Osmotic stress-induced gene expression in Saccharomyces cerevisiae requires Msn1p and the novel nuclear factor Hot1p. Mol. Cell Biol. *19*, 5474-5485.

Rep,M., Krantz,M., Thevelein,J.M., and Hohmann,S. (2000). The transcriptional response of Saccharomyces cerevisiae to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. *J Biol. Chem.* 275, 8290-8300.

Ribeiro, G.F., Côrte-Real, M. and Johansson, B. (2006). Characterization of DNA damage in yeast apoptosis induced by hydrogen peroxide, acetic acid, and hyperosmotic shock. Mol Biol Cell. 17:4584-91.

Ruis,H. and Schuller,C. (1995). Stress signaling in yeast. *Bioessays*. *17*, 959-965.

Schuller, C., Brewster,J.L., Alexander,M.R., Gustin,M.C., and Ruis,H. (1994). The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the Saccharomyces cerevisiae CTT1 gene. *EMBO J* 13, 4382-4389.

Schmitt, A.P. and McEntee,K. (1996). Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. *93*, 5777-5782.

Schmitz, I., Kirchhoff, S. and Krammer, P.H. Regulation of death receptor-mediated apoptosis pathways. (2000). *Int J Biochem Cell Biol.* 32:1123-36.

Schwob, E., Bohm, T., Mendenhall, M.D. and Nasmyth, K. (1994) The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae, *Cell* 79:233– 244.

Seol, J.H., Feldman, R.M., Zachariae, W., Shevchenko, A., Correll, C.C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., et al. (1999) Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. *Genes* & *Dev.* 13: 1614–1626.

Severin, F.F. and Hyman A.A. (2002). Pheromone induces programmed cell death in S. cerevisiae. Curr Biol. 12:R233-5.

Sheikh-Hamad and Gustin, 2004. MAP kinases and the adaptive response to hypertonicity: functional preservation from yeast to mammals. *Am J Physiol Renal Physiol.* 287(6):F1102-10. Review.

Shulewitz,M.J., Inouye,C.J., and Thorner,J. (1999). Hsl7 localizes to a septin ring and serves as an adapter in a regulatory pathway that relieves tyrosine phosphorylation of Cdc28 protein kinase in Saccharomyces cerevisiae. *Mol. Cell Biol.* 19, 7123-7137.

Sinclair, D.A. and Guarente, L. (1997) Extrachromosomal rDNA circles-a cause of aging in yeast. Cell. 7:1033-42.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J. and Harper, J.W. (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell*. 2:209-19.

Sohal, R.S. and Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. *Science*. 273:59-63.

Strahl-Bolsinger, S., Hecht, A., Luo, K. and Grunstein, M. (1997) SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev* 11: 83–93.

Tamas,M.J., Rep,M., Thevelein,J.M., and Hohmann,S. (2000). Stimulation of the yeast high osmolarity glycerol (HOG) pathway: evidence for a signal generated by a change in turgor rather than by water stress. FEBS Lett. *472*, 159-165.

Tatebayashi,K., Yamamoto,K., Tanaka,K., Tomida,T., Maruoka,T., Kasukawa,E., and Saito,H. (2006). Adaptor functions of Cdc42, Ste50, and Sho1 in the yeast osmoregulatory HOG MAPK pathway. *EMBO J*. *25*, 3033-3044.

Tatebayashi, K., Tanaka, K., Yang, H.Y., Yamamoto, K., Matsushita, Y., Tomida, T., Imai, M., and Saito, H. (2007). Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. *EMBO J.* 26:3521-33.

Teige,M., Scheikl,E., Reiser,V., Ruis,H., and Ammerer,G. (2001). Rck2, a member of the calmodulin-protein kinase family, links protein synthesis to high osmolarity MAP kinase signaling in budding yeast. *Proc. Natl. Acad. Sci. U. S. A* 98, 5625-5630.

Thrower, J.S., Hoffman, L., Rechsteiner, M. and Pickart, C.M. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J*. 1:94-102.

Treger, J.M., Magee, T.R., and McEntee, K. (1998) Functional analysis of the stress response element and its role in the multistress response of Saccharomyces cerevisiae. *Biochem. Biophys. Res. Commun.* 1:13-9.

Truckses,D.M., Bloomekatz,J.E., and Thorner,J. (2006). The RA domain of Ste50 adaptor protein is required for delivery of Ste11 to the plasma membrane in the filamentous growth signaling pathway of the yeast Saccharomyces cerevisiae. *Mol. Cell Biol. 26*, 912-928.

Uren, A.G., O'Rourke, K., Aravind, L.A., Pisabarro, M.T., Seshagiri, S., Koonin, E.V. and Dixit, V.M. (2000) Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol. Cell.* 6:961-7.

Uesono Y., and Toh-E. A. (2002) Transient inhibition of translation initiation by osmotic stress. *J Biol Chem.* 277(16):13848-55.

Van Wuytswinkel,O., Reiser,V., Siderius,M., Kelders,M.C., Ammerer,G., Ruis,H., and Mager,W.H. (2000). Response of Saccharomyces cerevisiae to severe osmotic stress: evidence for a novel activation mechanism of the HOG MAP kinase pathway. *Mol. Microbiol.* 37, 382-397.

Vaux, D.L. and Korsmeyer, S. J. (1999). Cell death in development. *Cell*. 96:245-54.

Vellai, T., Takacs-Vellai, K., Zhang, Y., Kovacs, A.L., Orosz, L., et al. (2003) Genetics: Influence of TOR kinase on lifespan in C. elegans. *Nature.* 426:620.

Vincent,A.C. and Struhl,K. (1992). ACR1, a yeast ATF/CREB repressor. *Mol. Cell Biol. 12*, 5394-5405.

Visintin, R., Prinz, S. and Amon, A. (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science* 278:460–463.

Warmka,J., Hanneman,J., Lee,J., Amin,D., and Ota,I. (2001). Ptc1, a type 2C Ser/Thr phosphatase, inactivates the HOG pathway by dephosphorylating the mitogen-activated protein kinase Hog1. *Mol. Cell Biol.* 21, 51-60.

Willems, A.R., Goh, T., Taylor, L., Chernushevich, I., Shevchenko, A. and Tyers, M. (1999) SCF ubiquitin protein ligases and phosphorylation-dependent proteolysis. *Philos Trans R Soc Lond B Biol Sci*. 354:1533-50.

Willems, A.R., Schwab, M. and Tyers, M. (2004) A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim Biophys Acta*. 1695:133-70. Review.

Wissing, S., Ludovico, P., Herker, E., Büttner, S., Engelhardt, S.M., Decker, T., Link, A., Proksch, A., Rodrigues, F., Corte-Real, M., Fröhlich, K.U., Manns, J., Candé, C., Sigrist, S.J., Kroemer, G. and Madeo, F. (2004). An AIF orthologue regulates apoptosis in yeast. *J. Cell. Biol.* 166:969-74.

Wood, J.G., Rogina, B., Lavu, S., Howitz, K., Helfand, S.L., et al. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature.* 430:686–689.

Wright, A.R. and Rees, S.A. (1998) Cardiac cell volume: crystal clear or murky waters? A comparison with other cell types. *Pharmacol Ther.* 80:89-121.

Wurgler-Murphy,S.M., Maeda,T., Witten,E.A., and Saito,H. (1997). Regulation of the Saccharomyces cerevisiae HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. *Mol. Cell Biol.* 17, 1289-1297.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. *Cell.* 124:471–484.

Yang, S.H., Sharrocks, A.D., and Whitmarsh, A.J. (2003) Transcriptional regulation by the MAP kinase signaling cascades. *Gene* 320:3-21.

Zuin, A., Gabrielli, N., Calvo, I.A., García-Santamarina, S., Hoe, K.L., Kim, D.U., Park, H.O., Hayles, J., Ayté, J., and Hidalgo, E. (2008). Mitochondrial dysfunction increases oxidative stress and decreases chronological lifespan in fission yeast. *PLoS ONE*. 3:e2842.