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Respuesta de las Levaduras al Estrés Osmótico Causado por Elevadas Concentraciones de Glucosa: Función de las Proteínas Hgi1 y Hot1

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Director: Marcel·lí del Olmo Muñoz

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Memoria presentada por Mercè Gomar i Alba
Para optar al grado de Doctora por la Universitat de València

Director: Marcel·lí del Olmo Muñoz

MARCEL·LÍ DEL OLMO MUÑOZ, Doctor en Ciencias Biológicas y Profesor Titular del Departament de Bioquímica i Biologia Molecular de la Universitat de València,

INFORMA: que Mercè Gomar i Alba, Licenciada en Biología por la Universitat de Valencia, ha realizado bajo su dirección el trabajo que con el título "Respuesta de las Levaduras al Estrés Osmótico Causado por Elevadas Concentraciones de Glucosa: Función de las Proteínas Hgi1 y Hot1" presenta para optar al grado de Doctora por la Universitat de València.

Burjassot, Julio de 2015

Dr. Marcel·lí del Olmo Muñoz

Als meus pares

En esta vida no hay nada incomprensible,
cuando quiere uno tomarse la molestia de comprender...

Joseph Rouletabille

Agraïments

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Agradecimientos

Índice

Abreviaturas

3AT: 3-amino-1,2,4-triazol

6-AU: 6-azauracilo

ADH: alcohol deshidrogenasa

ADP: adenosina difosfato

AMP: adenosina monofosfato

ATP: adenosina trisfosfato

BSA: seroalbúmina bovina

cAMP: AMP cíclico

CDK: quinasas dependientes de ciclinas

cDNA: *complementary DNA*

CFW: *Calcofluor White*

ChIP: inmunoprecipitación de cromatina

CHX: cicloheximida

CoIP: coinmunoprecipitación

Ct: carboxiterminal

CTD: *carboxy terminal domain*

CWI: integridad de la pared celular

Cy3/5: *cyanine Dye 3/5* (fluoróforo)

DAPI: diclorohidrato de 4', 6'-diamidino-2-fenilindol

DBD: dominio de unión al DNA

dCTP: desoxicitosina trisfosfato

DMA: dimetil adipimidato

DNA: ácido desoxirribonucleico

dNTPs: desoxinucleósidos trisfosfato

DTT: 1,4-ditioeritrol

EDTA: ácido etilén diamino tetra-acético

eEFs: factores de elongación traduccional eucariotas

EGS: ester etilenglicol disuccionato bis(sulfo-N-succinimidyl)

eIFs: factores de inicio de traducción eucariotas

EMSA: *Electrophoretic mobility shift assays*

ESR: respuesta a estrés ambiental

FAD^+: dinucleótido de flavina y adenina (forma oxidada)

FADH: dinucleótido de flavina y adenina (forma reducida)

FG: crecimiento filamentoso

FSR: respuesta a estrés fermentativo

GAE: elemento GA

GDP: guanosina difosfato

GEO: *Gene Expression Omnibus*

GFP: *green fluorescent protein*

GMP: guanosina monofosfato

GO: ontología génica

GST: Glutation-S-transferasa

GTFs: factores generales de transcripción

GTP: guanosina trifosfato

HA: hemaglutinina

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesufonic acid)

HK: histidina quinasa

HOG: *high osmolarity glycerol*

HoRE*: Hog1-Responsive Element*

Inr: elemento iniciador de transcripción

IP: inmunoprecipitado

iRES: *Internal Ribosome Entry Sequences*

Kb: kilobase

KDa: kilodalton

LB: medio *Luria-Bertani*

MAPK: *mitogen-activated protein kinase*

MAPKK: MAPK quinasa

MAPKKK: MAPKK quinasa

min: minutos

MOPS: ácido 3-(N-morfolino) propanosulfónico

mRNA: RNA mensajero

mRNPs: ribonucleoparticulas de mRNA

NAD^+: dinucleótido de nicotinamida y adenina (forma oxidada)

NADH: dinucléotido de nicotinamida y adenina (forma reducida)

NCBI: *National Center for Biotechnology Informaton*

NCR: represión por catabolito de nitrógeno

NPC: complejo del poro nuclear

Nt: aminoterminal

ºC: grado centígrado

OD$_{600}$: *optical density* (a 600nm)

ONPG: *ortho-Nitrophenyl-β-galactoside*

ORF: *open reading frame* (pauta abierta de lectura)

p/v o w/v: peso/volumen

PAP: peroxidasa anti peroxidasa

pb: par de bases

PBS: *Phosphate-Buffered Saline*

PCR: reacción en cadena de la polimerasa

PDS: *post-diauxic shift*

PEG: poletilenglicol

PH: feromonas

PIC: complejo de pre-inicio de la transcripción

PKA: proteína quinasa A

PKC: proteína quinasa C

PMSF: fluoruro de fenilmetilsulfonilo

PT: proteína total

RBPs: proteínas de unión a RNA

RNA: ácido ribonucleico

RNApolII: RNA polimerasa II

rNTP: ribonucleósido trifosfato

rpm: revoluciones por minuto

rRNA: RNA ribosómico

RT-PCR: PCR a tiempo real

RT: retrotranscripción

RTG: señalización retrógrada mitocondrial

SAPK: *stress-activated protein kinase*

SBDS: *Síndrome de Shwachman-Bodian-Diamond*

SC: *syntetic complete medium*

SCSIE: Servei Central de Suport a la Investigació Experimental

SDS-PAGE: electroforesis en gel de poliacrilamida con SDS

SDS: dodecilsulfato sódico

SGD: *Saccaromyces Genome Database*

STRE: elemento de respuesta a estrés

TAE: tris-acetato sódico EDTA

TAFs: factores asociados a TBP

TAP: *Tandem Affinity Purification*

TBE: tris-borato EDTA

TBP: proteína de unión a caja TATA

TBS: *Tris-Buffered Saline*

TCA: ácido tricloroacético

TEMED: N,N,N',N', tetrametileno-diamino

TOR: diana de rapamicina

Tris: Tris (hidroximetil) aminometano

tRNA: RNA detransferencia

UAS: *Upstream Activation Sequence*

UV: ultravioleta

v/v: volumen/volumen

WT: *wild type*, cepa silvestre

Y8RE: *Yap8 Response Element*

YP20: YPD glucosa 20% (p/v)

YP30: YPD glucosa 30% (p/v)

YPD: *yeast extract peptone dextrose medium* (medio rico con extracto de levadura, bactopeptona y glucosa)

YPE10: YPD etanol 10% (v/v)

YPNaCl: YPD cloruro sódico

YPS: YPD sorbitol 1M

Aminoácidos, código de tres y una letras

Cualquier aminoácido	Xaa	X			
Alanina	Ala	A	Leucina	Leu	L
Arginina	Arg	R	Lisina	Lys	K
Asparragina	Asn	N	Metionina	Met	M
Ácido Aspártico	Asp	D	Fenilalanina	Phe	F
Cisteína	Cys	C	Prolina	Pro	P
Glutamina	Gln	Q	Serina	Ser	S
Ácido Glutámico	Glu	E	Treonina	Thr	T
Glicina	Gly	G	Triptófano	Trp	W
Histidina	His	H	Tirosina	Tyr	Y
Isoleucina	Iso	I	Valina	Val	V

1. Introducción

1.1. La expresión génica y su regulación en *Saccharomyces cerevisiae*

La levadura como organismo modelo

Tradicionalmente la ciencia ha hecho uso de los organismos modelo, que proporcionan a la investigación tanto un marco en el que desarrollar y optimizar métodos analíticos como una salida a las limitaciones éticas y experimentales existentes en algunos organismos de destino (Karathia y col., 2011). Por definición, un organismo modelo debe ser representativo de un grupo más grande de seres vivos, en cuyos fenómenos y procesos hay un interés general. En ese sentido, la levadura *S. cerevisiae* representa una excelente opción para el estudio de procesos biológicos esenciales en eucariotas.

El hecho de ser un organismo unicelular, de rápido crecimiento y sencilla manipulación, con un genoma completamente secuenciado, y la posibilidad de trabajar con formas haploides del mismo, hacen de este un organismo ideal para poder llevar a cabo las aproximaciones experimentales pertinentes para analizar los principales procesos bioquímicos de eucariotas (Fields y Johnston, 2005). *S. cerevisiae* es usado como modelo de estudio de regulación de la expresión génica, transducción de señales, ciclo celular, envejecimiento, metabolismo, apoptosis y desordenes neurodegenerativos, entre otros. Pero a pesar de que el genoma completo de la levadura (con sus más de 6000 genes en 16 cromosomas) fue secuenciado en 1996, y que aproximadamente el 30% de los genes codificantes de proteínas tienen homólogos en mamíferos (Botstein y col., 1997), gran parte de los genes de levadura permanecen sin función conocida, por lo que aún estamos lejos de entender en toda su complejidad las bases moleculares de procesos esenciales para la vida.

Transmisión de la información genética en *S. cerevisiae*

En Biología Molecular el concepto de identidad celular va estricta e indiscutiblemente ligado a los genes, que definen las particularidades de los organismos. En 1953 Watson y Crick desvelaron la estructura tridimensional del DNA e identificaron su función como molécula responsable de la herencia. Poco después, al demostrarse que el DNA podía ser copiado, se postulaba el dogma central de la Biología Molecular, donde quedaba impreso el flujo de información en el proceso de expresión génica, desde el ácido nucleico a la proteína.

Transcripción por la RNA polimerasa II

Dentro del flujo de transmisión de la información genética, la transcripción es el proceso de síntesis enzimática de un RNA a partir de una secuencia de DNA complementaria. En eucariotas, la transcripción de genes que codifican proteínas es llevada a cabo por la RNA polimerasa II (RNApolII), que selecciona el ribonucleósido trifosfato (rNTP) adecuado complementario a la plantilla de DNA, cataliza la formación del enlace fosfodiéster y se transloca a la siguiente posición, permitiendo la repetición de este ciclo la formación de la cadena de RNA. Superpuesto a este ciclo enzimático, la transcripción tiene una serie de etapas, inicio, elongación y terminación, en las que la RNApolII actúa junto con varios factores auxiliares que regulan y promueven el proceso de expresión génica (Perales y Bentley, 2009).

Inicio

En la etapa de inicio, la RNApolII se une al promotor y forma, junto con otros factores generales de transcripción (GTFs), el Complejo de Pre-Inicio (PIC) (Perales y col., 2013).

Los promotores de los genes transcritos por la RNApolII tienen un núcleo o *core* formado por la caja TATA (secuencia rica en A/T situada a unos 25-30 nucleótidos en posición 5' respecto al sitio de inicio transcripcional) que es reconocida por la proteína de

unión a la caja TATA (TBP) (Smale y Kadonaga, 2003) y un elemento iniciador (Inr), rico en pirimidinas, que define y dirige un inicio de transcripción preciso (Breathnach y Chambon, 1981). Algunos genes no tienen caja TATA y poseen en su lugar otra secuencia que hace la misma función (el elemento GA, GAE, Seizl y col., 2011). Además de la caja TATA o GAE y del Inr, los promotores de levadura tienen otras secuencias y la combinación de ellas da lugar a una gran diversidad de promotores distintos. Todos estos elementos son secuencias características de DNA que hacen posible el correcto ensamblaje del PIC en el lugar adecuado del promotor.

El ensamblaje empieza con la unión de la TBP (que conforma, junto a los Factores Asociados a TBP o TAFs, el factor de inicio TFIID), mediada por activadores transcripcionales y en coordinación con la estructura global de la cromatina (Agalioti y col., 2002; Thomas y Chiang, 2006), tal como se muestra en la Figura 1.1.
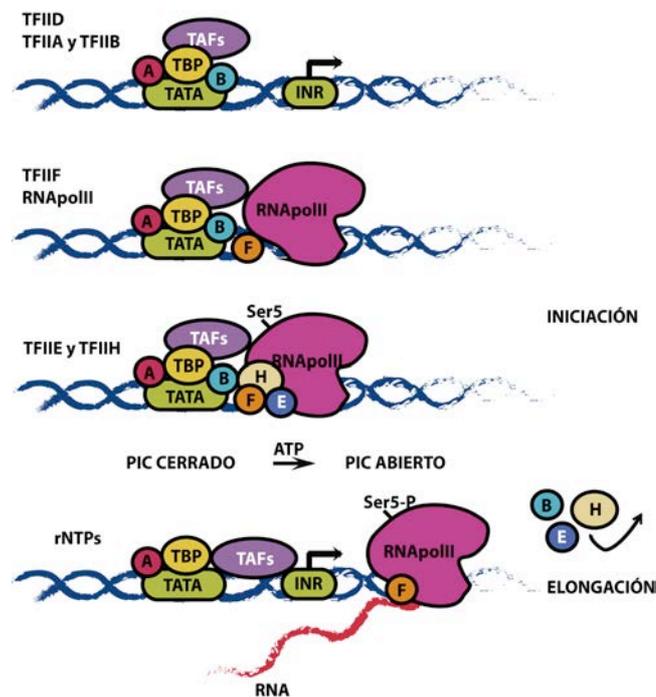
Figura 1.1. Modelo del ensamblaje y función del complejo de preinicio (PIC) durante la etapa de inicio de la transcripción. Los factores activadores de la transcripción dirigen al promotor la proteína de unión a la caja TATA (TBP). La RNApolII es reclutada al elemento iniciador Inr tras la unión de los factores TFIIA y B. El PIC se forma con la entrada de TFIIE y TFIIH, siendo este último el encargado de fosforilar la cola CTD de la RNApolII, lo que le permite iniciar su movimiento de alejamiento del promotor y dar paso a la elongación tanscripcional. Adaptado de Martínez (2002).

Recientemente se ha descrito que también pueden intervenir en esta etapa el Mediador, complejo co-activador que hace de puente entre activadores y maquinaria basal de transcripción y está presente en la mayoría de promotores dependientes de RNApolII (Thomas y Chiang, 2006) y SAGA, complejo acetil-transferasa que se recluta en los promotores con caja TATA e interacciona con activadores, TBP y la histona H3 (Sikorski y Buratowski, 2009). Cuando TBP se une al DNA, se incorpora TFIIA y TFIIB, y seguidamente la RNApolII. La entrada de TFIIE y TFIIH completará el ensamblaje del complejo de preinicio (Thomas y Chiang, 2006).

Una vez el PIC está formado, se produce la apertura de 11 a 15 pares de bases del DNA de doble cadena, de forma que una de las hebras se sitúa en el sitio activo de la polimerasa (Cramer, 2004) y la transcripción se inicia con la entrada de dos rNTPs y la formación del primer enlace fosfodiéster del RNA naciente. Para abandonar el promotor, los factores de elongación deben interaccionar con el dominio CTD de la RNApolII (Sims y col., 2004). El CTD es el dominio C-terminal de la subunidad mayor de la RNApolII, Rpb1, y está formado por 26 repeticiones en tándem del heptapéptido YSPTSPS (Allison y col., 1985; Corden y col., 1985; West y Corden, 1995; Eick y Geier, 2013). TFIIH debe fosforilar la serina en posición 5 de las repeticiones que incluye este dominio, y con la fosforilación, se reclutan las enzimas que introducen la caperuza al mRNA y finalmente la holoenzima puede salir del promotor (Thomas y Chiang, 2006).

Elongación

La elongación es el proceso en el que la RNApolII recorre la región codificante del gen, una vez iniciada la síntesis del RNA en el promotor, hasta formar el RNA mensajero completo.

En una etapa temprana de la elongación, cuando el pre-mRNA naciente cuenta con 22-25 nucleótidos, tiene lugar el proceso de *capping* o adición de la caperuza en el extremo 5' del transcrito naciente. Dicho proceso implica tres etapas enzimáticas: eliminación del extremo 5'-γ-fosfato del primer nucleótido, adición de una guanina monofosfato (lo que resulta en la formación de una caperuza de guanosina (GpppN)) y, finalmente, metilación de la guanina en posición N7 (m^7GpppN). Es a partir de la adición de la caperuza en el extremo 5' del RNA que se alcanza la fase de elongación productiva y la síntesis de RNA

se produce a una tasa constante (Krogan y col., 2002; Shilatifard y col., 2003). La caperuza protege el mRNA de la degradación y promueve transcripción, poliadenilación, eliminación de intrones, exportación y traducción del mRNA (Topisirovic y col., 2011).

El estado de fosforilación del dominio CTD (Figura 1.2) afecta a las distintas fases de actividad de la RNApolII y, junto a la adición de la caperuza, es también clave para alcanzar una elongación productiva. La fosforilación en Ser5 es necesaria para salir de la pausa en el promotor y se mantiene en la elongación durante las primeras decenas de nucleótidos, pero a medida que la polimerasa avanza a lo largo del gen esta fosforilación disminuye rápidamente y va aumentando la fosforilación en Ser2 del mismo dominio (Komarnitsky y col., 2000). La teminación se inicia con sólo Ser2 fosforilada hasta que las fosfatasas actúan para promover el reciclaje de la RNApolII (Bataille y col., 2012).
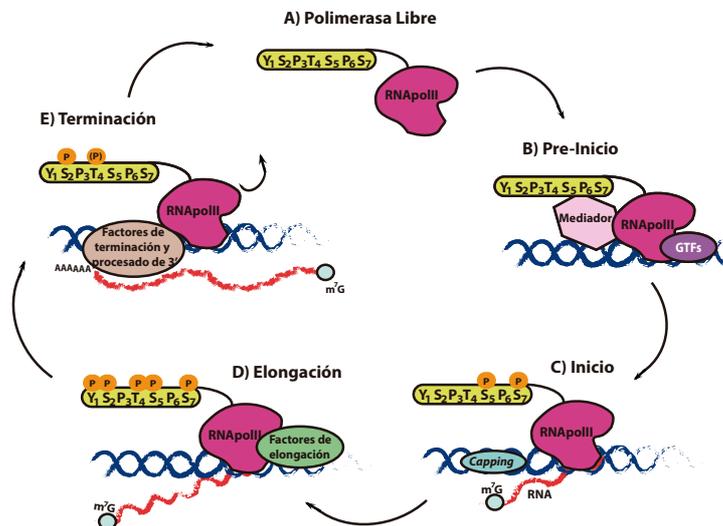
Figura 1.2. Modelo del ciclo de la RNApolII durante la transcripción. A) RNApolII libre e hipofosforilada, no unida al DNA. B) Los factores generales de transcripción (GTFs) se ensamblan en el complejo de preinicio en el promotor, reclutándose la RNApolII y el complejo Mediador. C) Primera onda de fosforilación de las Ser5 y Ser7 del dominio CTD y reclutamiento de las enzimas encargados de adicionar la caperuza. D) Dinámica de fosforilación y desfosforilación del dominio CTD durante la fase de elongación. E) Eliminación gradual de las fosforilaciones de la cola CTD llevada a cabo por fosfatasas, y liberación de la RNApolII y del transcrito del DNA molde. Adaptado de Eick y Geyer (2013).

Terminación

La terminación de la transcripción requiere dos procesos distintos: la formación en el RNA de un extremo 3' definido, o procesamiento en 3' del RNA naciente, que implica el

corte del mRNA precursor y la adición de la cola poliA al grupo 3'OH generado, y la liberación de la RNApolII del DNA molde. Ambos procesos están íntimamente conectados y son igualmente fundamentales en la producción de un RNA mensajero funcional (Mischo y Proudfood, 2013).

Exportación de mensajeros

En eucariotas, transcripción y traducción ocurren en compartimentos subcelulares distintos. La biogénesis del mRNA tiene lugar en el núcleo y la traducción de éste a proteínas en el citoplasma, estando ambos procesos, por tanto, fisiológicamente separados por la membrana nuclear. Es por eso que el mRNA maduro sintetizado en el núcleo, y asociado a su vez con distintas proteínas (ribonucleoparticulas de mRNA, mRNPs), deberá ser exportado a través del complejo del poro nuclear (NPC) hasta el citoplasma (Strambio-de-Castillia y col., 2010).

Existen numerosas proteínas implicadas en el proceso de exportación, pero la que juega el papel más importante es el heterodímero Mex67-Mtr2 (Figura 1.3). Mex67 promueve la unión de las mRNPs con la cesta del poro nuclear, vía la interacción secuencial de la propia Mex67 con las proteínas especificas del complejo del poro nuclear, llamadas nucleoporinas FG (Tutucci y Stutz, 2011; Rodriguez-Navarro y Hurt, 2011).
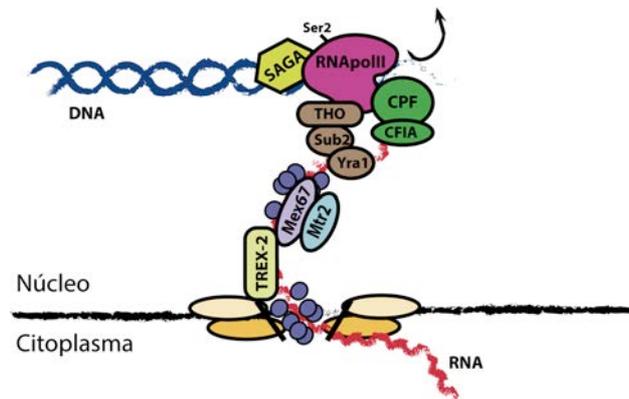
Figura 1.3. Modelo de los principales factores implicados en la exportación al citoplasma del mRNA. El esquema muestra las interacciones físicas y funcionales del complejo THO de *S. cerevisae* con la RNApolII, factores de exportación del mRNA (Yra1, Sub2 y el heterodímero Mex67/Mtr2) y la maquinaria de procesamiento del extremo 3' del mRNA (CFIA y CPF). Adaptado de Luna y col. (2012).

Pero a pesar de que Mex67 tiene un papel esencial en la exportación del mRNA, no puede interaccionar directamente con el mensajero, y requiere de proteínas adaptadoras, como Yra1 o su redundante funcional Yra2 (Zenklusen y col., 2001; Lund y Guthrie, 2005) que, además de actuar como adaptadores, facilitan el reclutamiento de Mex67 a las mRNPs. También funciona como adaptador la proteína de unión a la cola poli(A) Nab2, que forma complejo con Yra1 y Mex67 (Iglesias y col., 2010) y el complejo THO, que ayuda al reclutamiento temprano y cotranscripcional de Yra1 para formar el complejo TREX que, al igual que TREX-2, hace posible el acoplamiento entre transcripción y exportación (Fischer y col., 2004). Finalmente, la proteína Npl3, implicada en el proceso de eliminación de intrones, es otro importante factor de exportación capaz de actuar como adaptador entre Mex67 y el mRNA (Gilbert y Guthrie, 2004).

A pesar de que, tal como muestran las Figura 1.3 y 1.5, todos estos factores son reclutados co-transcripcionalmente a los transcritos nacientes, es la formación del extremo 3' del mRNA la que provoca la liberación de las mRNPs, el paso realmente crítico para que pueda tener lugar la exportación del mensajero al citoplasma. En ese sentido, la proteína esencial de unión a la cola poli(A) del mRNA, Pab1, es un elemento fundamental para conectar biogénesis de mRNA y exportación (Brune y col., 2005).

Una vez formado el mRNA maduro, junto con sus proteínas, la mRNP será translocada a través del NPC al citoplasma gracias a la acción combinada de los factores de exportación y las nucleoporinas de la cesta del poro nuclear, que participan directamente en la unión, translocación y liberación al citoplasma de la mRNP (Zenklusen y Stutz, 2001).

Traducción dependiente de caperuza

Una vez que el mensajero es exportado al citoplasma, la traducción en los ribosomas representa la última etapa en el proceso de expresión génica, y hace posible la formación del proteoma desde la información genómica.

Las características estructurales del mRNA son fundamentales en la traducción. Así, la caperuza en 5' (m^7GpppN) y la cola poli(A) en 3' son motivos canónicos que promueven potentemente su inicio, y algunas estructuras secundarias, como las horquillas, pueden bloquear el proceso. Finalmente, elementos de secuencia, como los IREs (*Internal*

Ribosome Entry Sequences) pueden desencadenar la traducción de forma independiente de la caperuza (Gebauer y Hentze, 2004).

Al igual que la transcripción, la traducción (Figura 1.4) también se puede subdividir en tres etapas claramente diferenciadas, inicio, elongación y terminación, siendo también fundamental la fase de reciclado de ribosomas.
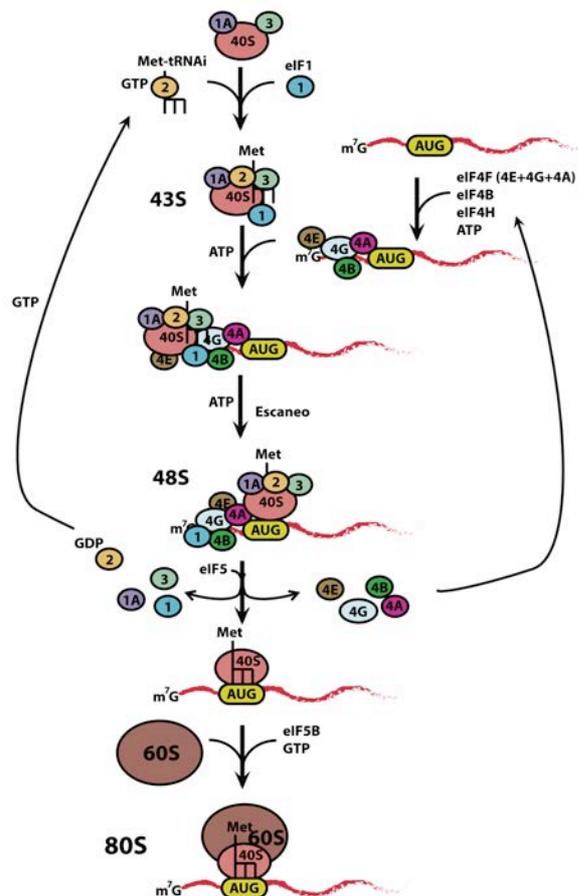
Figura 1.4. Modelo del ensamblaje del ribosoma funcional 80S. Se indican las principales etapas del inicio de la traducción, así como los elementos reguladores más importantes. Adaptado de Liwak y col. (2012).

El inicio de la traducción implica el posicionamiento de un ribosoma funcional 80S sobre el codón de inicio AUG. La subunidad pequeña del ribosoma, 40S, se une el extremo 5' del mRNA y escanea el transcrito en dirección 5'→3' hasta identificar el codón de inicio. En ese momento, la subunidad grande ribosoma, 60S se une a la 40S en esa posición para formar el ribosoma competentemente catalítico 80S.

Los factores de inicio de traducción (eIFs) 3, 1, 1A y 5 se ensamblan con la subunidad pequeña del ribosoma 40S (Figura 1.4), formando el Complejo Pre-Inicio (PIC) 43S al unirse el Met-tRNA$_i^{Met}$ iniciador. Por otro lado, el complejo formado por los factores eIF4B, eIF4F y eIF4H reconoce los mRNAs a través de la caperuza, mediante la interacción directa entre el factor eIF4E y dicha estructura (Figura 1.4). Otro componente de eIF4F, eIF4G, hace de andamio, interaccionando con eIF4E unido a la caperuza, con el PIC 43S y con la proteína de unión a la cola poli(A) Pab1. Así, el complejo de Pre-Inicio 43S queda posicionado con respecto al mRNA y empieza a escanear en dirección 5'-3' con consumo de ATP, hasta que reconoce el codón de inicio AUG por complementariedad de bases con el tRNA iniciador. A continuación, el GTP unido a eIF2 en el mismo tRNA es hidrolizado en un proceso de catálisis mediada por eIF5. Esta reacción y una segunda hidrólisis catalizada por eIF5B son necesarias para que la subunidad ribosomal 60S se una al complejo de inicio, se forme así el complejo 80S y pueda empezar la síntesis polipeptídica (Gebauer y Hentze, 2004; Valásêk, 2012; Beznoskova y col., 2013).

En el proceso de elongación desempeñan un papel fundamental los factores eEFs (*eukaryotic elongation factors*). eEF1A deposita el correspondiente aminoacil-tRNA en el sitio A del ribosoma, y eEF1B es el factor de intercambio de nucleótidos que permite regenerar la forma activa de los factores dependientes de GTP que participan en el proceso. Finalmente, eEF2A es el responsable de la translocación del complejo peptidil-tRNA·mRNA desde el sitio A al P, una vez formado el enlace peptídico, para iniciar un nuevo ciclo de elongación (Anand y col., 2003; Mateyak y Kinzy, 2010).

Es clave entender que, tal como muestra la Figura 1.5 de forma simplificada, varios ribosomas se posicionan sobre un mismo mRNA para llevar a cabo un proceso de elongación más eficiente (Orphanides y Reinberg, 2002).

La terminación de la traducción empieza con la entrada del codón de stop en el sitio A ribosomal, y culmina en la liberación del recientemente sintetizado polipéptido del ribosoma, y la disociación del complejo ribosoma-tRNA·mRNA (Alkalaeva y col., 2006). Esta etapa requiere también diversos factores proteicos y el consumo de GTP. La complejidad de los procesos de elongación y terminación es mucho mayor de lo que se ha descrito en este apartado, y existen diversos artículos de revisión en donde se analizan en profundidad (Mateyak y Kinzy, 2010; Valásêk, 2012; Beznoskva y col., 2013). Finalmente,

las proteínas recién sintetizadas deben ser plegadas para alcanzar su estado funcional, plegamiento que puede ser co o post-traduccional.

Mecanismos de control de la expresión génica en *S. cerevisiae*

Tal como se describió en el apartado anterior y en la Figura 1.5, la expresión eficiente de los genes de *S. cerevisiae* implica varias etapas: síntesis del mensajero, eventos de maduración en el núcleo, exportación del mensajero maduro al citoplasma, remodelación estructural para alcanzar un estado competente de traducción, y traducción para finalmente sintetizar el polipéptido correspondiente, con la posterior degradación del mensajero (Le Hir y col., 2001; Jensen y col., 2003; Luna y col., 2008). La mayoría de estos procesos están acoplados y tienen lugar a veces de forma simultánea, estando cada etapa conectada de forma continua con la siguiente.
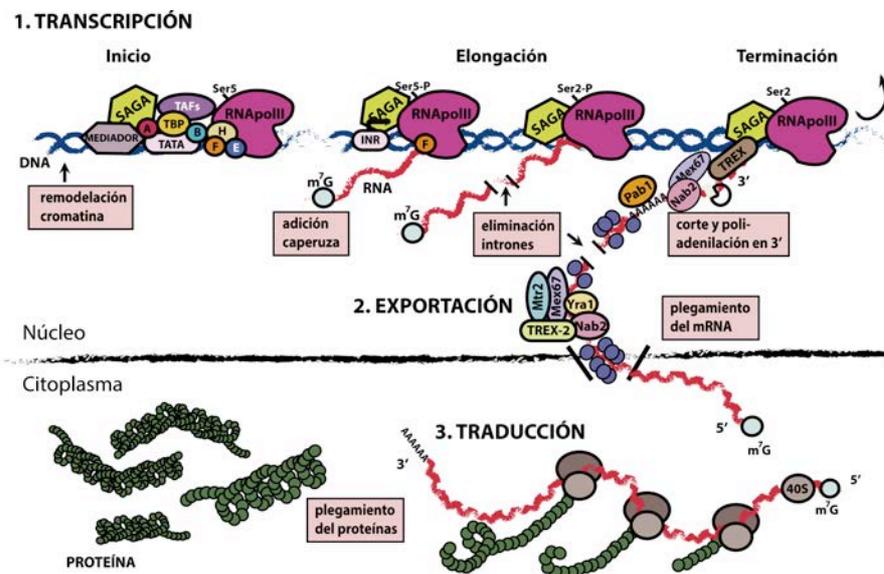
Figura 1.5. Modelización integrada de las etapas de la expresión génica. Cada etapa de la regulación de la expresión génica está subdividida en una serie de procesos continuos, estando cada etapa física y funcionalmente conectada a la siguiente. La Figura representa una simplificación dentro de la gran complejidad de los factores y proteínas implicados en cada fase, siendo solamente representados aquellos cuya importancia se resalta en el texto. Adaptado de Orphanides y Reinberg (2002) y García-Oliver y col. (2012).

Aunque en la levadura existen muchos genes de expresión constitutiva, cuyos niveles de mRNA se mantienen constantes a lo largo del tiempo e independientemente de las condiciones ambientales, esto no es lo habitual, y durante el ciclo de vida de la levadura, no siempre hay los mismos requerimientos proteicos. De este modo, en algunas etapas del ciclo celular es necesaria la biosíntesis de unas proteínas, o la degradación de otras, para poder progresar hacia la división celular. Y las condiciones ambientales, como la disponibilidad de nutrientes, la variación en las fuentes de carbono fermentables o no fermentables accesibles, la presencia de agentes genotóxicos o situaciones adversas para el crecimiento, hacen que sea necesario, en cada momento, expresar una batería de genes u otros. Es por ello que el proceso de expresión génica debe estar regulado de forma precisa, y esta regulación puede afectar a todas las etapas de la síntesis proteica, como muestra la Figura 1.6.
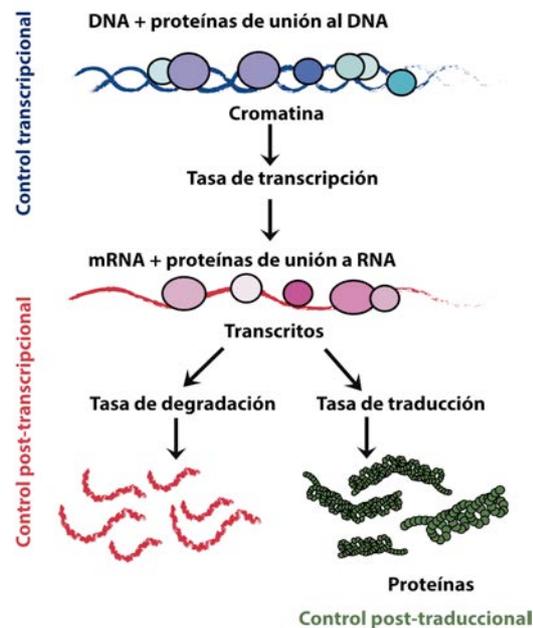
Figura 1.6. Etapas en el control de la expresión génica. La tasa de transcripción y la degradación del mRNA determinan en nivel estacionario de los niveles de transcrito, y los niveles de mRNA, junto con la tasa de traducción, determinan la cantidad de proteína producida. La etapa de la transcripción está controlada por proteínas de unión al DNA, mientras que la regulación post-transcripcional, como la tasa de degradación del mensajero o la traducción, está mediada por proteínas que se unen el RNA, y forman complejos ribonucleoproteicos con los transcritos. Existe un alto nivel de coordinación e interdependencia entre las distintas etapas del proceso de expresión génica, ya que la expresión génica post-transcripcional está también regulada a otros niveles, como procesamiento, exportación y localización de mRNA. Las proteínas están reguladas a nivel post-traduccional, vía modificación y degradación (Adaptado de Mata y col., 2005).

De este modo, la tasa de transcripción génica y la degradación del mRNA determinan el estado estacionario de niveles de transcrito. Y estos niveles, junto con la tasa de traducción, la cantidad de proteína sintetizada. De forma simplificada, podríamos decir que la regulación transcripcional viene determinada por la estructura de la cromatina y proteínas de unión al DNA. El control post-transcripcional, como estabilidad de mRNA y traducción, se lleva a cabo por proteínas de unión al RNA y complejos ribonucleoproteicos. Finalmente, el control post-traduccional estaría relacionado con la modificación y degradación de proteínas (Mata y col., 2005).

Control transcripcional de la expresión génica

Tradicionalmente, la regulación de la transcripción ha sido el mecanismo de control considerado más importante y, en consecuencia, más estudiado. Esta consideración se debe a ser la etapa más intuitiva de regulación y estar situada en primera posición dentro de las distintas fases de expresión génica, lo que implica que la célula no desperdicia energía en los pasos posteriores. En eucariotas, de los miles de genes codificantes de proteínas, cada uno de ellos puede tener su propio programa transcripcional de control. Este control está ejercido por proteínas, generalmente factores de transcripción, que se unen al DNA, en secuencias concretas dentro o cerca del promotor, y que transmiten esa información de secuencia del DNA a factores y cofactores reguladores, y a la maquinaria general de la RNApolII (Kadonaga, 2004). Estos factores específicos de secuencia actúan, pues, como intermediarios entre la información genética de regulación y el sistema de transcripción.

Por tanto, además de la maquinaria general de inicio de transcripción, en el proceso de regulación de la expresión génica intervienen proteínas reguladoras específicas de gen o grupo de genes, que van a determinar con su unión al promotor que se exprese un gen concreto en un momento determinado (Stam y Laurie, 1996). Estas harán posible el reclutamiento de la maquinaria general de inicio de la transcripción, y también tienen sus secuencias de reconocimiento específicas en los promotores, como se explicará después en más detalle con ejemplos concretos. Aunque existen proteínas represoras, en esta introducción nos ocuparemos principalmente de las que actúan como activadoras.

El otro nivel fundamental de control transcripcional hace referencia a la estructura y plegamiento del DNA en la cromatina. Lo ejecutan los factores remodeladores de la

cromatina que mobilizan los nucleosomas, y una gran variedad de enzimas que catalizan la modificación covalente de histonas y otras proteínas mediante reacciones de acetilación, desacetilación, fosforilación, metilación, ubiquitinación, deubiquitinación y ADP-ribosilacion (Kadonaga, 2004).

Regulación de la transcripción por Factores de Transcripción de unión al DNA específicos de secuencia

Características estructurales de los dominios de unión al DNA

A nivel estructural, los factores transcripcionales específicos de secuencia suelen estar organizados en módulos. En 1985, Brent y Ptashne demostraron, mediante la fusión de ciertas regiones del factor transcripcional Gal4 de levadura con el dominio de unión al DNA de la proteína bacteriana LexA, que el dominio de activación transcripcional de Gal4 puede funcionar autónomamente y activar la transcripción bajo la secuencia de reconocimiento de LexA. Con ello, además de introducirnos en las fusiones génicas, se demostraba la existencia de dos módulos independientes, uno de unión al DNA y otro de activación transcripcional o transactivación, que se pueden combinar de forma efectiva aún viniendo de proteínas y organismos distintos, pero que son imprescindibles ambos en conjunto para que se produzca la transcripción.

El módulo de unión al DNA puede estar formado por motivos helice-vuelta-helice, el mejor conocido en factores de transcripción procariotas, aunque también hay otros motivos característicos de eucariotas como su variante homeodominio hojas β, o los dedos de zinc (Figura 1.7).
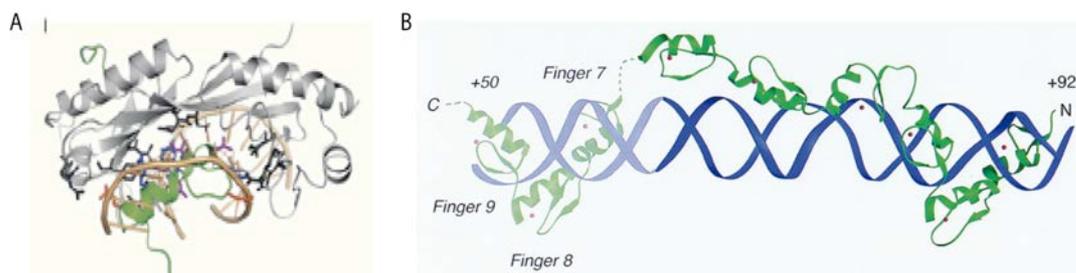
Figura 1.7. Interacciones específicas entre factores de transcripción de levadura y DNA. A) Interacción de TBP (gris) y TAF1 (verde) con la caja TATA. B) Modelo del complejo formado por los nueve dedos de Zn de TFIIIA con el DNA. Imágenes procedentes de Anandapadamanaban y col. (2013) y Nolte y col. (1998) respectivamente.

Otros elementos estructurales, como las cremalleras de leucina o las hélice-lazo-hélice (Pabo y Sauer, 1992) reconocen regiones básicas en el DNA y posibilitan la dimerización. En ese sentido, otro módulo que puede existir en los factores de transcripción es el motivo de dimerización o multimerización. Este no siempre está presente, y cuando lo está puede ser, o un módulo independiente, o (como en los casos citados) un mismo módulo que ejerce la acción dual de unión al DNA y dimerización (Murre y col., 1989).

En cuanto al módulo de transactivación, puede contener distintos motivos. En numerosas proteínas se han encontrado regiones ricas en aminoácidos ácidos, aunque hay mucha variabilidad, y se han detectado dominios de este tipo ricos en glutaminas (Courey y Tjian 1988), prolinas (Mermod y col., 1989) u hojas β hidrofóbicas (Van Hoy y col., 1993). Como ya se ha comentado con anterioridad, el módulo de transactivación se puede asociar en *trans*, vía interacciones proteína-proteína con los módulos de unión al DNA.

Mecanismo de actuación

Los factores transcripcionales suelen realizar su función principalmente mediante el reclutamiento vía interacción proteína-proteína de coactivadores o correpresores al DNA (Ptashne y Gann, 1997). Estos cofactores podrán, a continuación, regular de forma directa o indirecta la actividad de la RNApolII y la maquinaria transcripcional en el promotor. Es importante destacar que estos correguladores pueden ser, por ejemplo, factores de unión a la TBP o las mismas subunidades del componente TFIID (Horikoshi y col., 1988). Pero también hay otra clase distinta de cofactores, relacionados con la cromatina, los ya mencionados remodeladores de nucleosomas y modificadores covalentes de histonas o DNA molde. Hay, por tanto, una notable diversidad y complejidad en la regulación transcripcional.

Regulación

Este punto es especialmente interesante, porque los factores de transcripción, reguladores por excelencia del proceso de expresión génica, también tienen que estar, a su vez, convenientemente controlados. Así, pueden ser activados o reprimidos, generalmente a través de modificaciones post-traduccionales, como fosforilación, acetilación o

ubiquitinación (Kadonaga, 2004). Aún se desconoce gran parte de la complejidad de los mecanismos de regulación, así como sus implicaciones en el proceso de expresión génica.

Sitios de reconocimiento

Se ha demostrado que los factores transcripcionales de eucariotas suelen unir el DNA con especificidad relativamente baja (Walter y col., 1994). Un control preciso de la transcripción génica requiere un grado mayor de especificidad que la otorgada por la unión de un único factor transcripcional específico de secuencia y esto se consigue con la existencia de múltiples sitios de reconocimiento en *cis*. Las secuencias, de 6-8 pb, tienden a encontrarse agrupadas, y aunque lo común es que cada factor reconozca una única secuencia consenso, la especificidad de la activación génica se logra precisamente al tener múltiples sitios de unión en *cis* reconocidos por factores con estructura funcional en forma de dímero, o conteniendo varios elementos capaces de unirse a distintos sitios. Es importante señalar, además, la mejora sinérgica que se logra en muchos genes al tener múltiples factores distintos actuando en *trans*, que logran activar la transcripción de forma más fuerte (Laybourn y Kadonaga, 1992).

De este modo se consigue que, de forma perfectamente regulada, en un momento determinado se exprese un gen, o una batería concreta de genes, bajo el control de una serie de factores de transcripción específicos que reconocen secuencias determinadas en el promotor de los genes que regulan.

Control post- transcripcional de la expresión génica

La regulación post-transcripcional incluye procesamiento, exportación y traducción de mRNAs, así como localización y degradación de los productos génicos, y está mediada por varias combinaciones de proteínas de unión al RNA (RBPs) que determinan el destino de los transcritos y parecen regular coordinadamente subconjuntos específicos de RNAs (Keene and Tenebaum, 2002; Hieronimus y Silver, 2003; Keene y Lager, 2005).

La estabilidad del mRNA es una etapa clave y la tasa de degradación del mismo viene definida por elementos de control localizados normalmente dentro de las regiones 3' UTR (no traducidas) de los mRNAs, y que serán reconocidos por RBPs específicas. La

degradación de los transcritos tiene lugar en el citoplasma, en los cuerpos de procesamientos (o *P-bodies*) (Wilusz y Wilusz, 2004).

El control traduccional se puede resumir en dos categorías. En la regulación global, la traducción de la mayoría de mRNAs de la célula es controlada de forma conjunta, mediante modificaciones en los factores de inicio de la traducción. En la regulación específica de mRNA, la traducción de un grupo definido de mensajeros es modulada, sin afectar ni alterar la biosíntesis de proteínas en su conjunto, mediante complejos proteicos reguladores que reconocen elementos presentes en las regiones 5' y 3' UTR de los mRNAs diana.

1.2. La Respuesta a Estrés en la levadura *Saccharomyces cerevisiae*

El éxito de supervivencia de un organismo en un ambiente determinado está vinculado a su habilidad para adaptarse a los cambios que se producen a su alrededor. Así, para un organismo eucariota unicelular y no móvil, como *S. cerevisiae*, adaptarse es esencial para sobrevivir (Simpson y Ashe, 2012).

Se considera estrés cualquier factor ambiental que compromete o dificulta el óptimo desarrollo o supervivencia de una célula (Hohmann y Mager, 2003). En respuesta a esta situación desfavorable, la levadura activa mecanismos implicados en la detección del estrés y la transducción de la señal, produciéndose una serie de modificaciones transcripcionales y post-transcripcionales que harán posible la acumulación de agentes protectores y actividades reparadoras (Mager y De Kruijff, 2005). Los cambios en la expresión génica en respuesta a estrés van a ser, por tanto, fundamentales para que la célula pueda sobrevivir, por lo que el estudio de la regulación transcripcional es un elemento clave para entender los mecanismos de supervivencia.

La Respuesta General a Estrés ambiental, ESR

Independientemente de cual sea el agente causante del estrés *S. cerevisiae* tiene una respuesta común, llamada Respuesta General a Estrés ambiental (*Environmental Stress Response* (ESR)). De este modo, bajo distintos tipos de estrés, se activa inmediata (aunque transitoriamente) una misma respuesta, que afecta a aproximadamente 900 genes (Gasch y col., 2000). Estos genes van a sufrir alteraciones en su tasa de transcripción, pudiendo estar sobreexpresados o reprimidos respecto a la condición de no estrés, lo que se manifiesta en cambios significativos en sus niveles de mRNA.

Algunos de los genes que forman parte de la respuesta ESR presentan también una regulación común, independientemente de la condición ambiental cambiante. Contienen en

su promotor un elemento llamado STRE (*Stress Response Element*) con una secuencia consenso definida: AGGGG (Kobayashi y McEntee, 1990; Marchler y col., 1993). Este elemento de secuencia es reconocido por los factores transcripcionales Msn2 y Msn4, homólogos y parcialmente redundantes (Martínez-Pastor y col., 1996), regulados por la ruta de la proteína quinasa A (PKA) (Marchler y col., 1993) y la ruta TOR (Helliwell y col., 1998), que controlan el crecimiento celular en función de la disponibilidad de nutrientes, como se explicará después con mayor detalle. Estos factores transcripcionales son, por tanto, los mayoritariamente responsables de la reprogramación de la expresión génica común en respuesta a estrés.

No obstante, el control de los genes ESR es más complejo y, junto a Msn2/4, existen un gran número de factores transcripcionales que también están implicados en la misma. Del mismo modo, los niveles de mRNA de estos genes, no sólo están regulados a nivel de transcripción, sino también por la estructura de cromatina y la estabilidad de mensajeros (Gasch, 2002; Hohman y Mager, 2003). Toda esta regulación, en su conjunto, hace posible una respuesta rápida y precisa para hacer frente a las necesidades celulares comunes causadas por cualquier tipo de estrés o condición ambiental desfavorable.

La Respuesta a Estrés Osmótico

Además de la Respuesta General a Estrés, existen respuestas específicas para hacer frente a condiciones adversas particulares. De este modo, la levadura dispone de varios mecanismos frente a tipos de estrés concretos, que implican rutas y factores de transcripción específicos. Del mismo modo que Msn2/4 son responsables de la respuesta a general a estrés, la defensa a choque térmico o altas temperaturas está mediada por las proteínas de choque térmico (*Heat Shock Proteins*, Hsp), controladas por el factor transcripcional Hsf1. La respuesta a estrés oxidativo requiere la familia de proteínas YAP, especialmente los factores de transcripción Yap1 y Yap2. Y la respuesta a estrés osmótico esta mediada por la ruta HOG (*High Osmolarity Glicerol*) que implica una cascada de MAP quinasas (MAPK, *Mitogen-activated protein kinase*) (Hohmann y Mager, 2003).

Estres hiperosmótico

Las células, tanto de organismos unicelulares, como de pluricelulares, son capaces de sentir, responder y adaptarse a los cambios en la actividad de agua de su entorno (Hohmann, 2015). Al disminuir la disponibilidad de agua libre en el medio que las rodea, van a sufrir deshidratación, causada por la pérdida de agua interna, que, siguiendo su gradiente de concentración, difunde hacia el exterior (Hohmann 2002; Wood, 2011).

Esto es justamente lo que ocurre cuando las células de *S. cerevisiae* son expuestas a estrés hiperosmótico, es decir, cuando la osmolaridad externa es superior al rango fisiológico interno de la levadura. Esta situación puede estar producida por sal, glucosa, sorbitol o cualquier otro osmolito en el medio, y es importante remarcar que, en su ambiente natural, la levadura de gemación crece en frutos en descomposición, como la uva, donde la abundancia de azúcares (glucosa, sacarosa y fructosa) se acerca al punto de saturación. La falta de nutrientes compromete la supervivencia celular, pero la abundancia de los mismos va a suponer en la mayoría de casos un incremento en la osmolaridad del medio, en detrimento también, por tanto, del óptimo desarrollo de la levadura.

La salida por difusión del agua interna en condiciones de incremento en la osmolaridad externa afecta severamente a la forma y tamaño de la célula, produciéndose encogimiento y pérdida de turgencia de la misma (Hohmann, 2002). Además de alterar la morfogénesis, aumenta la concentración de iones citosólicos (especialmente Na^+), y se altera en general la dinámica intracelular (Wood, 2011).

La osmoregulación será, por tanto, fundamental para mantener la forma, tamaño, turgencia y morfogénesis de la célula, pero también para asegurar un óptimo ambiente de dinámica intracelular (Mika y Poolman 2011; Wood, 2011). La principal estrategia para evitar la deshidratación masiva, y contrarrestar la alta osmolaridad del medio para poder sobrevivir, es producir y acumular en el interior celular osmolitos compatibles (Hohmann y col., 2007; de Nadal y Posas, 2011). Se entiende por osmolito compatible aquel que es inerte respecto a los procesos intracelulares, es decir, que no altera la dinámica interna de la célula, y que reemplaza el agua perdida y/o revierte el gradiente de concentración y conduce el agua de nuevo al interior celular (Yancey y col., 1982). La naturaleza del

osmolito compatible es muy distinta según el organismo y aunque algunos, como trehalosa, aminoácidos, e incluso iones, pueden contribuir de forma diferencial a adaptarse a la situación de osmoestrés, en *S. cerevisiae* el más importante es claramente el glicerol (Hohmann, 2007). La acumulación intracelular de glicerol es esencial en la respuesta adaptativa al estrés y será clave para el crecimiento y la supervivencia celular. La Figura 1.8 muestra el metabolismo del glicerol en la levadura.
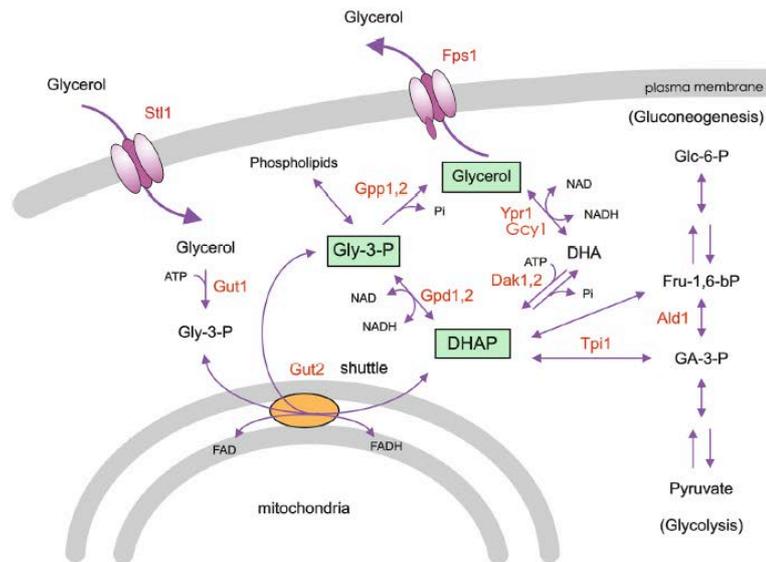
Figura 1.8. Metabolismo del glicerol en *S. cerevisiae*. El glicerol se produce a partir del intermediario glicolítico dihidroxiacetona fosfato (DHAP), en dos etapas catalizadas por las glicerol-3-fosfato deshidrogenasas dependientes de NAD Gpd1 y Gpd2 y por las glicerol-3-fosfato fosfatasas Gpp1 y Gpp2. El glicerol es exportado al exterior celular por el canal Fps1, mientras que es asimilado en el interior celular vía la incorporación mediante Stl1. El glicerol se fosforila mediante la acción de la glicerol quinasa Gut1 y puede ser de nuevo convertido en DHAP mediante la glicerol-3-fosfato-deshidrogenasa dependiente de FAD Gut2, para entrar en glicolisis/gluconeogénesis. La utilización y producción de glicerol son dos procesos que probablemente no ocurren nunca simultáneamente, debido a la ruta de represión por glucosa, que será explicada después con detalle. Figura procedente de Hohmann (2015).

De acuerdo con esto, cuando aumenta la osmolaridad del medio, la levadura inicia un muy complejo programa de adaptación, que incluye la parada temporal del ciclo celular (y por tanto del crecimiento hasta que ocurra la adaptación), ajustes en los patrones de transcripción y traducción, y la síntesis y retención del glicerol. Y todos estas respuestas adaptativas están principalmente dirigidas por la ruta de señalización HOG, cuyo centro es la MAPK Hog1 (Saito y Posas, 2012).

La ruta HOG

Muchas rutas de señalización intracelular implican cascadas de MAPK, estando sus módulos o unidades de señalización evolutivamente conservados en varios organismos eucariotas, incluidos hongos y levaduras (Zhon y col., 1999).

Cada cascada de MAPK está compuesta por tres quinasas (Figura 1.9) que se van activando de forma secuencial en respuesta a estímulos específicos que son captados por sensores o receptores de membrana, pudiendo haber entre estos sensores y la primera quinasa de la ruta varias proteínas encargadas de ejercer el control primario de la misma. En el caso de la ruta HOG, la primera quinasa, la MAPK quinasa quinasa (MAPKKK) tiene un dominio N-terminal regulador, que en ausencia de estrés, bloquea al C-terminal catalítico, el dominio quinasa. En condiciones de osmoestrés, esta quinasa puede ser activada bien por otra quinasa localizada aún más arriba en la ruta, o bien por la unión a una proteína activadora.
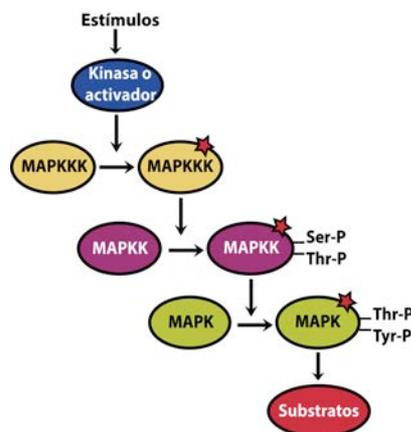
Figura 1.9. Diagrama esquemático del módulo MAPK. Activación secuencial de las distintas MAPK que forman la cascada del módulo. La estrella roja representa la forma activada, y por tanto catalítica, de la correspondiente quinasa. Adaptado de Saito y Posas (2012).

Una vez activada, la MAPKKK activará a la MAPK quinasa (MAPKK) mediante la fosforilación de residuos de Ser y Thr, localizados dentro de la parte conservada del dominio quinasa de la proteína. De forma similar, la MAPKK activará a la tercera quinasa de la ruta, la MAPK, por fosforilación dual en los residuos conservados de Thr y Tyr, localizados también dentro del bucle de activación del dominio catalítico (Saito y Posas,

2012). La activación de la tercera quinasa de la ruta resulta en la translocación al núcleo de la misma y, en este compartimento celular, puede a su vez activar, mediante fosforilación en Ser y Thr a proteínas nucleares, como factores de transcripción, aunque también existen otras posibilidades. También en el citoplasma puede ejercer su acción catalítica para regular, por ejemplo, a otras quinasas. Finalmente, con esto, se consigue activar las respuestas adaptativas necesarias para hacer frente a la situación de estrés, mediante cambios en los niveles de mRNAs y de proteínas.

La parte inicial de la ruta HOG implica dos ramas que, siendo mecanísticamente distintas, son funcionalmente redundantes, y llevan el nombre de sus respectivos osmosensores de membrana, receptores que captan la señal de alta osmolaridad en el medio. De este modo, tanto la rama Sln como la rama Sho confluyen, tal como se muestra en la Figura 1.10, en la activación de la MAPKK Pbs2, que a su vez activa por fosforilación a la MAPK Hog1 (Brewster y col., 1993; Maeda y col., 1994).
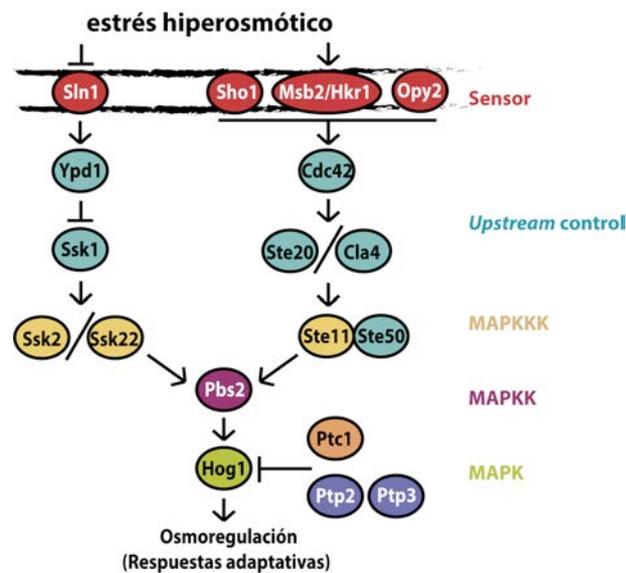
Figura 1.10. Diagrama esquemático de la ruta HOG en *S. cerevisiae*. La rama Sln está inhibida en condiciones fisiológicas, eliminándose esta represión en presencia de osmoestrés, mientras que la rama Sho se activa en presencia del mismo. De este modo, bajo condiciones de alta osmolaridad, ambas vías confluyen en la activación de la MAPKK Pbs2, que a su vez activará por fosforilación a la MAPK Hog1, principal responsable de llevar a cabo las respuestas adaptativas necesarias para hacer frente a la situación desfavorable. Las fosfatasas Ptc1 y Ptp2/3 desactivan la ruta mediante la inhibición de la MAPK Hog1 cuando el balance osmótico celular ha sido restablecido o cesan las condiciones adversas. / indica proteínas funcionalmente redundantes. Adaptado de Saito y Posas (2012).

En respuesta al estrés y una vez activada, Hog1 se transloca al núcleo, donde regulará las respuestas adaptativas de transcripción y ciclo celular, aunque también tiene dianas citosólicas. Una vez restablecido el balance osmótico, Hog1 es, de nuevo, exportada de vuelta al citoplasma.

Es importante destacar que tener activada la ruta Hog1 en ausencia de estrés es nocivo para la célula, por lo que los mecanismos de control van a ser fundamentales, tanto para evitar que la ruta esté activa en condiciones fisiológicas como para la inhibición de Hog1 una vez que las condiciones de estrés han pasado y el equilibrio ha sido restablecido (Posas y Saito, 1997).

Además, también es relevante mencionar que, como se explicará después con mayor detalle, hay otras rutas de señalización en *S. cerevisiae* que implican cascadas de MAPK, que participan en la respuesta a feromonas, crecimiento filamentoso e invasivo y biogénesis de la pared celular. Algunas de estas rutas presentan elementos de señalización comunes, como la MAPKKK Ste11, lo que nos muestra la existencia de una conexión entre las rutas de señalización, así como la necesidad de una regulación recíproca dentro de esta compleja red. Este aspecto será analizado con detalle posteriormente.

La rama Sln

La rama Sln es una variante del sistema de sensores de dos componentes, formados por una molécula sensor (con dominio sensor o *input*, dominio catalítico histidina quinasa (HK), y sitio de autofosforilación en histidina) y una molécula reguladora de la respuesta (con un dominio efector u *output* y un dominio receptor). La parte N-terminal del osmosensor Sln1 es el dominio sensor, compuesto por dos fragmentos transmembrana (Maeda y col., 1994). La parte C-terminal lo componen el dominio histidina quinasa y el dominio receptor. Así, al ser activada, Sln1 se autofosforila en la His576, cerca del dominio HK (Posas y col., 1996) y el grupo fosforilo se transfiere al Asp1144 del dominio receptor, para iniciar a su vez la respuesta mediante la transferencia del grupo fosforilo a la proteína Ypd1, en la His64. Ypd1 es una proteína que hará de intermediaria catalizando la transferencia específica del grupo fosforilo entre, en este caso, dos proteínas con dominio receptor, siendo, por tanto, transferido dicho grupo a continuación al Asp554 del dominio receptor de Ssk1.

Bajo condiciones normales y ausencia de estrés, el dominio HK de Sln1 está catalíticamente activo, con lo que Ssk1 está fosforilada, y por tanto, inactiva. Así se impide que en ausencia de estrés la ruta HOG esté funcionando. La disrupción del gen *SLN1* es letal para la célula, ya que resulta en la activación constitutiva de la ruta HOG (Maeda y col., 1994). Bajo condiciones de estrés osmótico, Sln1 no está activo, por lo que se acumula Ssk1 sin fosforilar, que entonces es capaz de unir y activar a Ssk2/Ssk22, las MAPKKK, que una vez activadas inician la cascada de fosforlación, primero sobre la MAPKK Pbs2 y, finalmente, la MAPK Hog1, tal como se muestra en la Figura 1.10.

Ypd1 también transfiere el grupo fosforilo a otra proteína, no implicada en la ruta HOG, sino en la biogénesis de la pared celular, Skn7 (Brown y col., 1994). Mientras que Ssk1 es activado en alta osmolaridad, Skn7 (implicado entre otros procesos en la respuesta a estrés oxidativo, (Krems y col., 1996)) se activa bajo condiciones de ausencia de estrés o hipo-osmolaridad evidenciando de nuevo la interconexión entre distintas rutas de señalización.

La rama Sho

Al contrario que en el caso anterior, el mecanismo de activación de esta rama de la ruta sólo ha sido hasta el momento vagamente definido.

Los putativos osmosensores de membrana de esta rama son Msb2 y Hkr1, proteínas altamente glicosiladas en su dominio exterior y con un único dominio transmembrana (Tatebayashi y col., 2007). Sho1 sería el co-osmosensor y, aunque el mecanismo de interacción de éste con los dos anteriores aún permanece desconocido, se sabe que esta respuesta conduce a la activación de las quinasas Ste20 y Cla4 al inducir su asociación con la proteína de membrana Cdc42 (Lamson y col., 2002). Cuando Ste20/Cla4 están activadas, fosforilan y activan a la MAPKKK Ste11 (Drogen y col., 2000), que a su vez fosforila y activa a la MAPKK Pbs2, que está asociada al receptor de membrana Sho1 (Tatebayashi y col., 2006).

Dado que ambos complejos, Sho1-Pbs2 y Cdc42-Ste20 estan localizados en la membrana, Ste11, activado por Ste20 y activador de Pbs2, debe a su vez estar localizado en este compartimento. Ste50 es la proteína que hace de andamio entre Ste11 y la

membrana, formandose un complejo estable entre ambas (Posas y col., 1998; Wu y col., 1999), gracias tanto a la asociación de Ste50 con la proteína de anclaje a membrana Opy2 (Yamamoto y col., 2010) como por las interacciones Ste50-Cdc42 y Ste50-Sho1 (Tatebayashi y col., 2006).

Finalmente, Pbs2 activará a la MAPK Hog1, del mismo modo que ocurre en la rama Sln1, y tal como se muestra en la Figura 1.10.

Transporte al núcleo de la MAPK Hog1

Una vez activada por fosforilación en sus residuos Thr174 y Tyr176, Hog1 se transloca rápidamente al núcleo. Tras la adaptación a la alta osmolaridad del medio, o si desaparece la condición externa de estrés, es exportada de nuevo al citoplasma (Ferrigno y col., 1998). Su estancia transitoria en el núcleo está directamente correlacionada con la fosforilación de la proteína (Reiser y col., 1999). En este sentido, aunque Hog1 puede fosforilarse a sí mismo, esta autofosforilación no es suficiente para que se acumule en el núcleo, por lo que su localización nuclear es dependiente de la fosforilación por parte de la MAPKK Pbs2 (Mattison y Ota, 2000).

La cinética de localización nuclear depende de la severidad de las condiciones de estrés del medio. Así, un osmoestrés causado por NaCl 0.4 M resulta en la fosforilación y translocación al núcleo casi inmediata, en 1-2 minutos, y se mantienen ambas situaciones hasta aproximadamente 30 min. Condiciones de estrés osmótico más severas retardan el inicio del proceso, pero mantienen su duración más tiempo (Van Wuytswinkel y col., 2000).

Además del estrés osmótico, otros tipos de estrés pueden activar la ruta HOG. Es el caso de estrés por frío (Hayashi y Maeda, 2006), choque térmico (Winkler y col., 2002), hipoxia (Hickman y col., 2011), arsenito (Thorsen y col., 2006), acido acético (Mollapour y Piper, 2006), y pH bajo (Kapteyn y col., 2001), entre otros. En la mayoría de estos casos, Hog1 es activado sólo de forma moderada, y la cinética de fosforilación, y por tanto de localización nuclear, es distinta a la observada bajo osmoestrés. Estas condiciones de estrés a veces activan una u otra rama de la ruta, pero nunca las dos.

Aunque la rápida acumulación de Hog1 sugiere que la mayor parte de la acción reguladora de la MAPK tiene lugar en el núcleo, una parte de la proteína activada permanece en el citosol, donde también va a mediar importantes efectos reguladores, que al igual que las respuestas nucleares, tienen como finalidad última la osmoadaptación de la levadura.

Inactivación de la ruta

Existen varios mecanismos de desregulación de la ruta HOG, pero el más importante en la regulación negativa es la acumulación interna en la levadura de glicerol (Brewster y col., 1993). Dado que la inducción transcripcional de los genes implicados en la biosíntesis de glicerol tarda al menos 15 min, más que regular directamente la deslocalización de Hog1, estos genes están implicados en la desregulación de la ruta a más largo plazo (Hirayama y col., 1995). Por eso se cree que es la acción de Hog1 sobre el canal de glicerol Fps1 (veáse las Figuras 1.8 y 1.11) y sobre enzimas implicadas en el metabolismo del glicerol lo que media una respuesta más rápida (Beese y col., 2009; Bouwman y col., 2011).

Otro mecanismo importante de desregulación de la ruta es el ejercido por las fosfatasas porque, aunque la señalización a los osmosensores de la ruta se detenga al restablecerse el equilibio osmótico por la producción de glicerol, es necesario inactivar las quinasas por desfosforilación. Las proteínas Ptc1, Ptc2 y Ptc3 desfosforilan la Thr174 de Hog1, teniendo la primera el papel más relevante en la desactivación de la MAPK (Warmka y col., 2001). Por otra parte, Ptp2 y Ptp3 desfosforilan la Tyr176 (Jacoby y col., 1997), siendo de nuevo la primera, de localización nuclear (veáse las Figuras 1.10 y 1.11), la más relevante en el proceso. Las fosfatasas que inactivan las otras quinasas de la ruta permanecen aún sin identificar.

Además, Hog1 activado también participa en la desregulación de la ruta mediante la fosforilación de algunos elementos superiores de la misma, como Sho1 y Ste50 (con lo que reduce la afinidad de la interacción entre esta y Opy2 (Yamamoto y col., 2010)).

Finalmente, como se explicará después con mayor detalle, la interconexión entre rutas de señalización de MAPK también está implicada en la inhibición de la ruta HOG. La

rama Sho presenta muchos elementos comunes con las rutas de feromonas y crecimiento filamentoso (Saito, 2010).

Respuestas adaptativas

En respuesta a la alta osmolaridad del medio, la activación de la MAPK Hog1 dirige una serie de respuestas adaptativas para hacer frente a la condición desfavorable y fomentar la supervivencia de la levadura. Las respuestas citoplasmáticas incluyen el control de los flujos de iones, el transporte de glicerol, el de la actividad de enzimas metabólicos y el control del proceso de traducción. En cuanto a las respuestas nucleares, modula la progresión del ciclo celular y el control de la expresión génica.

Restablecimiento del balance osmótico: Acumulación de glicerol

La acumulación interna de glicerol y el restablecieciemiento del equilibrio iónico son respuestas adaptativas a corto plazo (Albertyn y col., 1994). La Figura 1.11 muestra los efectos de la MAPK Hog1 sobre los niveles de glicerol. En respuesta a osmoestrés, desde el primer minuto empieza a acumularse este osmolito compatible, manteniéndose de forma significativa al menos hasta los 30 minutos (Klipp y col., 2005). Esta regulación implica, por tanto, una rápida respuesta inicial, llevada a cabo por 1) cambios en el metabolismo, que redirije las fuentes de carbono hacia la producción de glicerol, mediante el control de la glicolisis y la producción del osmolito compatible (Hohmann 2007), y 2) cambios en el transporte de glicerol, en el que intervienen, dada la baja permeabilidad al mismo de la bicapa lipídica, canales específicos de importación y exportación. Stl1 (Figuras 1.8 y 1.11) es una proteína similar a los transportadores de azúcar (*sugar transporter-like)* que participa en la importación de glicerol al interior celular. El gen *STL1* se induce rápidamente en respuesta a estrés y su expresión está regulada por la MAPK Hog1. No obstante, el mecanismo más rápido para asegurar la acumulación interna de glicerol se produce vía Fps1, canal de exportación del osmolito, que en respuesta a estrés se cierra por acción de la MAPK (Tamás y col., 1999; Hohmann, 2015).

Del mismo modo que en la respuesta a estrés se induce la expresión de *STL1*, se activa también de forma dependiente de Hog1 la transcripción de genes implicados en la biosintesis de glicerol (Figuras 1.8 y 1.11), como glicerol-3-fosfato deshidrogenasas

(*GPD1/2*) y glicerol-3-fosfato fosfatasas (*GPP1/2*). La disrupción de estos genes afecta severamente al crecimiento de *S.cerevisiae* en condiciones de alta osmolaridad (Hohmann, 2002a). Esta respuesta implica regulación transcripcional, por lo que no es tan rápida como las dos anteriores. La expresión de la mayoría de los genes implicados en la biosíntesis de glicerol, tal como se muestra en la Figura 1.11, es dependiente del factor transcripcional de respuesta a estrés Hot1, del que se hablará después en profundidad.
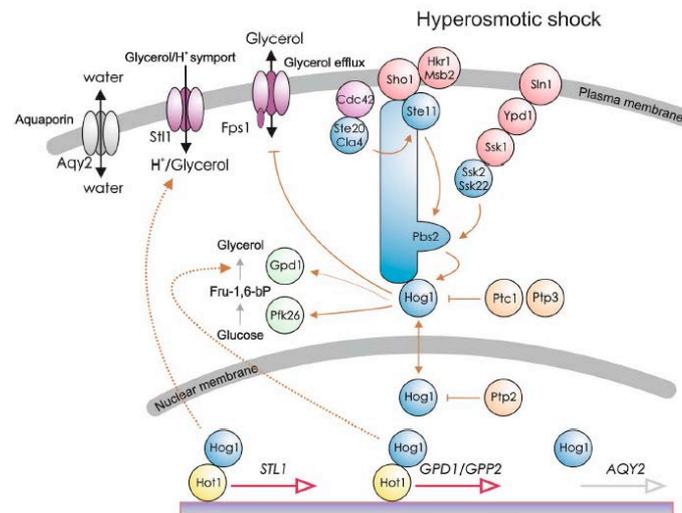
Figura 1.11. Efectos de la ruta HOG en la acumulación de glicerol. En respuesta a estrés, la MAPK Hog1 actúa cerrando el canal de exportación de glicerol Fps1 y estimula indirectamente la producción de glicerol (mediante el control de la actividad de Gpd1) y el flujo glicolítico (vía la activación de Pfk26). Hog1 también media la expresión de genes relacionados con la producción de glicerol, como *GPD1/2* y *GPP1/2*, y su incorporación al interior celular, como *STL1*. Finalmente, también parece regular la expresión del gen *AQY2* que codifica la acuaporina, proteína que restringe la pérdida celular de agua. Figura procedente de Hohmann (2015).

Restablecimiento del balance iónico

En cuanto al restablecimiento del equilibrio iónico, Hog1, en respuesta a estrés, fosforila al menos dos proteínas localizadas en la membrana plasmática (Proft y Struhl 2004): Nha1 (responsable del antiporte Na^+/H^+) y Tok1 (canal de potasio). La adaptación a largo plazo en el control del flujo de iones está determinada por el control de la expresión del gen *ENA1*, que codifica una ATPasa dependiente de Na^+. Por tanto, de nuevo, una única MAPK coordina distintas respuestas de estrés que son temporal, espacial y mecanísticamente distintas, para finalmente adaptarse a los cambios del medio (Saito y Posas, 2012).

Control del proceso de traducción

En respuesta a estrés osmótico, se produce una parada transitoria de la traducción (Melamed y col., 2008), viéndose la elongación traduccional inhibida mediante una ruta de señalización lineal (Teige y col., 2001). No obstante, esta reducción general de la síntesis proteica es perfectamente compatible con la expresión de genes específicos de respuesta a estrés, cuyos productos están implicados en distintas funciones del proceso de adaptación, por lo que su correcta traducción debe estar asegurada. Esto lleva implícito que debe haber una traducción preferencial de un subgrupo de mRNAs en ciertas condiciones, aunque las bases moleculares de la misma permanecen aún desconocidas.

En la inhibición de la elongación de la traducción en respuesta a estrés, Rck2, miembro del grupo de las proteín quinasas de calmodulina (Melcher y Thorner, 1996), fosforila e inhibe al factor de elongación EF-2. Rck2 es una diana directa de Hog1 y ambas son necesarias para la inhibición de la traducción que se produce en respuesta a estrés (Teige y col., 2001).

Control del ciclo celular

Coordinar el control de la progresión del ciclo celular es uno de los papeles clave de la MAPK Hog1, ya que esta parada transitoria es esencial en el proceso de adaptación, por ejemplo para que las células sean capaces de modificar su programa transcripcional. Hog1 induce un retraso rápido y transitorio en varias fases del ciclo celular para asegurar que la progresión del mismo se detenga hasta que se complete el desarrollo de las respuestas adaptativas (Clotet y Posas, 2007; Yaakov y col., 2009).

En *S. cerevisiae* hay una única quinasa dependiente de ciclina (CDK), Cdc28, cuya actividad se regula a través de la síntesis y degradación de varias ciclinas e inhibidores que se unen a ella (Clotet y Posas, 2007). Hog1 media la parada en G2 mediante un mecanismo dual. Por una parte, disminuye los niveles de la ciclina Clb2, inhibiendo su transcripción, y por otra parte, aumenta la concentración del inhibidor de Cdc28 Swe1 (Lim y col., 1996) mediante la fosforilación de la quinasa Hsl1 (Cid y col., 2001). Cuando Hsl1 es fosforilada, se deslocaliza del cuello de la gema, lo cual impide el reclutamiento a esta localización de Swe1. Al no reclutarse, Swe1 no se fosforila, requisito para su degradación, por lo que se

acumula. La presencia de este inhibidor de Cdc28, junto con los bajos niveles de ciclina Clb2 hacen disminuir la actividad del complejo Clb2-Cdc28, necesario para el paso de G2 a mitosis, por lo que la célula queda temporalmente parada en G2. Un mecanismo dual explica también la parada en G1 mediada por la MAPK Hog1, afectando a la regulación de la expresión de la ciclina y a la proteína reguladora de ciclo Sic1 (Zapater y col., 2005).

Control de la expresión génica

Las células de levadura necesitan llevar a cabo de forma rápida, precisa y eficiente una reprogramación transcripcional para hacer frente a la alta osmolaridad del medio.

Una de las funciones más estudiadas en este sentido, es la capacidad de Hog1, y de otras MAPK de levadura, de regular el inicio de transcripción mediante el control de factores de respuesta a estrés. Estos factores de transcripción, no relacionados entre si, controlan cada uno la expresión de una batería de genes concretos de respuesta a estrés (Romero-Santacreu y col., 2009; Miller y col., 2011).

Los factores de respuesta a estrés controlados por Hog1 son los activadores transcripcionales Hot1, Smp1, Msn1, Gcn4, Skn7, Rtg1/3 así como Msn2 y Msn4 (Rep y col., 1999a,b; Posas y col., 2000; De Nadal y Posas, 2008; Ni y col., 2009). Hog1 también regula al represor transcripcional Sko1 (de Nadal y Posas, 2008). Estos factores de transcripción, se unen de forma individual o combinada a los promotores de los genes que regulan.

El mecanismo por el cual Hog1 controla a estos factores de transcripción es distinto en cada uno de los casos. Mientras que Sko1 y Smp1 están regulados mediante fosforilación por la MAPK (Nehlin y col., 1992; Vincent y Struhl, 1992), se ha demostrado que la fosforilación de Hot1 no es esencial ni necesaria para la expresión de los genes que regula (Alepuz y col., 2003), y dado que la transcripción de los mismos es dependiente de la activación por la MAPK, debe ser otro mecanismo distinto el que active a este factor de transcripción. La fosforilación tampoco es necesaria para la regulación por Hog1 de los factores generales de respuesta a estrés Msn2 y Msn4 (Alepuz y col., 2003).

Hot1 (que de entre todos los factores de transcripción regulados por la MAPK Hog1, presenta una destacable relevancia en este trabajo, especialmente en los Capítulos 2,

3 y 4) fue identificado en un rastreo mediante doble híbrido en el que regiones codificantes de Hog1 se usaban como cebo. Presenta homología de secuencia con otros factores transcripcionales de levadura, Msn1 y Gcr1 (Rep y col., 1999b), y regula la expresión de una serie de genes relacionados, en su mayoría, con la biosíntesis y acumulación de glicerol (Figuras 1.8 y 1.11), como *STL1*, *GPD1* y *GPP2* (Rep y col., 1999b; Ferreira y col., 2005). En un estudio reciente llevado a cabo por Gomar-Alba y col., en 2012, y que conforma el Capítulo 1 de esta Tesis, se descubrió otro gen cuya expresión es dependiente de Hot1, *HGI1/YHR087W*. Hot1 se une al promotor de los genes que regula, aunque las particularidades de la unión varían en función del gen: en algunos casos (*CTT1* y *HSP12*) es completamente dependiente de la presencia de estrés osmótico, mientras que en otros, como *STL1* y *GPD1,* puede estar unido en ausencia de estrés (Alepuz y col., 2001). Del mismo modo, el reclutamiento de Hog1 al promotor de estos dos genes es dependiente de la presencia de Hot1 en el mismo, mientras es independiente de la presencia de dicho factor transcripcional en el caso de *CTT1* y *HSP12* (Alepuz y col., 2001).

En cualquier caso, la presencia de Hog1 en los promotores de los genes inducibles en respuesta a estrés hace pensar que la MAPK tiene un papel en el inicio de la transcripción (Proft y Struhl, 2002). Precisamente, el reclutamiento de la maquinaria de la RNApolII a estos genes es dependiente tanto de la MAPK activada como de la presencia de los factores de transcripción específicos. Se ha demostrado que Hog1 interacciona con la subunidad grande de la polimerasa (Alepuz y col., 2003) y participa en el reclutamiento de la maquinaria básica de transcripción, como SAGA, el Mediador, etc (Zapater y col., 2007).

El papel de Hog1 en la regulación de la transcripción va más allá del inicio de la misma, participando también en la elongación transcripcional de los genes de respuesta a estrés. La MAPK interacciona con la RNApolII y con los componentes generales de la maquinaria de elongación (Proft y col., 2006), de forma que acompaña a la RNApolII elongante a lo largo de toda la región codifcante de los genes de respuesta a osmoestrés (Pokholok y col., 2006; Proft y col., 2006).

Además, Hog1 también regula la expresión génica mediante el control de la remodelación de la cromatina (Mas y col., 2009; Pelet y col., 2011; de Nadal y Posas, 2015) y del procesado, exportación y estabilidad del mRNA (Molin y col., 2009; Romero Santacreu y col., 2009; Miller y col., 2011).

Interconexión entre rutas de señalización

Aunque la ruta HOG es probablemente la mejor caracterizada hasta el momento, existen otras rutas de señalización de gran relevancia para el crecimiento y supervivencia de la levadura de gemación.

Todas ellas tienen en común la existencia de receptores o sensores de membrana, que captan el estímulo externo e inician señales intracelulares específicas. Proteínas efectoras amplifican y transmiten la señal a través de cascadas de transducción. En el caso de algunas rutas, como en la vía HOG, estas moléculas efectoras son los módulos MAPK, siendo algunos de ellos a veces comunes entre las distintas cascadas de señalización, como se explicará después.

A una señal extracelular concreta, le corresponde una respuesta adaptativa específica. En *S. cerevisiae* existen 5 cascadas de MAPK funcionales (Figuras 1.12 y 1.13), implicadas en crecimiento filamentoso, respuesta a feromonas (o *mating*), integridad de la pared celular, esporulación y respuesta a estrés osmótico. Cada una de estas rutas responde a señales determinadas y posee una direccionalidad e individualidad para hacer posible una respuesta concreta. No obstante, entender el funcionamiento de las rutas y los mecanismos mediante los cuales se mantiene esta especificidad entraña una notable complejidad, ya que habitualmente varios módulos MAPK tienen dos o más componentes en común entre diferentes rutas, y aun así mantienen su individualidad. Actualmente existe un gran interés en entender cómo se asegura que la señal no siga una direccionalidad errónea en estos módulos superpuestos y cómo se regula (incluso a veces se evita) la interconexión entre rutas (Saito, 2010).

Rutas de MAPK en *S. cerevisiae*

Tal como se observa en la Figura 1.12, tanto la ruta de respuesta a feromonas (PH) como la de crecimiento filamentoso (FG) muestran como elemento común a la vía HOG la MAPKKK Ste11, de la rama Sho. Del mismo modo, comparten los elementos reguladores de esta Ste50, Ste20 y Cdc42.
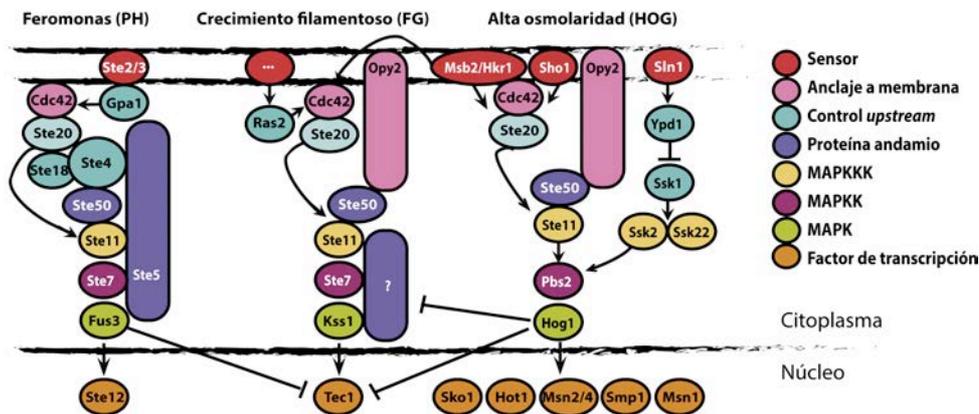
Figura 1.12. Representación esquemática de las vías de señalización PH, FG y HOG en *S. cerevisiae*. En respuesta a distintos estímulos, la levadura activa cascadas de señalización de MAPK. Algunas de ellas presentan elementos comunes, aunque cada una produce respuestas adaptativas específicas a su estímulo particular. Se muestran las distintas interconexiones entre las tres rutas, así como los factores de transcripción que son activados por las respectivas MAPK en el núcleo. Adaptado de Saito (2010) y Waltermann y Klipp (2010).

La vía de respuesta a feromonas (PH)

En levaduras haploides esta ruta detecta moléculas de feromonas (señal extracelular) mediante los receptores de membrana Ste2 y Ste3. Ste2 detecta el factor α en células *MATa*, mientras que Ste3 reconoce el factor a en células *MATα*. Ambos receptores están acoplados a la proteína G heterotrimérica Gpa1-Ste4-Ste18 (Chen y Thorner, 2007). Al detectarse las feromonas, Ste4 y Ste18 se disocian de Gpa1, lo que permite el reclutamiento de los factores Ste20 y Ste50 alrededor de Ste4 en la membrana. En esta ruta, existe una proteína andamio, Ste5, que interacciona con Ste4 y con las MAPKs Ste11, Ste7 y Fus3, que se unen al complejo y se activan de forma secuencial mediante fosforilación.

La MAPK Fus3 activa la transcripción de genes específicos de *mating* y promueve la parada temporal del ciclo celular para formar las prolongaciones celulares o *shmoos*, produciéndose así cambios en la pared y membrana celular para preparar la fusión con la célula del otro tipo sexual (Schwarts y Madhani, 2004).

La vía de crecimiento filamentoso (FG)

Esta ruta se activa en ambientes donde hay limitación de nutrientes, produciéndose en las células cambios morfológicos: se vuelven más alargadas (en lugar de su estado

óptimo ovalado) y proliferan sólo por un polo de la célula. Así, las gemas de las células hijas emergen únicamente por el polo opuesto al que finalizó el nacimiento de la madre. Se desconoce la molécula que actúa como receptor en esta ruta y su mecanismo de actuación, aunque se ha descrito que algunas proteínas de membrana como Sho1, Msb2 (Cullen y col., 2004), Mep2 (Lorenz y Heitman, 1998) y Gpr1 (Van Nuland y col., 2006) son necesarias para que se de el crecimiento filamentoso. Aunque no se conoce el mecanismo de activación de la misma, la proteína G Ras2 es en este caso la encargada de activar a Cdc42, aunque a diferencia de la vía PH, no se forma ningún complejo en la membrana, ni hay proteína que actúe como andamio. Ste11 activa a la MAPKK Ste7 (también presente en la ruta PH), que a su vez activa a la MAPK Kss1, responsable de la inducción de genes de respuesta del crecimiento filamentoso (Waltermann y Klipp, 2010). Es importante destacar, que para que se produzca un crecimiento filamentoso óptimo, además de Kss1, se necesitan al menos dos proteín quinasas más, efectores de rutas implicadas en la disponibilidad de nutrientes (que serán posteriormente explicadas con mayor detalle): Snf1 (proteína quinasa dependiente de 5'-AMP) y Tpk2/Pka2 (proteína quinasa dependiente de AMP cíclico) (Truckses y col., 2004; Verstrepen y Klis, 2006). Las otras PKAs, Tpk1 y Tpk3, son reguladores negativos del crecimiento filamentoso.

La vía de la PKC o Integridad de la pared celular (CWI)

También conocida como de estrés de la pared celular, esta ruta (Figura 1.13) se activa bajo distintas condiciones de estrés que alteran la estructura y función de la pared celular, como calor o choque térmico, hipo-osmolaridad, glucanasas, agentes de unión a quitina (Calcofluor, Congo Red), estrés oxidativo… (Harrison y col., 2004; Levin, 2005).
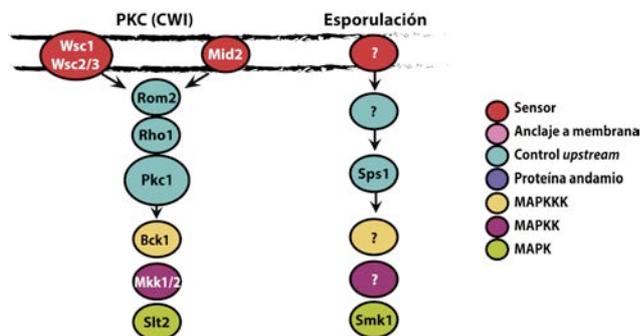
Figura 1.13. Representación esquemática de las vías de señalización CWI y esporulación en *S. cerevisiae*. Adaptado de Waltermann y Klipp (2010).

La vía de la PKC es una cascada simple de MAPK y no presenta elementos comunes con la vía HOG. Tampoco se da, tal como se observa en las Figuras 1.12 y 1.13, la interconexión observada entre las vías PH/FG, aunque sí existen entre la vía HOG y la de la PKC (o CWI) algunos mecanismos de conexión, revisados entre otros en Bermejo y col. (2008) y Fuchs y Mylonakis (2009).

La vía de Esporulación

Es la más desconocida de las 5 rutas de MAPK (Figura 1.13). En ausencia de fuentes de carbono fermentables y de nutrientes esenciales como nitrógeno, sulfuro o fosfato, los diploides *MATa/MATα* realizan meiosis para formar esporas resistentes. Los mutantes en la MAPK Smk1, aunque son capaces de realizar esta meiosis, son defectivas en el ensamblaje de las capas de la pared de las esporas (Huang y col., 2005). Sólo se conoce de la ruta los activadores de la MAPKKK: Ama1, Cak1 y Sps1.

Ejemplos de interconexión entre rutas

Dada la gran cantidad de elementos comunes entre las distintas rutas de señalización, la interconexión entre ellas debe estar estrechamente regulada, para prevenir su activación indeseada.

Se han descrito hasta el momento varios mecanismos para prevenir la interconexión entre rutas indeseada: El primer de ellos se basa en interacciones físicas (*docking*) proteína-proteína, directas y específicas, entre dos elementos consecutivos de la ruta (Bardwell, 2006) o interacciones vía una proteína andamio que mantiene juntos dos o más componentes de la misma ruta (Dard y Peter, 2006). Ambas variantes limitan el flujo de señalización dentro de una misma vía. Otra posibilidad es la inhibición cruzada, donde un elemento activado de la ruta (MAPK, por ejemplo) inhibe elementos de otra ruta, impidiendo que la transducción de la señal se haga extensiva a rutas no deseadas (Schwarts y Madhani, 2004). Finalmente, la cinética de activación de los elementos también es un factor de regulación importante, ya que algunos se activan mediante pulsos rápidos y transitorios mientras que otros lo son por señales intensas y más prolongadas (Behar y col., 2007). Algunos ejemplos de los mecanismos mencionados se describen a continuación.

La vías PH y FG presentan varios elementos comunes (Figura 1.12): la MAPKKK, la MAPKK (Ste11→Ste7) y algunos de los elementos reguladores de Ste11, como Cdc42 y Ste20. Las feromonas, sin embargo, activan la MAPK Fus3 y no la Kss1 debido a la proteína andamio Ste5 (Elion, 2001), que además de mantener las MAPKs Ste11→Ste7→Fus3 juntas, es imprescindible para que se den las dos reacciones de activación mediante fosforilación entre ellas (Flatauer y col., 2005). La vía FG se activa en las condiciones de limitación de nutrientes detectadas por Msb2, aunque también están implicadas Sho1, Opy1, Ste50, Cdc42 y Ste20 (Yang y col., 2009) pero no Ste5, y en estas condiciones se activan respuestas dependientes de Kss1, y no de Fus3.

Del mismo modo, si bien la vía PH es dependiente de la proteína andamio Ste5, la vía HOG depende de Sho1 y Pbs2. Además, Ste7 no tiene afinidad por la MAPK Hog1, por lo que es sencillo de entender que, aunque compartan la MAPKKK Ste11, ambos módulos están separados de forma segura tanto por interacciones directas proteína-proteína, como por sus respectivas proteínas andamio (Zarrinpar y col., 2004; Reményi y col., 2005).

Tal como se ha señalado anteriormente, las rutas HOG y FG muestran, además de Ste11, muchos elementos reguladores comunes, situados en la ruta antes de las MAPK, incluidos los sensores Sho1 y Msb2 y la proteína de anclaje a membrana Opy2 (Figura 1.12). No obstante, el osmoestrés activa Kss1 sólo de forma débil y transitoria (Shock y col., 2009) mientras que los defectos de glicosilación (que activan Kss1) no activan a Hog1 (Yang y col., 2009). El hecho de que en mutantes *Δpbs2* o *Δhog1* bajo condiciones de osmoestrés se active Kss1 y el crecimiento celular polarizado (O'Rourke y Herskowitz, 1999), así como que la activación de Hog1 (vía osmoestrés o sobreexpresión de Pbs2) inhiba las respuestas FG (Pitoniak y col., 2009) sugiere un mecanismo de inhibición entre las dos rutas, mediado, al menos, por la MAPK Hog1 (Westfall y Thorner, 2006), aunque de momento se desconoce la identidad del sustrato de Hog1 en la vía FG. Saito (2010) sugiere que podría ser una supuesta proteína andamio de esta vía, tal como se muestra en la Figura 1.12.

Otro ejemplo de inhibición cruzada es el llevado a cabo por la MAPK Fus3 que, en respuesta a feromonas, fosforila el factor de transcripción Tec1 (Figura 1.12), diana de Kss1 y responsable de la activación de genes de respuesta a crecimiento filamentoso. Al ser

fosforilado, Tec1 es rápidamente ubiquitinado y degradado, evitándose de nuevo que bajo el estímulo de feromonas se active una respuesta de FG (Sabbagh y col., 2001). También se ha propuesto que en respuesta a osmoestrés Hog1 inhibe a este nivel la vía FG, no mediante la fosforilación y degradación de Tec1, sino afectando a la unión de este factor de transcripción al DNA (Shock y col., 2009).

Esto son sólo alguno de los varios mecanismos existentes y que dejan patente la evidente complejidad de los mecanismos de regulación de las redes de señalización en la levadura de gemación.

Otras rutas de transducción de señales importantes en *S. cerevisiae*

Los nutrientes proporcionan a las células la energía necesaria para crecer, reproducirse y construir todas las estructuras necesarias. No es de extrañar, por tanto, que regulen también las principales rutas de señalización de los organismos. El mecanismo de represión por glucosa, la vía de la PKA (Proteína quinasa A) y la vía TOR (*Target of Rapamicine*) son las principales rutas implicadas en crecimiento celular y disponibilidad de nutrientes.

Mecanismo de represión por glucosa: Snf1

La glucosa es la fuente de carbono fermentable preferida por la levadura *S. cerevisiae* para obtener energía. Cuando este azúcar (u otros similares rápidamente fermentables como fructosa o manosa) está presente en el medio se activa una compleja red de señalización para inactivar el transporte y metabolismo de fuentes de carbono alternativas menos eficientes (Petrenko y col., 2013), y se reprime la transcripción de genes implicados en la respiración celular (ciclo de Krebs y cadena de transporte electrónico), gluconeogénesis, ciclo del glioxilato y transporte y degradación de fuentes de carbono alternativas (Gancedo, 1998).

La quinasa activada por AMP Snf1 y el represor transcripcional Mig1 desempeñan un papel fundamental en el mecanismo de represión por glucosa. En condiciones de ausencia o bajos niveles de glucosa, esta quinasa se activa mediante fosforilación en el

residuo Thr210 (Carlson y col., 1981). Esta modificación es dependiente de tres quinasas parcialmente redundantes: Sak1, Tos3 y Elm1 (Hong y col., 2003; Sutherland y col., 2003). Una vez activada, Snf1 promueve el metabolismo respiratorio, la acumulación de glucógeno, la gluconeogénesis, la autofagia, el ciclo del glioxilato, la biogénesis de peroxisomas y el envejecimiento (Lin y col., 2003; Hedbacker y Hedbak, 2008; Usaite y col., 2009). Su mecanismo de actuación, tal como se muestra en la Figura 1.14, es llevado a cabo mediante la activación de los factores de transcripción Adr1, Sip1 y Cat8, y la inactivación de la proteína represora Mig1.
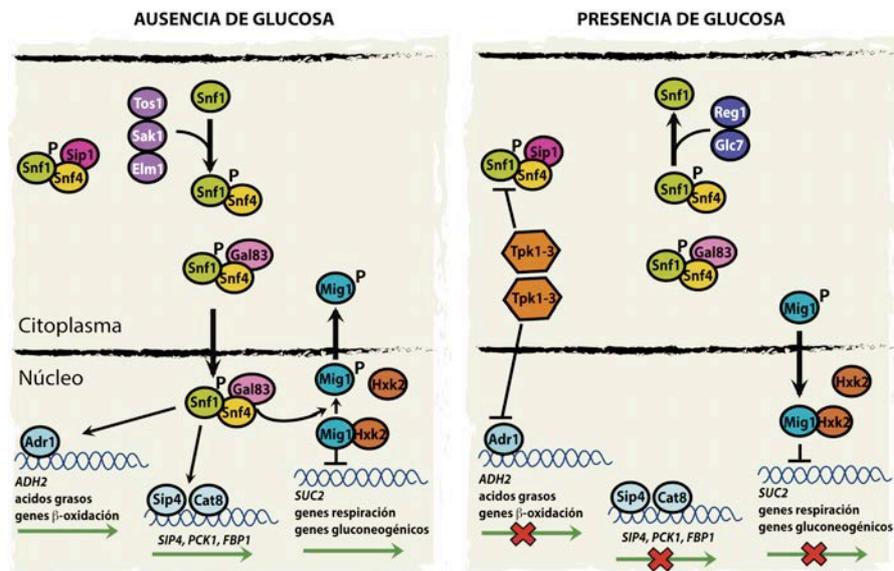
Figura 1.14. Esquema del mecanismo de represión por glucosa en *S. cerevisiae*. La quinasa Snf1 orquesta la represión por glucosa de fuentes de carbono alternativas y de genes implicados en respiración y gluconeogénesis. Adaptado de Conrad y col. (2014).

Mig1, al ser fosforilada, se disocia de Hxk2 y se transloca al citoplasma, por lo que deja de ejercer en el núcleo su acción sobre la transcripción de genes respiratorios y gluconeogénicos, que, al igual que *SUC2*, podrán ser ahora expresados para hacer frente a las condiciones de ausencia de glucosa.

En condiciones de presencia o abundancia de glucosa, Snf1 está inactiva mediante la acción de las fosfatasas Reg1 y Glc7, y en consecuencia, Mig1 está activa y unida a Hxk2 en el núcleo, por lo que ejerce, junto con el corepresor Cyc8-Tup1 (McCartney y Schmidt, 2001), el mecanismo de represión por glucosa.

Vía de la PKA

La vía de la proteína quinasa A es otra ruta importante que detecta la presencia de glucosa extracelular, condición óptima de nutrientes que reprime la adaptación y respuesta al estrés en general, y estimula la fermentación y el crecimiento y proliferación celular (Smets y col., 2010).

Tal como se muestra en la Figura 1.15, *S. cerevisiae* dispone de un sistema dual de detección de glucosa. Por una parte, el receptor Gpr1, reconoce la glucosa extracelular, mientras que el sistema sensor Cdc25-Sdc25-Ras1/2-Ira1/2 (Rolland y col., 2000) detecta la glucosa intracelular fosforilada por la hexoquinasa Hxk1/2, implicada en la glicolisis. Ambos modos de detección de glucosa convergen en la activación de la adenilato ciclasa (AC), cuyo incremento de actividad resulta en un augmento de los niveles intracelulares de cAMP (Colombo y col., 1998; Versele y col., 2001). El cAMP se une a la subunidad reguladora de la PKA, Bcy1, haciendo que se disocie del complejo y que queden libres y activas las subunidades catalíticas Tpk1-3.
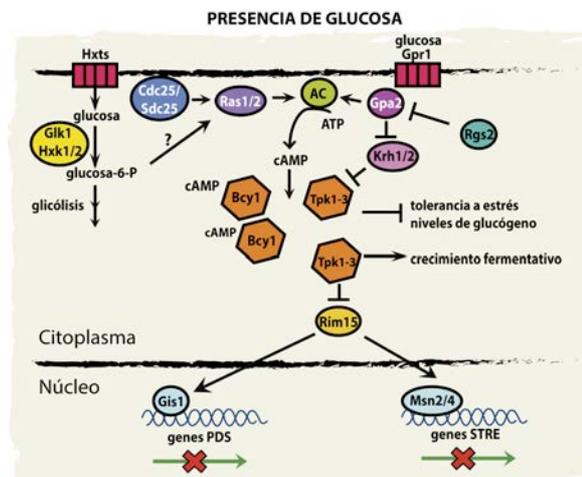
Figura 1.15. Esquema de la ruta de la PKA en *S. cerevisiae*. En presencia de glucosa se activa la adenilato ciclasa (AC) lo que resulta en un direccionamiento de la expresión génica hacia el crecimiento fermentativo rápido. Adaptado de Conrad y col., (2014).

Las Tpks ejercen ahora una serie de acciones, entre ellas, inactivar la quinasa Snf1 (Figura 1.14), lo que promueve, como se ha explicado anteriormente, la represión de genes implicados en gluconeogénesis, respiración, uso de sustratos alternativos, etc. (Thevelein y col., 2000). Las Tpks, en general, van a favorecer un rápido crecimiento fermentativo y a

inhibir el crecimiento más lento, dependiente de respiración o de fase estacionaria. El mecanismo de actuación es a través de la fosforilación directa de enzimas citosólicos (Schepers y col., 2012) y de la regulación de la expresión génica. En este sentido, PKA fosforila e inhibe Rim15, regulador positivo de importantes factores de transcripción, como Gis1 o Msn2/4 (Cameroni y col., 2004). De este modo, en condiciones óptimas de glucosa y nutrientes, se evita que se active la respuesta general a estrés (ESR), asegurando así el óptimo crecimiento de la célula.

Vía TOR (*Target of Rapamicine*)

La ruta TOR (Figura 1.16) regula el crecimiento celular en función de la disponibilidad de nutrientes, activándolo si hay condiciones óptimas de fuentes de carbono y nitrógeno (Thomas y Hall, 1997). Por tanto, permite interconectar los mecanismos de represión por glucosa y la PKA con el metabolismo del nitrógeno. La inactivación de TOR afecta a múltiples procesos del metabolismo celular, crecimiento y longevidad (Inoki y col., 2011).
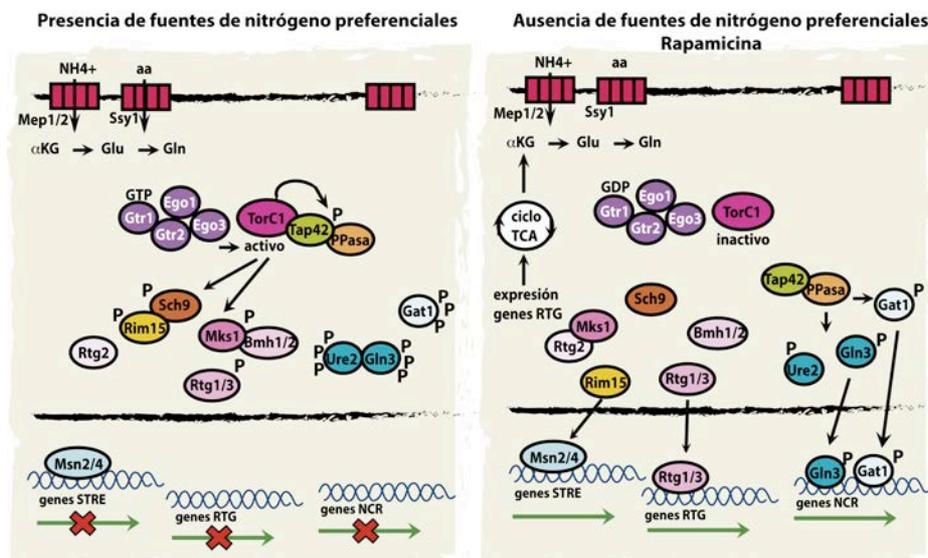
Figura 1.16. Esquema de la vía TOR en *S. cerevisiae*. Adaptado de Conrad y col. (2014).

En levadura existen dos proteínas TOR, Tor1 y Tor2. Ambas actúan en complejos con otras proteínas. Se denomina TORC1 al complejo formado por Tor1 y Tor2 asociadas con Kog1, Lst8 y Tco89 (Reinke y col., 2004) y TORC2 al constituido por Tor2 junto con

Avo1-3, Bit61 y Lst8 (Loewith y col., 2002). TORC1 es sensible a rapamicina, y controla la expresión de genes implicados en la síntesis de proteínas (rRNAs, tRNAs y proteínas ribosomales), en la utilización de nitrógeno (sometidos a represión por catabolito de nitrógeno, NCR) y en la vía de señalización retrógrada mitocondrial (RTG). TORC2 no es sensible a rapamicina y afecta a la organización del citoesqueleto de actina, endocitosis, síntesis lipídica y supervivencia celular.

TORC1 está regulado en la membrana vacuolar por la acción de cuatro proteínas, Ego1, Ego3, Gtr1 y Gtr2 (Dubouloz y col., 2005). Como muestra la Figura 1.16, en presencia de fuentes de nitrógeno preferentes, es decir, aquéllas que pueden ser fácilmente convertidas en glutamato (Glu) y glutamina (Gln), los principales precursores de la biosíntesis de aminoácidos, TORC1 está activo y causa la represión de genes implicados en el metabolismo de fuentes de nitrógeno menos preferentes (mecanismo NCR, *Nitrogen Catabolite Repression*) (Rohde y col., 2008). TOR1C ejerce su control vía dos efectores, Sch9 y el complejo Tap42 fosfatasa (Loewith y Hall, 2011). TORC1 activo fosforila y activa a su vez a Sch9, que modula la traducción y el tamaño celular obtenido antes de la división celular (Jorgensen y col., 2004). Pero además, Sch9 fosforila y secuestra en la membrana a Rim15, encargada de activar, como se indicó anteriormente, a los factores transcripcionales Gis1 y Msn2/4 (Wanke y col., 2008). Así, de nuevo, bajo óptima disponibilidad de nutrientes (esta vez fuentes de nitrógeno preferentes) se evita la respuesta general a estrés mediante el control de la actividad de Msn2/4.

En presencia de rapamicina o ausencia de fuentes de nitrógeno preferentes, TORC1 está inactivo, Sch9 se desfosforila rápidamente (como sucede también en ausencia de fuentes de carbono, Binda y col., 2009) y ante esta situación desfavorable sí estaría activa la respuesta general a estrés. Estas condiciones también promueven la desfosforilación de las fosfatasas Tap42 (Yan y col., 2006), que dejan de estar asociada a TORC1 y, al liberarse en el citoplasma, reducen la fosforilación de Ure2, Gln3 y Gat1, lo que promueve la localización nuclear de las dos últimas, y por tanto la expresión de genes controlados por represión de catabolito de nitrógeno (Duvel y col., 2003), para suplir la ausencia de nitrógeno en el medio. Además, tanto la rapamicina como la ausencia de nutrientes activan la vía de integridad de la pared celular (CWI) que como se describió anteriormente es una importante ruta de respuesta a estrés.

La respuesta a alta glucosa

Tal como se ha señalado en el apartado anterior, la glucosa es la fuente de carbono fermentable preferida por *S. cerevisiae* para obtener energía. El estrés osmótico es una condición adversa que también se ha descrito ampliamente a lo largo de esta introducción, y uno de los osmolitos causantes del mismo puede ser la glucosa, cuando está en el medio de crecimiento en concentraciones superiores a las óptimas.

En estas condiciones la célula entra en un paradógico dilema pues, por una parte, la alta osmolaridad del medio hace que se active la vía de respuesta a osmoestrés HOG (Remize y col., 2003; Jiménez-Martí y col., 2011), con todo lo que ello supone para la célula en cuanto a la puesta en marcha de los mecanismos adaptativos a esta situación desfavorable. Pero, por otro lado, el exceso de nutrientes en forma de glucosa en el medio hace que se active el mecanismo de represión por glucosa, así como la vía de la PKA (Conrad y col., 2014), ya que ambas rutas responden a la glucosa extracelular, y entienden su presencia como una condición favorable de disponibilidad (aunque en exceso) de nutrientes.

Las células de levadura están sometidas a estrés por altas concentraciones de azúcar durante su utilización en importantes procesos biotecnológicos como, por ejemplo, la producción de bebidas alcohólicas (Attfield, 1997; Myers y col., 1997; Pretorius y Bauer, 2002). Es por ello que el osmoestrés causado por glucosa ha sido ampliamente estudiado en el contexto de la fermentación, tanto al inicio de la vinificación (Perez-Torrado y col., 2002a; Zuzuarregui y del Olmo, 2004b) como en fases avanzadas de la misma (Riou y col., 1998).

Estudios transcriptómicos en presencia de elevadas concentraciones de glucosa

Aunque en este trabajo no se reproducen las condiciones de fermentación, ni se realizan análisis con cepas vínicas, hay algunos estudios interesantes al respecto que pueden ayudar a entender la complejidad de la respuesta al osmoestrés causado por glucosa. La concentración de este azúcar considerada más frecuentemente en estos análisis ha sido

del 20% (p/v) o 1.1 M, puesto que se aproxima a la que se suele encontrar en los mostos al inocular las levaduras. Esta concentración equivale en términos de estrés osmótico a sorbitol 1M y NaCl 0.6M (actividad del agua 0.981; Chen, 1989).

Como se ha comentado anteriormente, en los estudios transcriptómicos llevados a cabo para entender la respuesta a osmolitos como sal o sorbitol (Gasch y col., 2000; Posas y col., 2000; Rep y col., 2000; Causton y col., 2001; Hirasawa y col., 2006) se han descrito cambios en la expresión de genes implicados en el metabolismo de glucosa, trehalosa y/o glucógeno, la regulación redox y la respuesta a estrés. La respuesta molecular de la levadura a alta glucosa presenta muchos aspectos necesariamente comunes con estas condiciones de osmoestrés, ya que, como se ha comentado con anterioridad, también en presencia de dicho osmolito se activa la vía HOG (Remize y col., 2003; Jiménez-Martí y col., 2011). Así, los estudios transcriptómicos globales llevados a cabo por Kaeberlein y col. (2002) describieron un incremento en la expresión de genes relacionados con la biosíntesis de glicerol y trehalosa bajo condiciones de 20% (p/v) de glucosa. Erasmus y col., (2003) llevaron a cabo otro análisis transcriptómico en la cepa vínica Vin13 comparando 40% con 20% de glucosa y observaron una mayor expresión en la primera condición de los genes implicados en las rutas glicolítica y de los fosfatos de pentosa, así como en la formación de ácido acético a partir de acetaldehído; la expresión era menor en genes relacionados con la biosíntesis de *novo* de purinas, pirimidinas, histidina y lisina. Marks y col. (2008) identificaron 223 genes inducidos fuertemente en distintas etapas de la fermentación, designados como FSR (*Fermentative Stress Response*), e implicados también en transporte, organización subcelular, modificación de proteínas, metabolismo del mRNA, respuesta a estrés y transcripción.

Otros estudios, realizados durante procesos de fermentación alcohólica con mostos con alta concentración de azúcares, describen cambios inesperados en la expresión génica que afectan, por ejemplo, a genes regulados por represión por catabolito de carbono (Rossignol y col., 2003; Zuzuarregui y col., 2006; Mendes-Ferreira y col., 2007) o algunos genes de respuesta a estrés (Pérez-Torrado y col., 2002a).

En un estudio más reciente, llevado a cabo en 2011 en nuestro grupo por Jiménez-Martí y colaboradores se observó que los genes que mostraban una expresión superior a dos veces en 20% de glucosa respecto a YPD (medio óptimo de crecimiento de la levadura, que

contiene un 2% de glucosa) se podían clasificar en tres categorías: i) genes implicados en el metabolismo del glicerol, ii) genes que participan en la respuesta a estímulos químicos y iii) genes de función desconocida. Además, la mayoría de genes que presentaban el comportamiento contrario, es decir, reprimidos bajo estas condiciones de estrés, estaban relacionados con la fosforilación oxidativa, y el ciclo de Krebs y de la urea.

En 2011, Zakrzewska y colaboradores realizaron un rastreo con una colección de mutantes por deleción para identificar genes implicados en la supervivencia a estrés y encontraron una correlación inversa entre tasa de crecimiento y resistencia a condiciones adversas. Demostraron, utilizando medios con elevadas concentraciones de glucosa, que la reducción de la tasa de crecimiento favorecía una distribución de los recursos hacia las funciones de tolerancia a estrés, y sugirieron un importante papel de la histona desacetilasa Rpd3 en la adquisición de tolerancia a ácido acético y estrés por calor.

A pesar de toda esta información y de la proporcionada por otros estudios relacionados, realizados con cepas vínicas o de laboratorio en presencia de elevadas concentraciones de glucosa, todavía no se ha llevado a cabo un estudio comparativo directo que permita una buena comparación con respecto a lo que ocurre en presencia de los osmolitos más analizados, sorbitol y sal.

Particularidades de la respuesta a alta glucosa

Los estudios transcriptómicos descritos anteriormente revelan diferencias en los patrones de expresión génica derivadas de la naturaleza del osmolito considerado. Estas diferencias están fundamentalmente relacionadas con el mecanismo de represión por glucosa y el funcionamiento de la ruta de la proteín quinasa A.

Si bien algunos estudios de expresión génica llevados a cabo tras una corta incubación en presencia de 20% (p/v) de glucosa (Pérez-Torrado y col., 2002a; Jiménez-Martí y col., 2011) parecen sugerir un efecto represor adicional debido al aumento de glucosa presente en el medio, existen otros datos que apuntan en otra dirección. Así, en 2008, Marks y colaboradores describieron una atenuación parcial del mecanismo de represión por glucosa durante el proceso de fermentación que podría ser consecuencia de la

respuesta al incremento de la concentración de etanol en el medio, pues la producción de éste durante la vinificación es otro elemento clave que afecta al crecimiento de las células.

Por otra parte, dado que en presencia de glucosa está activa la vía de la PKA, se ha sugerido que las diferencias en la respuesta a alta glucosa deben estar relacionadas con la inhibición de la actividad de Msn2/4 (Capaldi y col., 2008), por lo que estos autores han propuesto un cambio en el programa de expresión dependiente de Hog1 hacia los genes regulados por Sko1 y Hot1, factores transcripcionales que ganarían, por tanto, un mayor protagonismo en esta condición que en el osmoestrés causado por sal o sorbitol.

Con todo, la activación y entrada en el núcleo de Hog1 bajo el osmoestrés por azúcar debe permitir a estos factores de transcripción ser reclutados a las regiones reguladoras de los genes de respuesta a estrés, para direccionar y reclutar a su vez el complejo Hog1-RNApolII que haga posible su transcripción (Cook y O'Shea, 2012; Nadal-Ribeles y col., 2012).

El gen de respuesta a estrés *YHR087W*

Uno de los genes de función desconocida encontrados en el estudio transcriptómico citado anteriormente (Jiménez-Martí, 2011), que se induce aproximadamente 5 veces bajo condiciones de 20% (p/v) de glucosa es *YHR087W/RTC3,* que codifica una proteína pequeña de 111 aminoácidos (12kDa), cuyos niveles también se incrementan de forma perfectamente correlacionada a la inducción del gen en estas condiciones.

La expresión de este gen también se incrementa en presencia de los osmolitos sal y sorbitol, así como bajo otras condiciones adversas distintas al estrés hiperosmótico, como choque térmico, daño oxidativo, etanol, pHs ácidos o básicos y fase estacionaria (Gasch y col., 2000; Causton y col., 2001; Capaldi y col., 2008).

Resultados obtenidos en estudios recientes han aportado evidencias acerca de la importancia de este gen en la respuesta a altas concentraciones de glucosa. Por una parte, su sobreexpresión en cepas vínicas mejora su comportamiento fermentativo y su tolerancia a estrés (Jiménez-Martí y col., 2009). Además, cepas con mayor resistencia a osmoestrés presentan niveles más altos de mRNA de este gen (Jiménez-Martí y col., 2011). Finalmente, su disrupción en cepas de laboratorio causa problemas de crecimiento, menor

viabilidad y disminución en el consumo de glucosa en presencia de concentraciones de dicho azúcar del 20% y 30% (Jiménez-Martí y col., 2011).

Desde el punto de vista estructural, Yhr087w presenta homología con la familia de proteínas relacionadas con SBDS (Savchenko y col., 2005). SBDS es la proteína humana cuya mutación provoca el Síndrome de Shwachman-Bodian-Diamond, una enfermedad rara autosómica recesiva entre cuyos síntomas clínicos destacan disfunción hematológica, insuficiencia pancreática y anormalidades esqueléticas, presentando los enfermos que la padecen una mayor predisposición a desarrollar mielodisplasia y leucemia (Bodian y col., 1964; Shwachman y col., 1964; Ginzberg y col., 1999). El ortólogo en levadura de la SBDS humana es Sdo1 (Boocock y col., 2006) y, dada la similitud estructural que presenta Yhr087w con la parte N-terminal de Sdo1 (Figura 1.17), se ha propuesto que podría realizar una función similar o redundante a la de Sdo1, que se une el RNA, interacciona con factores que procesan el rRNA y está implicada en la maduración de la subunidad ribosomal 60S (Savchenko y col., 2005; Menne y col., 2007; Luz y col., 2009).
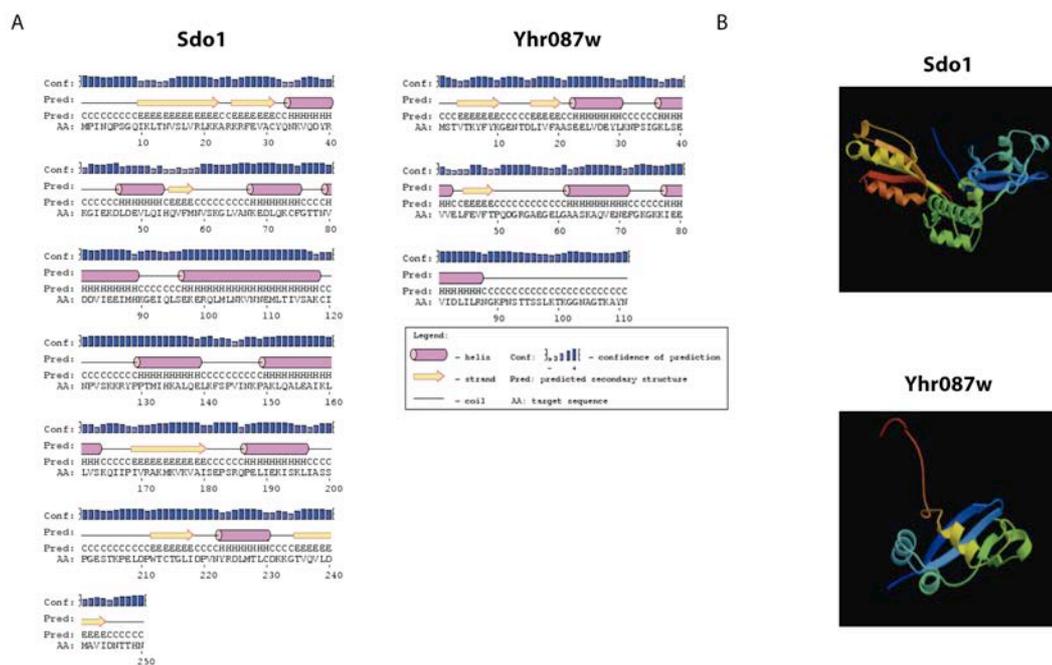
Figura 1.17. Modelización informática de la estructura de las proteínas de *S. cerevisiae* Sdo1 y Yhr087w. Realizada mediante las herramientas A) Geno3d (Pôle Bioinformatique Lyonnais (PBIL) del paquete Expasy (SIB Bioinformatics Resourse Portal)) y B) PSIPRED (University College London, Department of Computer Science. Bioinformatics group) del paquete ExPASy (www.expasy.org/proteomics)**.**

Además, resultados obtenidos en varios estudios de otros autores, relacionan la función de Yhr087w con distintas etapas del proceso de expresión génica, pudiéndosele asignar a esta proteína funciones muy diversas, en el contexto de la transcripción y su control (Costanzo y col., 2010), procesamiento y transporte del RNA, o traducción y procesos post-traduccionales (Savchenko y col., 2005). Incluso se le ha llegado a atribuir una posible función relacionada con la estabilidad telomérica (Adinall y col., 2008). La mayoría de estas evidencias provienen de estudios globales de interacciones genéticas, por lo que por el momento se desconoce la función de la proteína y su relación con la respuesta a estrés.

2. Objetivos

Teniendo en cuenta los antecedentes comentados en la sección de Introducción, en esta Tesis Doctoral se pretende profundizar en cómo responden las levaduras al estrés osmótico provocado por elevadas concentraciones de glucosa y qué papel desempeñan en dicha respuesta las proteínas Hot1 e Yhr087w.

Los objetivos concretos a desarrollar son los siguientes:

1.- Análisis funcional de Yhr087w/Rtc3, proteína de *S. cerevisiae* cuyos niveles se incrementan en respuesta a distintos tipos de estrés y, especialmente, en condiciones de alta osmolaridad. Se analizará la regulación diferencial de la expresión del gen en distintas condiciones de estrés. Además, se pretende conocer las interacciones físicas que establece con otras proteínas y su función en el contexto de la respuesta a estrés osmótico.

2.- Estudio de los elementos de secuencia del factor transcripcional de respuesta a estrés osmótico Hot1 esenciales para su funcionalidad y, por tanto, para la expresión de sus genes diana. En particular, se tratará de entender el mecanismo de activación llevado a cabo por la MAPK Hog1 sobre Hot1 en condiciones de osmoestrés. En estos análisis se utilizarán *YHR087W* y *STL1* como modelo de genes de respuesta a estrés osmótico regulados respectivamente parcial y completamente por Hot1.

3.- Análisis de la interacción de Hot1 con el DNA. Se pretende determinar el dominio de unión al DNA del factor transcripcional, así como la secuencia de reconocimiento en los promotores de sus genes diana. Para ello, se utilizarán diversas estrategias. Por un lado, se procederá al estudio transcriptómico del mutante por deleción *Δhot1* para determinar la totalidad de los genes regulados por Hot1 en condiciones de osmoestrés, así como al análisis *in silico* de la secuencia de los promotores de dichos genes para hallar posibles secuencias consenso. También se intentará demostrar *in vitro* e *in vivo* la unión de Hot1 a su secuencia UAS de reconocimiento.

4.- Profundizar en la función del factor transcripcional Hot1 en la expresión génica mediante el estudio de las interacciones físicas proteína-proteína de Hot1. Se prestará especial atención al análisis de la fosforilación del factor transcripcional en diversas condiciones de osmoestrés en mutantes de quinasas diferentes a la MAPK Hog1. También se estudiará la implicación de la interacción de Hot1 con el co-activador transcripcional Sub1 y la posible participación de Hot1 en la elongación transcripcional.

5.- Entender las peculiaridades que presenta el estrés osmótico causado por elevadas concentraciones de glucosa en comparación con el producido por otros osmolitos estudiados tradicionalmente, como sal o sorbitol. Para cumplir este objetivo se recurrirá al análisis molecular de la implicación de los factores transcripcionales de respuesta a estrés Msn2/4 y Hot1 en respuesta a alta glucosa. Además, se llevará a cabo un análisis transcriptómico global para identificar genes inducidos y reprimidos de forma diferencial en sorbitol y alta glucosa. Se determinarán las implicaciones fisiológicas a nivel celular del estrés causado por elevadas concentraciones de glucosa.

3. Publicaciones

3.1. Capítulo 1

The *Saccharomyces cerevisiae* Hot1p regulated gene *YHR087W* (*HGI1*) has a role in translation upon high glucose concentrations stress

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The *Saccharomyces cerevisiae* Hot1p regulated gene *YHR087W* (HGI1) has a role in translation upon high glucose concentration stress

M Gomar-Alba[1], E Jiménez-Martí[1,2] and M del Olmo[1*]

Abstract

Background: While growing in natural environments yeasts can be affected by osmotic stress provoked by high glucose concentrations. The response to this adverse condition requires the HOG pathway and involves transcriptional and posttranscriptional mechanisms initiated by the phosphorylation of this protein, its translocation to the nucleus and activation of transcription factors. One of the genes induced to respond to this injury is *YHR087W*. It encodes for a protein structurally similar to the N-terminal region of human SBDS whose expression is also induced under other forms of stress and whose deletion determines growth defects at high glucose concentrations.

Results: In this work we show that *YHR087W* expression is regulated by several transcription factors depending on the particular stress condition, and Hot1p is particularly relevant for the induction at high glucose concentrations. In this situation, Hot1p, together to Sko1p, binds to *YHR087W* promoter in a Hog1p-dependent manner. Several evidences obtained indicate Yhr087wp's role in translation. Firstly, and according to TAP purification experiments, it interacts with proteins involved in translation initiation. Besides, its deletion mutant shows growth defects in the presence of translation inhibitors and displays a slightly slower translation recovery after applying high glucose stress than the wild type strain. Analyses of the association of mRNAs to polysome fractions reveals a lower translation in the mutant strain of the mRNAs corresponding to genes *GPD1*, *HSP78* and *HSP104*.

Conclusions: The data demonstrates that expression of Yhr087wp under high glucose concentration is controlled by Hot1p and Sko1p transcription factors, which bind to its promoter. Yhr087wp has a role in translation, maybe in the control of the synthesis of several stress response proteins, which could explain the lower levels of some of these proteins found in previous proteomic analyses and the growth defects of the deletion strain.

Keywords: *Saccharomyces cerevisiae*, High glucose osmotic stress, Gene *YHR087W*, Gene expression, Translation, Hot1p, Hog1p, Polysomes

Background

Stress response in the yeast *Saccharomyces cerevisiae* involves the detection of adverse conditions (high or low osmolarity, nutrient limitation, ethanol exposure, increased level of oxidant reagents, variations in pH, etc.), activation of signal transduction pathways, and transcriptional and posttranscriptional regulation, resulting in the accumulation of protective agents and repairing activities [1]. All these mechanisms are intended to allow yeast cells to adapt to environmental changes.

Under several different stress conditions, *S. cerevisiae* displays a common response, the so-called *Environmental Stress Response* (ESR), characterized by changes in the expression of approximately 900 genes [2]. Most of these genes contain the AGGG consensus sequence in their promoter [3], which is recognized by transcription factors Msn2p and Msn4p [4]. The activity of these factors is regulated by two pathways that control cell growth: *Protein Kinase A* (PKA, [5]) and *Target of Rapamycin* (TOR, [6]).

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Besides this general response, yeast cells display specific mechanisms to resist particular adverse conditions. In the case of hyperosmotic stress, produced by a high concentration of salt, sorbitol, glucose or of any other osmolyte, the response is mediated by the *High Osmolarity Glycerol* (HOG) pathway. High osmolarity is detected by osmosensors located in the membrane, that activate MAP quinases which, finally, permit the phosphorylation of MAPK Hog1p, which results in its translocation to the nucleus. Once inside this compartment, Hog1p activates several transcription factors (Hot1p, Msn1p, Smp1p, Gcn4p, Skn7p, Sko1p, Msn2p and Msn4p) [1,7,8]. These factors determine an induction in the expression of about 10% of the yeast genes under conditions of stress caused by high salt or sorbitol concentrations [2,9-12]. One of the consequences of these changes in gene expression is an increase in the intracellular concentration of glycerol, the osmolyte that yeast cells produce to counteract hyperosmotic stress.

One of the conditions of hyperosmotic stress that can affect yeast cells in particular environments (for instance, during the production of alcoholic beverages) is that produced by sugar concentrations of 20% (w/v) or even higher. Several transcriptomic analyses have been carried out to understand the particularities of the response to this form of stress [13-15]. All these studies have indicated that three groups of genes display higher expression levels under these conditions: i) genes involved in glycerol metabolism, ii) genes participating in response to chemical stimulus, and iii) several genes of unknown function. One of them, *YHR087W*, is induced approximately 5 times under 20% glucose and encodes for a protein of 111 amino acids (12 kDa). The expression of this gene also increases under other hyperosmotic stress conditions (salt and sorbitol) and in response to other adverse conditions such as heat shock, oxidative damage produced by H_2O_2 or diamide, ethanol, acid or basic pHs and the stationary phase [2,11,16]. The increase in the *YHR087W* mRNA levels under 20% glucose is also followed by higher content of the corresponding protein [15].

Some of the data obtained in recent years have demonstrated the relevance of the *YHR087W* gene expression in the response to high sugar concentrations. On the one hand, its overexpression in wine yeast strains results in an improved stress response and fermentative behavior [17]. Besides, strains with a high resistance to osmotic stress show higher mRNA levels corresponding to this gene [15]. Finally in laboratory strains, its disruption results in growth delay, lower viability and reduced glucose consumption under 25% and 30% glucose concentrations [15].

From the structural point of view, Yhr087wp presents a strong homology with the protein family related with human SBDS [18]. SBDS is the human protein whose mutation provokes the Shwachman-Bodian-Diamond syndrome, a rare autosomal recessive disorder with clinical features, including haematological dysfunction, pankreatic exocrine insufficiency and skeletal abnormalities, as well as a significant predisposition to the development of myelodysplasia and leukemia [19-21]. The yeast orthologue of SBDS is Sdo1p [22]. Yhr087wp contains the same structural elements as Sdo1p in the N-terminus region and, according to sequence analyses [18,23], they are distant homologues.

Sdo1p binds RNA, interacts with nuclear rRNA-processing factors [24], and is involved in the maturation of the ribosomal 60 S subunit required for the translational activation of ribosomes [25]. There is a report describing an aberrant regulation of Btn1p in the absence of Sdo1p, which suggests that portions of the ribosome maturation pathways survey the vacuolar function, presumably as a means to adjust protein levels for optimal cellular homeostasis [26]. Due to the structural relationship between Yhr087wp and Sdo1p, it has been proposed that the role of that protein in yeast cells would be associated in some way with the RNA metabolism [18]. Actually, synthetic lethality has been described between *yhr087w* and the mutants in genes encoding proteins involved in RNA processing and transport, translation and posttranslational processes (*nat3* –acetylation of ribosomal proteins-, *nsr1* -synthesis of rRNA 18 S and its precursor 20 S-, *air1* –nuclear RNA processing-, *npl3* and *yra2p* -mRNA export [18]).

However, the data found by other authors suggest that Yhr087wp could be involved in other cellular processes. The *YHR087W* gene has also been named *RTC3* (from *Restriction of Telomere Capping*) because its null mutant suppresses the phenotype of termosensitive mutant *cdc13-1* [27]. In this mutant, at the non-permissive temperature, telomeric DNA is degraded and cell cycle progression is impaired. On the other hand, Costanzo et al. [28] described genetic interactions between *YHR087W* and several genes encoding proteins related with transcription and its control, such as Nut1p (a component of the RNA polymerase II mediator complex), Set3p (a member of a histone desacetylase complex), Npl3p (RNA polymerase II transcription elongation), Cna1p (a component of calcineurin) or Bcy1p (a regulatory subunit of the PKA). Finally, the results of a recent proteomic comparison made in our laboratory between deletion mutant Δ*yhr087w* and its corresponding wild type strain [15] under 20% glucose showed lower levels of two proteins involved in protein folding (Hsp104p and Hsp78p) in the mutant strain, which appeared as an overrepresented category. In this sense, genetic interactions have been described between *yhr087w* and *hsp82* [29].

In this work we carry out several experiments in order to gain more insights into the role of Yhr087wp in yeast cells. The data obtained confirms once more the relationship between this protein and the stress response, provides new information about its transcriptional regulation, and points to a role in translation under adverse growth conditions.

Results

Yhr087wp is distributed throughout the cell

Data from a global study of protein localization by GFP fusions [30] indicate that protein Yhr087wp is distributed throughout the cell.

In order to verify this information and to determine whether the subcellular localization could be affected in some way when yeast cells are subjected to hyperosmotic stress conditions due to high glucose, a GFP tag was introduced into the chromosomal copy of gene *YHR087W* in the 3' coding region.

The fluorescence microscopy analysis of the exponentially growing cells (Figure 1) showed that Yhr087wp is located in the nucleus and cytoplasm, and that this location is not affected by changes in glucose concentration. Nonetheless, as previously described [15], an important increase in protein levels takes place when cells are transferred to SC 20% glucose. This increase is particularly important after 30 min.

YHR087W is differentially regulated by transcription factors Msn2/4p and Hot1p depending on the stress conditions considered

According to the microarray analyses carried out by Capaldi et al. [16], the increased expression of *YHR087W* under several conditions of osmotic stress is regulated through the HOG pathway. As this pathway is also involved in the response to high osmolarity due to 20% glucose [15] we determined if under this stress condition HOG1

controls *YHR087W* expression. For this purpose, cells exponentially growing in YPD medium from the strains described in Additional file 1: Table S1 TM141 (wild type) and TM233 (*hog1Δ*) were incubated for 30 min in YP20. RNA levels of gene *YHR087W* were determined by Northern analysis using a probe resulting from the amplification of genomic DNA by oligonucleotides YHR087W-1 and YHR087W-2 (Additional file 1: Table S2). As shown in Figure 2, *YHR087W* mRNA levels increase when cells are incubated for 30 min in growth medium containing 20% glucose (compare lanes C and 20% in WT in panel A), but the expression of the gene in the mutant is about ten times lower than in the wild type strain under these conditions (compare lanes 20% *Δhog1* and 20% WT and see the corresponding bar in panel B), thus demonstrating a role of the HOG pathway in the control of the transcription of this gene also when high glucose is used as an osmolyte.

The Msn2/4p transcription factors involved in the general stress response are activated by the HOG pathway under osmotic stress conditions. An analysis of the *YHR087W* sequence indicates the presence of STRE elements at positions -301 and -199, which could be recognized by these proteins. Besides, Msn2/4p proteins have been reported to be involved in the control of the *YHR087W* expression under certain conditions, such as zymolyase-induced cell wall stress [31], weak acid stress [32] and hyperosmolarity caused by high KCl concentrations [16]. To determine whether or not induction of *YHR087W* under high glucose concentrations follows the same pattern, RNA samples were obtained from exponentially growing cultures in YPD affected or not by 20% glucose or heat shock. These experiments were carried out with mutant strains in several of the transcription factors involved in the response to osmotic stress (Msn2/4p, Hot1p and Sko1p) using in each case its corresponding wild type strain as reference. As seen in Figure 2
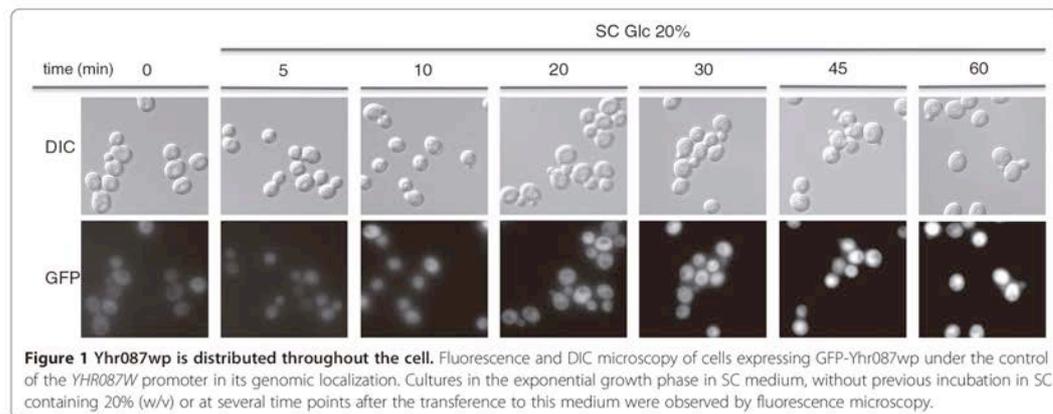
Figure 1 Yhr087wp is distributed throughout the cell. Fluorescence and DIC microscopy of cells expressing GFP-Yhr087wp under the control of the *YHR087W* promoter in its genomic localization. Cultures in the exponential growth phase in SC medium, without previous incubation in SC containing 20% (w/v) or at several time points after the transference to this medium were observed by fluorescence microscopy.

Figure 2 Regulation of the *YHR087W* gene expression by the HOG pathway, the Msn2/4p, Hot1p and Sko1p transcription factors, and the Bcy1p subunit of the PKA. Cells from the exponential cultures at 30°C in YPD medium of several wild type and mutant strains were maintained under these conditions (control, C), transferred to YP20 for 30 min (20%) or were affected by a 30-min heat shock at 39°C (39°C). Then were collected and total RNA was isolated. Gene expression was determined by Northern analysis using a specific probe. Data have been normalized with the *SRC1* gene. Images (panel **A**) show a representative example of the results obtained. Quantification of some of these results (panel **B**) has been carried out from the data obtained in three independent experiments; the average value and the standard deviation appear. In this quantification, the mRNA level found in each mutant under each stress condition is represented as the percentage of that detected in the corresponding wild type strain in the same situation, which is considered 100.

panel A, the induction of this gene under heat shock occurs in all the wild type strains considered, and is only drastically reduced (to about 24% compared to its reference strain, panel B) in the Δ*msn2,4* mutant. However, under high glucose stress conditions, although the induction is also detected in all the wild type strains used in this analysis (panel A) the factors mainly involved in the regulation of the expression of this gene are Sko1p, and particularly Hot1p, being the reduction in the expression to about 40% and 20% respectively when compared to the reference strains (panel B).

Chromatin immunoprecipitation experiments carried out with a wild type strain and its corresponding Δ*hog1* mutant both containing a Hot1-TAP tagged version (Figure 3 panel A) indicate that Hot1p binds to a region of the *YHR087W* gene promoter located between positions -360 and -181 under high glucose stress conditions and that this interaction depends on the presence of Hog1p. Accordingly, the involvement of Hot1p in the regulation of *YHR087W* expression under 20% glucose is similar to that found for other osmostress-induced genes, such us *CTT1, HSP12* and *STL1*, with exposure to NaCl 0.4 M [33]. Similar experiments have been carried out with the corresponding strains containing a Sko1p-TAP tagged version (Figure 3 panel B) and demonstrate that this transcription factor also binds to the region considered of *YHR087W* promoter in a Hog1p dependent manner.

Role of Yhr087wp in transcription and its control

Costanzo et al. [28] created a genome-scale genetic interaction map by examining 5.4 million gene-gene pairs for synthetic genetic interactions. With this approach, these

Figure 3 Hot1p and Sko1p bind to the *YHR087W* promoter under high sugar stress in a Hog1p- dependent manner. Panel **A1** shows the relative occupation of Hot1p over the region of the *YHR087W* promoter between -360 and -181, determined by chromatin immunoprecipitation (ChIP) followed by Real-Time PCR, in a Δ*hog1* mutant and its corresponding wild type strain, both of which contained a TAP-tagged version of Hot1p. Data were obtained as described in the *Materials and Methods* section. Experiments were carried out in triplicate; the average and standard deviation data are indicated. Panel **A2** contains the result of a representative ChIP experiment analyzed by semiquantitative PCR. In this case, a mix of three pairs of primers was used for each PCR reaction, which amplify the region corresponding to the *YHR087W* promoter described above and, those located between positions -750 and -420 of gene *CLN2* and -822 to -567 of gene *CLB2*. In this panel WCE indicates the whole cell extract prior to the inmunoprecipitation. For these experiments, cells from the exponentially growing cultures were also incubated for 10 min in YP20 (YP20 in panel A.1, + in panel A.2) or not (Control in panel A.1, - in panel A.2) to determine the dependence of Hot1p binding on high glucose stress. Panels **B1** and **B2** correspond to the same experiments in the case of a Δ*hog1* mutant and its corresponding wild type strain, both of which contained a TAP-tagged version of Sko1p.

authors described the interactions between *YHR087W* and several genes related to transcription and its control, such as *BCY1*, *CNA1*, *NUT1*, *SET3*, and *NPL3*. In order to corroborate these interactions and to determine their relevance, growth of serial dilutions of yeast cells in YPD solid medium at 37°C, or containing 30% glucose, was determined. This glucose concentration was selected according to previous data obtained in our laboratory [15] and to the results shown in Additional file 2: Figure S1, which indicate that a growth defect occurs in strain Δ*yhr087w* at this glucose concentration, which can be followed by growth in such plates. For these experiments, we focused on the determination of the relationship between *YHR087W* and *BCY1* (coding for the regulatory subunit of the PKA) and *CNA1* (which encodes for calcineurine). For this purpose *YHR087W* disruption was carried out in mutant strains in all of those genes.

Regarding gene *CNA1*, it was not possible to detect any genetic relationship under 37°C or at a high glucose concentration (Figure 4), maybe because of the different growth conditions (compared to those used by Constanzo et al. [28]) used in this work in order to get high

expression of gene *YHR087W*. However, with gene *BCY1*, a partial supression of its growth defects at 37°C was detected in the double mutant with Δ*yhr087w*. As shown in Figure 4, a double mutant grows better than the Δ*bcy1* single mutant at 37°C. Despite this interaction, the *YHR087W* expression is not affected in the Δ*bcy1* mutant strain under heat shock stress or at high glucose concentrations (Figure 2). Being Bcy1p the regulatory subunit of PKA, this result reinforces the Yhr087wp's involvement in the stress response.

The genetic interactions commented above suggest that this protein could indeed play some direct role in transcription. ChIP analyses were carried out to test the possible binding of Yhr087wp to DNA, particularly to the stress-induced gene *HSP104*. For this purpose, a short incubation at 37°C, or under 20% (w/v) glucose, was applied to the exponentially growing cells carrying fusion protein Yhr087w-TAP, created by genetic manipulation in the chromosomal copy of gene *YHR087W*, as described in the *Materials and Methods* section. The results indicate that Yhr087wp does not bind to any of the regions considered in *HSP104* DNA under any of the study conditions because the absolute

Figure 4 Genetic interactions between *YHR087W* and *BCY1* and *CNA1*. Growth of mutants Δ*yhr087w*, Δ*bcy*, *yhr087wbcy*, *cna1* and Δ*yhr087w*Δ*cna1* under osmotic (30% glucose) and heat shock (37°C) stress. Serial dilutions from the cultures of these strains were spotted and growth was followed for 3 days at 30°C or 37°C, as described in the *Materials and Methods* section.
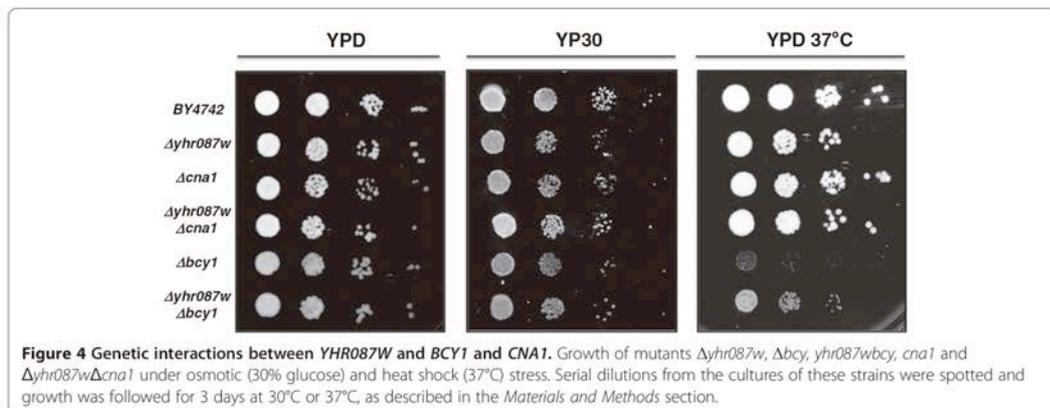
values are very similar to those found under the same conditions with a non labeled strain used as a negative control (Additional file 2: Figure S2). Additional ChIP experiments described in the Materials and Methods section indicated that the binding of the RNA polymerase II to *HSP104* promoter is not affected in the Δ*yhr087w* mutant (data not shown).

Yhr087wp interacts with proteins involved in stress response and ribosomal function

Of the various experimental methods available for identifying protein-protein interactions, this work considers the tandem affinity purification (TAP) of TAP-tagged Yhr087p expressed from its natural chromosome location (strain BY4741 *YHR087W:: TAP:: kanMX4*) to carry out a study on this protein.

In the global analysis of the protein complexes developed a few years ago by Krogan et al. [34], Yhr087wp appeared to physically interact with Eki1p (ethanolamine kinase) and Suc2p (invertase). An interaction with ribosomal protein Rpp0p was previously described [35]. As these data were obtained under non stress growth conditions, a TAP purification experiment was carried out after a 90-min incubation in YP20 of YPD-growing cells. Previous experiments carried out in our group [15] indicated that protein levels significantly increased under this condition when compared to control growth.

The analysis showed 45 proteins with the MASCOT software (Mascot Daemon, Matrix Science) that fulfill the conditions so they could be considered candidates for the interaction with Yhr087wp, whereas this number rose to 138 when the Protein Pilot software (AB Sciex), version 2.0 was used. After excluding from the analysis the proteins that appear with a high frequency in successful protein purifications with this procedure ([34]; Pamblanco, personal communication, Rodriguez-Navarro, personal communication) a total of 10 proteins, that are coincident

in both tests, were finally considered (Table 1) to be potential Yhr087wp-interacting proteins. Most of the proteins identified belong to two categories: stress response and translation. Actually, according to the FuncAssociate tool (http://llama.mshri.on.ca/funcassociate/) two statistically significant categories emerge: calmodulin-dependent protein kinase activity (with a p-value of 0.000003438) and eukaryotic translation 4F complex initiation factor (with a p-value of 0.00003427). These results demonstrate once again the relationship between Yhr087wp and stress response. Besides, the detection of interactions with factors involved in translation initiation (such as eIF4E, eIF4G and eIF3) suggests a putative role for the ribosomal function.

The co-immunoprecipitation experiments carried out with those strains carrying the TAP-tagged version of Yhr087wp and an HA-tagged version of Sti1p, Tif4631p, Cdc33p or Tif32p confirm the interactions found with the standard TAP protocol (Figure 5). As this Figure depicts, these proteins appear in the retained fraction after precipitation of the TAP-tagged protein with the α-PAP antibody.

Ribosome isolation experiments and detection of Yhr087wp protein location by Western blot have indicated that this protein is found, at least partially, in the ribosomal fraction (Figure 6). In these experiments, two control proteins were considered: α subunit of tubulin, which is completely found in the non ribosomal fraction, and the ribosomal protein Rpl5p, mainly detected in the ribosomes.

The *YHR087W* deletion mutant shows an increased sensitivity to translation inhibitors

The TAP experiment results suggest the possible implication of Yhr087wp in the ribosomal function. To gain more evidences about this hypothesis, sensitivity to translation inhibitors was determined in the deletion mutant of the coding gene. Two drugs were considered for this purpose: cycloheximide (which interferes with the peptidyl-

Table 1 Yhr087wp interacting proteins according to the TAP experiments carried out in this work

Protein	Function[1]
Atp7p	Subunit d of the stator stalk of mitochondrial F1F0 ATP synthase
Osh7p	Member of an oxysterol-binding protein family with seven members in *S. cerevisiae*; family members have overlapping, redundant functions in sterol metabolism and collectively perform a function essential for viability
Cmk1p	Calmodulin-dependent protein kinase; may play a role in stress response
Cmk2p	Calmodulin-dependent protein kinase; may play a role in stress response
Sti1p	Hsp90 cochaperone, interacts with the Ssa group of the cytosolic Hsp70 chaperones and activates Ssa1p ATPase activity
Tfg1p	TFIIF (Transcription Factor II) largest subunit; involved in both transcription initiation and elongation of RNA polymerase II;
Cdc33p	Cytoplasmic mRNA cap binding protein and translation initiation factor eIF4E; the eIF4E-cap complex is responsible for mediating cap-dependent mRNA translation via interactions with translation initiation factor eIF4G (Tif4631p or Tif4632p)
Scp160p	Essential RNA-binding G protein effector of mating response pathway, mainly associated with nuclear envelope and ER, interacts in mRNA-dependent manner with translating ribosomes via multiple KH domains, similar to vertebrate vigilins
Tif32p	eIF3a subunit of the core complex of translation initiation factor 3 (eIF3), essential for translation
Tif4631p	Translation initiation factor eIF4G, subunit of the mRNA cap-binding protein complex (eIF4F) that also contains eIF4E (Cdc33p); interacts with Pab1p and with eIF4A (Tif1p); also has a role in biogenesis of the large ribosomal subunit

[1] The description according to *Saccharomyces Genome Database* (SGD) is shown.

transferase activity) and hygromycin (which stabilizes the tRNA ribosomal acceptor site, thus preventing translocation). These inhibitors were tested not only with this strain, but also with mutants in *STI1* and in genes encoding the proteins involved in translation which appeared as candidates for the interaction with Yhr087wp according to the TAP analysis. Besides, double mutant strains with each one of these genes and *YHR087W* were obtained as described in the Materials and methods section and were considered for this analysis.

As Figure 7A shows, the mutants in the genes somehow related with the ribosomal function display sensitivity to one and/or another drug. With *Δscp160*, high sensitivity to cycloheximide was found, while growth was affected to a lesser extent by the presence of hygromycin. *Δtif4631* displayed total lack of growth in hygromycin, and very pronounced growth defects in cycloheximide. Growth in the presence of these drugs was not affected, however, by the deletion of the gene *STI1*, which does not participate in translation. With the *Δyhr087w* mutant, sensitivity to cycloheximide was

greater than to hygromycin. The opposite result was described for mutants in *SDO1* such as *sdo1*-K118A [25]. The introduction of the *YHR087W* deletion into these backgrounds did not result in enhanced or diminished sensitivity to the inhibitors tested.

Experiments were also carried out with thermosensitive mutants in *TIF32* and in *CDC33* and the corresponding double mutants with *Δyhr087w*. In the case of *tif32^{ts}* no sensitivity was detected to the inhibitors under the conditions considered and no additional effect was displayed by the double mutant (data not shown). For *cdc33^{ts}*, as can be seen in Figure 7B, the sensitivity to hygromycin of the single mutant in translation initiation factor eIF4E when incubated at the non permissive temperature at a concentration of 10 μg/mL increased in the double mutant with *Δyhr087w*. For a more accurate determination of the differences between these single and double mutants, the number of colony forming units and the viability under this concentration of hygromycin were determined (Table 2). The data obtained confirms the results of the serial dilution analysis and indicates that the variations between strains are statistically significant. It is worth mentioning that similar experiments were also carried out in plates contatining 20 μg/mL of hygromycin; in this case the viability was very low being four times lower in the double mutant than in the single *cdc33^{ts}* (1% compared to 4%).

The sensitivity to cycloheximide of the *Δyhr087w* mutant and the interaction between Cdc33p and Yhr087wp provide new insights about the role of this protein in translation.

Translation recovery after osmotic stress due to high glucose concentrations is slightly affected in the *Δyhr087w* mutant

The separation of the fractions of the translation complexes associated with mRNAs by sucrose gradients is a powerful methodology to study of global protein synthesis and the physiological functions of the individual factors involved in this process [36]. The analysis of the polysome profiles has also shown that when yeast cells are affected by high salinity stress, a temporary shutdown of translation in short times occurs, followed by a subsequent recovery in time [37].

To determine whether increased sensitivity to the translation inhibitors detected in strain *Δyhr087w* is associated with defects in this process, we analyzed the polysome profiles of this mutant and the corresponding wild type strain. For these experiments, the exponentially growing cultures of these strains were transferred to an OD_{600} of 0.4 to YP20. Samples were collected at different times to analyze the changes in the translational activity noted during the first minutes of incubation under 20% glucose, as well as the effect of the

Figure 5 Demonstration of the physical interaction between Yhr087wp, Sti1p, Tif4631p, Cdc33p and Tif32p by coimmunoprecipitation.
Cells of the strains carrying a TAP-tagged version of the Yhr087wp and an HA-tagged version of Sti1p, Tif4631p, Cdc33p or Tif32p proteins were exponentially grown in YPD at 30°C. After a 90-minute incubation in YP20 at the same temperature, cells were lysed and Yhr087p was immunoprecipitated as described in the *Materials and Methods* section. This Figure shows the Western analysis carried out with the total protein samples (TP), the non-retained fraction (NR) and the retained fraction (R). Yhr087wp is detected with α-PAP antibody, while the other four proteins were visualized with α-HA antibody. The results of the control experiments carried out with a FY86-derived strain carrying HA-tagged versions of the Sti1p, Tif4631p, Cdc33p and Tif32p proteins are also shown.

mutation in gene *YHR087W*. The time 0 sample was obtained before the transfer to the high glucose concentration medium took place.

Figures 8A and B show the polysome profiles obtained in these experiments. In order to gain a better understanding of the information provided by our analyses, Table 3 presents the percentage of area corresponding to the polysomal fraction in the profiles. From these data, it is possible to determine the relationship between the polysome area (P) under stress (P_S) and under normal growth conditions (P_N). This parameter represents the relative change in mRNA associated with the ribosomes participating presumably in translation [37]. The values in Table 3 also allow us to determine the relationship between the nonpolysomal (free and monosomal, FM) fraction under both conditions (FM_S/FM_N), which represents the relative change in mRNA that is not translated. If we consider all these data, it is possible to estimate the percentage of translation, determined as the relationship of the polysomal fraction between a particular timepoint compared to the time of the stress application (Figure 8C).

As shown in Figure 8A, the introduction of osmotic stress due to high glucose concentration determines a similar effect in the wild type yeast cells to that described for 1 M NaCl by Melamed et al. [37]. Translation markedly lowers during the first minutes, which can be clearly detected at 15 min. At this time, the translation rate drops almost a half (Figure 8C), and the P_S/P_N ratio is 0.52, while the FM_S/FM_N ratio shows a value of 2.6. Melamed et al. [37] revealed descreases in the polysomal fraction, which are higher for 1 M NaCl (around 3.5 times). Thirty min after

applying the high glucose concentration, the wild type strain is capable to recover around 84% of the initial level of translation, and the situation is very similar to that before applying stress (97%) after 45 min, suggesting that the translational effect of this kind of osmotic stress is lower than that provoked by high salinity (more than 3 h, [37]).

For strain *Δyhr087w*, a slightly higher decrease in translation was noted at 15 min after introducing the high sugar stress, with values for the P_S/P_N and FM_S/FM_N ratios of 0.45 and 2.79, respectively. Besides, as shown in Figure 8B for the 30 and 45 min timepoints, the recovery of translation shows a delay when compared to the wild type strain (78% at 30 min and 91% at 45 min). After 60 minutes, this strain had already recovered the initial levels of translation.

Although the differences between mutant and wild type strains are low, they are statistically significant at time points 15, 30 and, particularly, 45 min, which provides another result that points to an involvement of Yhr087wp in the ribosomal function. Unlike the results described for the *SDO1* mutants [25], this role would not seem to be related to the maturation of the 60 S subunit, as the ratio of the areas corresponding to 60 S/40 S fractions (Table 3) does not consistently decrease in mutant *Δyhr087w*.

The translation of several mRNAs is affected in the *Δyhr087w* deletion mutant under high glucose stress conditions

According to the results of a proteomic analysis [15], levels of several proteins (Hsp104p among them) in the *Δyhr087w* deletion mutant are lowered at the 20% glu-
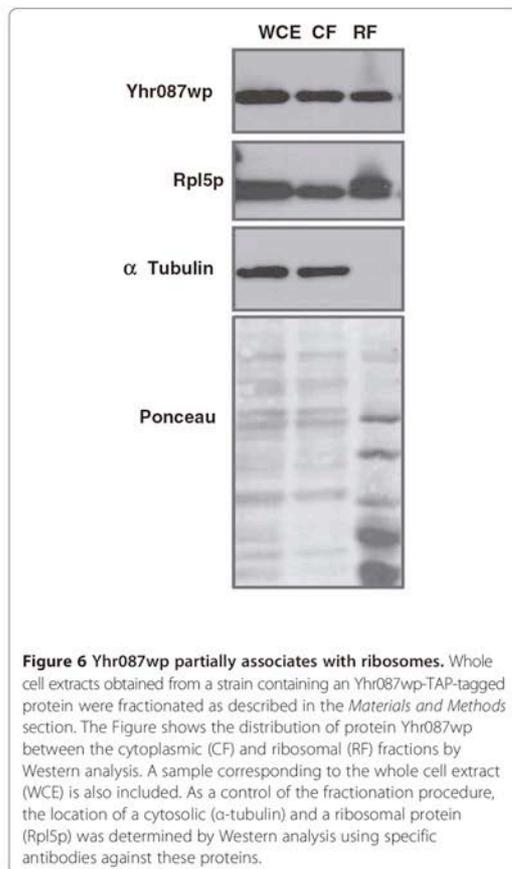
Figure 6 Yhr087wp partially associates with ribosomes. Whole cell extracts obtained from a strain containing an Yhr087wp-TAP-tagged protein were fractionated as described in the *Materials and Methods* section. The Figure shows the distribution of protein Yhr087wp between the cytoplasmic (CF) and ribosomal (RF) fractions by Western analysis. A sample corresponding to the whole cell extract (WCE) is also included. As a control of the fractionation procedure, the location of a cytosolic (α-tubulin) and a ribosomal protein (Rpl5p) was determined by Western analysis using specific antibodies against these proteins.

cose concentration. Additional file 2: Figure S3 confirms these results and indicates that the amount of this protein did not increase at 1 h after applying high glucose stress to exponential cultures of this mutant. These differences in protein levels cannot be explained by transcriptional control because the *HSP104* mRNA levels (relative to *ACT1*) under this stress condition, determined by Real-Time RT-PCR, are 5.55 and 5.51, respectively.

The slightly lower translation recovery found in the *Δyhr087w* mutant strain after applying the high glucose stress prompted us to determine if the distribution of *HSP104* mRNA between the polysomal and monosomal fractions could be affected in this strain. As shown in Figure 9, the P/FM ratio, is 1.5 times lower in the *Δyhr087w* mutant than in the corresponding wild type strain at 45 min after applying the adverse condition. This difference is in the threshold to be considered statistically significant (p-value of 0.049), is consistently found in all the replicates of the experiment and could explain the

differences noted in the Hsp104p levels between these two strains in the proteomic and Western blot analyses. This difference in mRNA translation is more clear and significant from the statistical point of view in the case of genes coding for the proteins involved in the stress response *HSP78* (p-value of 0,033) and, particularly, *GPD1* (p-value of 0,002).

Figure 9 indicates that not all the mRNAs in this mutant are affected in the same way. Genes *IPP1* and *PDA1* do not display differences in their P/FM ratio. Moreover, two other genes induced by osmotic stress because of high glucose concentrations, *YGR052W* and *YJL107C* [13-15], appear to be better translated in the mutant strain at this timepoint, although without statistically significant differences.

Yhr087wp has homologues in other yeasts

The application of the BLASTP tool has allowed the identification of Yhr087wp homologous proteins in many yeasts and fungi (Table 4). So far little is known about these proteins and their role, but global transcription analyses carried out in *Schizosaccharomyces pombe* [38] have shown that the ortholog in this microorganism (SPBC21C3.19) is induced also by several stress conditions, including osmotic stress (1 M sorbitol), heat shock (39 deg;C), and oxidative injuries (0.5 mM H_2O_2).

The sequence alignment among some of these homologue proteins (Additional file 2: Figure S4) shows four particularly conserved regions located between positions 30-53, 63-75, 80-98 and 102-114. Residues K34, V42, V67, V72, F73, A88, V93, F97, G98, E105, V106, T110, L111 and G114 are identical or conserved with SBDS proteins [18].

Discussion

In this work we carry out a functional analysis of gene *YHR087W*. To date this gene has been related to many different cellular processes, and determining its main role in yeast cells is proving to be a difficult task. The results previously obtained in our laboratory indicate an involvement of Yhr087wp in the osmotic stress caused by high glucose concentrations, although the gene expression data published by other authors suggest that it may also participate in the response to other forms of stress [2,11]. The link between Yhr087wp and response to stress (particularly osmotic stress caused by high glucose concentrations) is based on the following aspects: i) deletion of the coding gene results in a lower growth rate in the presence of 25% and 30% glucose concentrations [15] ii) wine yeast strains displaying better tolerance to osmotic stress have high levels of the mRNA of this gene [15]; iii) its overexpression improves the osmotic stress resistance and fermentative behavior of these strains [17]; iv) the proteomic study carried out with the *Δyhr087w* deletion mutant [15] provides a

Figure 7 Sensitivity to translation elongation inhibitors of the mutants in *YHR087W* and/or other genes encoding proteins that physically interact with Yhr087wp (according to the TAP experiment) and are involved, most of them, in the ribosomal function: *STI1, SCP160, TIF4631* (**Panel A**) and *CDC33* (**Panel B**). Serial dilutions were spotted on YPD, YPD cycloheximide (0.1 μg/mL) and YPD hygromycin (20 μg/mL or 10 μg/mL) plates, which were incubated for 48-72 h at 30°C. Incubation with mutants *cdc33ts* and Δ*yhr087w cdc33ts* was carried out at 33°C until growth was detected.

strong link between the levels of this gene expression under high glucose concentrations and the Hsp104p and Hsp78p levels; v) genetic interactions between *YHR087W* and *HSP82*, another gene encoding an Hsp protein [29] have been described. This work demonstrates another interesting interaction which was previously suggested by Constanzo et al. [28]: Δ*yhr087w* is capable of partially suppressing the growth defects displayed by the Δ*bcy1* mutant

Table 2 Viability of single and double mutant strains in YHR087W and CDC33 under hygromycin 10 μg/mL

Strain	Viability
BY4741	$95,33 \pm 8,08$
Δ*yhr087w*	$73,67 \pm 0,58$
cdc33ts	$41,71 \pm 8,33$
Δ*yhr087w cdc33ts*	$27,34 \pm 3,45$

Dilutions from cultures in exponential phase of growth were plated on YPD or YPD + hygromycin 10 μg/mL plates and the colony forming units appeared 48-72 hours later were counted. Viability was determined as the percentage between the CFU in hygromycin plates and in YPD plates. Application of the *t*-test to the data obtained for *cdc33ts* and Δ*yhr087w cdc33ts* mutant strains gave a *P* value (calculated for one tail using Microsoft Office Excel) of 0.0025.

at 37°C. As Bcy1p encodes the regulatory subunit of Protein Kinase A, involved in the control of cell growth and stress response, the connection of Yhr087p with this process becomes more evident.

On the other hand, results shown in Figure 2 demonstrate that the expression of *YHR087W* is controlled mainly by Msn2/4p transcription factors under heat shock, and by Hot1p and Sko1p (in a HOG-dependent manner) when osmotic stress due to high glucose concentration is applied. This differential regulation depending on the stress conditions could be explained by inhibition under 20% glucose of Msn2/4 activity and hence by an overall decrease in the activation of the general stress response in these conditions, according to Capaldi et al. [16].

Once the relationship between Yhr087wp and stress response is established, the question posed would be how this protein is involved in it and why is there a lower expression of several stress response proteins in this mutant. One possibility is that it would develop some transcriptional (by binding to the chromatin or by participating in the RNA polymerase II recruitment) or posttranscriptional (mRNA export or stability, for

Figure 8 Polysome profiles in the wild type (BY4742) and Δ*yhr087w* strains suggest an involvement of protein Yhr087wp in the translation process. The overlapping polysome profiles found for the wild type strain at several timepoints during the first hour after addition of 20% (w/v) glucose to the exponentially growing cells compared to the previous situation (panel **A**). Comparison made between the polysome profiles of the wild type and mutant strains at 45 min after introducing high sugar stress (panel **B**). Percentage of the transcription rate (relative to time 0) found for both strains during the time course of the experiment (panel **C**). In this panel asterisks indicate statistically significant differences (* p-value < 0.05, ** p-value < 0.005). More details about the experiment are included in the *Materials and Methods* section.

instance) role. This could explain the described genetic interactions with Mdm20p/Nat3p, Nsr1p, Npl3p, Yra2p and Air1p [18]. However, the transcriptomic experiments carried out in our laboratory (Jiménez-Martí and del Olmo, unpublished results) indicate a very low number of genes that are transcriptionally affected in the *Δyhr087w* mutant (38 genes more expressed in the mutant and 8 in the wild type strain) and they cannot be included in any statistically significant category. Besides, other studies also carried out by our group indicate that Yhr087wp is not involved in the mRNA export (Jiménez-Martí and del Olmo, unpublished results), the binding to yeast promoters (Additional file 2: Figure S2), the recruitment of RNA polymerase II to them or the stability of the messengers (data not shown).

The results obtained in the TAP experiments described in this work seem to assign a protein translation-related function to Yhr087wp. The physical interactions found in these experiments with several of the factors involved in translation (Table 1), its partial association with the ribosomal fraction (Figure 6), the fact that the deletion mutant displays lower levels of proteins associated with ribosomal function [15], and the genetic interactions noted in this work (Figure 7) with the gene encoding translation initiation factor eIF4E (Cdc33p) and those described by other authors with Nat3p and Nsr1p [18], all support this potential role of the protein. It is worth mentioning that many other proteins relating to the ribosome structure and function were also detected in the TAP experiments, but they were not considered because

Table 3 Analysis of the polysome profile obtained for the wild type and the Δyhr087w strains

	time (min)	0	15	30	45	60
BY4742	P (1)	77.12 ± 3.67	40.37 ± 1.60	64.65 ± 3.54	75.07 ± 1.95	75.74 ± 2.98
	P/(F + M)(2)	3.43 ± 0.71	0.68 ± 0.04	1.84 ± 0.28	3.03 ± 0.33	3.15 ± 0.51
	60/40 (3)	1.49 ± 0.19	1.73 ± 0.13	1.37 ± 0.39	1.23 ± 0.06	1.55 ± 0.26
Δyhr087w	P (1)	76.34 ± 2.89	34.06 ± 2.31	59.32 ± 0.55	69.33 ± 1.46	75.25 ± 0.01
	P/(F + M) (2)	3.26 ± 0.52	0.52 ± 0.05	1.46 ± 0.03	2.27 ± 0.16	4.54 ± 0.00
	60/40 (3)	1.23 ± 0.14	1.66 ± 0.05	1.52 ± 0.28	1.15 ± 0.12	1.63 ± 0.12

(1) The data shown correspond to the percentage of the polysome area relative to the total area of polysomal plus free plus monosomal fractions. The amount of rRNA in each fraction was measured by UV detection at 260 nm. Three independent experiments were carried out: average and standard deviation are shown.
(2) Ratio between the area of the polysomal and free plus monosomal fractions.
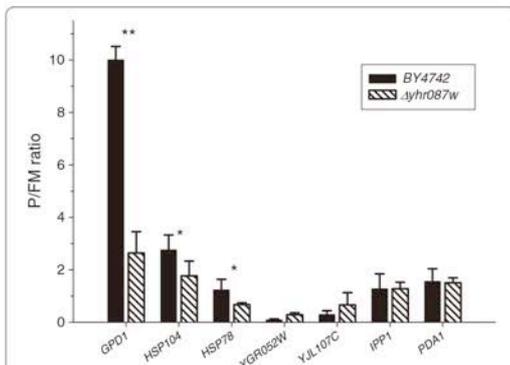(3) Ratio between the area corresponding to the 60 S and 40 S subunits.

they can be found in more than 3% of the successful protein purifications when this method is followed [34].

The experimental approach chosen to assess the role of this protein in ribosomal function also aimed to test whether a functional relationship with Sdo1p could be found, given the great structural similarity between these two proteins. The Yhr087wp protein shares with Sdo1p - and with other members of the family of proteins related to human SBDS- the FISH domain, characterized by a specific and highly-defined secondary structure: 2 β - 2 α - β - 2α. Prediction studies of the secondary and three-dimensional structure conducted by several authors [18,22] reveal that Yhr087wp is very similar to the Nt region of Sdo1p, where

Figure 9 The translation of several stress-induced genes is affected in strain Δyhr087W. Cultures of the WT and Δyhr087W strains growing exponentially in YPD were incubated for 45 min in 20% glucose and polysomal profiles were obtained. The fractions corresponding to free and monosomal (FM) and polysomal (P) were collected, RNA was isolated and cDNA was obtained. The expression of the indicated genes was analyzed by Real-Time RT-PCR using specific probes. Signals were normalized to *Bacillus subtilis LysA* mRNA, which was added to each group of fractions in equal amounts. The expression values shown are relative to those found in the *ACT1* gene. The quantified signals from three independent replicates were used to determine the average change in P/FM ratios. Asterisks indicate statistically significant differences (* p-value < 0.05, ** p-value < 0.005).

this domain is located. In evolutionary terms, Yhr087wp and Sdo1p have been proposed to have a relationship of a distant homology, and must result from a divergent evolution process [18]. The results presented in this work (Figure 8 and Table 3) indicate that, although Sdo1p is involved in translation and Yhr087wp could participate in this process, each one of these proteins would have its particular role in it, being that of Yhr087wp related with stress response. Actually Sdo1p is not regulated by adverse growth conditions [2].

Our studies done to determine the relationship of Yhr087wp with the ribosomal function provide some interesting results. On the one hand, the deletion mutant shows sensitivity to cycloheximide (and to hygromycin to a lesser extent), which also occurs in the mutants in proteins Scp160 and Tif4631p (Figure 7), both of which interact with Yhr087wp and have an unambiguous role in translation. On the other hand, the polysome profiles show that the *Δyhr087w* mutant is slightly more affected in translation activity than the wild type strain when high glucose osmotic stress is applied to exponentially growing cells. It is worth mentioning that our results indicate that the repression of translational activity described by Melamed et al. [37] under high salinity conditions also occurs with an osmotic stress due to 20% glucose, although during a shorter time.

The translation defect found in mutant *Δyhr087w* under 20% glucose does not result in a reduced translation of all the mRNAs and maybe for this reason the overall effect is low. Instead, some specific mRNAs involved in the stress response, such as those corresponding to *HSP104, HSP78* and *GPD1*, are affected. It is noteworthy that the levels of translation of the *IPP1* or *PDA1* mRNAs (usually employed for normalization purposes in gene expression analyses under osmotic stress and growth in different carbon sources respectively [41,42]) are similar in both the wild type and mutant strains. On the other hand, our analyses also included *YGR052W* and *YJL107C*, two genes induced by osmotic stress [13-15]. In this case, and at the time considered, the P/FM ratio was higher in the mutant strain.

Table 4 Yhr087wp homologue proteins[1]

Accession number	Protein	Max score	Total score	Query coverage	E-value
NP_011955	Rtc3p (*Saccharomyces cerevisiae* S288c)	221	221	100%	5e-74
XP_002494671	ZYRO0A06974p (*Zygosaccharomyces rouxii*)	133	133	89%	3e-39
XP_001646516	Hypothetical protein Kpol_1055p14 (*Vanderwaltozyma polyspora* DS 70294)	121	121	88%	1e-34
XP_446886	Hypothetical protein (*Candida glabrata* CBS 138)	120	120	89%	4e-34
XP_456050	Hypothetical protein (*Kluyveromyces lactis* NRRL Y-1140)	119	119	99%	7e-34
CCC71492.1	Hypothetical protein NCAS_0H01820 (*Naumovozyma castellii* CBS 4309)	108	108	81%	1e-29
XP_002553632	KLTH0E03454p (*Lachancea thermotolerans*)	106	106	80%	1e-28
CCD23842.1	Hypothetical protein NDAI_0C01810 (*Naumovozyma dairenensis* CBS 421)	101	101	76%	2e-26
XP_002615723	Hypothetical protein CLUG_04605 (*Clavispora lusitaniae* ATCC 42720)	68.2	68.2	89%	1e-13
EGV64915.1	Hypothetical protein CANTEDRAFT_121094 (*Candida tenuis* ATCC 10573)	67	67	86%	4e-13
XP_001212546	Conserved hypothetical protein (*Aspergillus terreus* NIH2624)	60.1	60.1	83%	3e-10
NP596599	DUF1960 family protein (*Schizosaccharomyces pombe* 972 h-)	59.3	59.3	77%	4e-10
XP_001386005.2	Hypothetical protein PICST_36865 (*Scheffersomyces stipitis* CBS 6054)	57.8	57.8	80%	1e-09
XP_001804429	Hypothetical protein SNOG_14233 (*Phaeosphaeria nodorum* SN15)	58.2	58.2	90%	1e-09
EFW98381.1	Hypothetical protein HPODL_0061 (*Pichia angusta* DL-1)	58.2	58.2	80%	2e-09
XP_461344.1	DEHA2F23078p (*Debaryomyces hansenii* CBS767)	57	57	81%	3e-09
XP_713165.1	Hypothetical protein CaO19.9418 (*Candida albicans* SC5314)	57	57	89%	4e-09
XP_001935065.1	Conserved hypothetical protein (*Pyrenophora tritici-repentis* Pt-1 C-BFP)	57	57	83%	4e-09
EDK_36318.2	Hypothetical protein PGUG_00416 (*Meyerozyma guilliermondii* ATCC 6260)	56.6	56.6	94%	6e-09
XP_001525927.1	Conserved hypothetical protein (*Lodderomyces elongisporus* NRRL YB-4239)	56.6	56.6	75%	6e-09
XP_001819636.1	RNA binding protein (*Aspergillus oryzae* RIB40)	56.2	56.2	89%	7e-09
XP_002418668.1	Protein involved in RNA metabolism, putative (*Candida dubliniensis* CD36)	55.8	55.8	88%	1e-08
XP_001594882.1	Hypothetical protein SS1G_04690 (*Sclerotinia sclerotiorum* 1980)	55.5	55.5	76%	1e-08
XP_002543973.1	Conserved hypothetical protein (*Uncinocarpus reesii* 1704)	54.7	54.7	85%	2e-08
XP_001393111.1	RNA binding protein (*Aspergillus niger* CBS 513.88)	54.7	54.7	82%	3e-08
XP_003347541.1	Hypothetical protein SMAC_04847 (*Sordaria macrospora* k-hell)	54.3	54.3	84%	4e-08
EGW33008.1	Hypothetical protein SPAPADRAFT_60332 (*Spathaspora passalidarum* NRRL Y-27907)	54.3	54.3	80%	4e-08
XP_003296114.1	Hypothetical protein PTT_04921 (*Pyrenophora teres* f.teres 0-1)	54.3	54.3	81%	8e-08
EGO53727.1	Hypothetical protein NEUTE1DRAFT_119291 (*Neurospora tetrasperma* FGSC 2508)	53.1	53.1	75%	1e-07

Table 4 Yhr087wp homologue proteins[1] (Continued)

XP_964260.1	Hypothetical protein NCU02765 (Neurospora crassa OR74A)	52.4	52.4	75%	2e-07
CBX97830.1	Similar to RNA binding protein (Leptosphaeria maculans)	52	52	83%	3e-07
XP_002793737.1	Conserved hypothetical protein(Paracoccidioides brasiliensis Pb01)	50.8	50.8	82%	8e-07
XP_001560959.1	Hypothetical protein BC1G_00044 (Botryotinia fuckeliana B05.10)	50.8	50.8	86%	8e-07
XP_002564167.1	Pc22g01230 (Penicillium chrysogenum Wisconsin 54-1255)	50.8	50.8	74%	9e-07

[1]BLASTP2.2.25 was used for the search [39,40]. Data show only the homologue proteins with E-values lower than exp-07 from a total of 84 blast hits.
All the non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF, excluding the environmental samples from WGS projects, were considered.

The differences found with the other contemplated stress-related genes could be due to the function developed (which still remains unknown for *YGR052W* and *YJL107C*) or to the kinetic variations in the P/FM ratio during the translation recovery to the applied osmotic stress. It is important also to note that the cDNA levels corresponding to these genes are lower under the tested conditions than those found for the others considered.

The lower polysomal/free plus monosomal ratio found for the *HSP104* and *HSP78* mRNAs in the mutant strain, if compared with the wild type, is consistent with the lower levels encountered for these proteins (Additional file 2: Figure S3) and with previous proteomic analyses [15]. Moreover, it can explain, together with the results found for *GPD1*, the growth defects displayed by that strain under high glucose stress [15]. The relationship found for YHR087wp between a role in translation recovery after osmotic stress treatment and loss of viability and growth reduction in its mutant strain has been recently observed for Cbc1p, which encodes the nuclear cap-binding protein [43]. Nevertheless, elucidating the particular role of each one of these proteins in translation recovery will require further analyses. The detection of the protein homologues of protein Yhr087wp in fungi (Table 4 and Additional file 2: Figure S4) suggests that this protein peforms a relevant role, particularly in stress response according to the data available from *Schizosaccharomyces pombe* [38]. Taking into account the function of Yhr087wp in the response to high sugar concentrations and the high mRNA levels found under these conditions we propose *HGI1* (High Glucose Induced 1) as a possible name to design the encoding gene in the future.

Conclusions

Our results lead us to several conclusions: (1) Yhr087wp protein is distributed throughout the cell, independently of the growth conditions, and is partially found in the ribosomal fractions. (2) The expression of the coding gene is controlled by the transcription factors Msn2/4p, Sko1p and Hot1p in a way depending on the stress

conditions considered, being particularly highly relevant the latter in the response to high glucose conditions. (3) Several experiments carried out in this work provide new insights about the involvement of this protein in the response to stress: it interacts with Cmk1/2p and Sti1p and displays a genetic relationship with the Bcy1p subunit of the PKA. (4) According to TAP experiments, it also interacts with several proteins participating in translation, including translation factors eIF4E and eIF4G. This result and the increased sensitivity of the deletion mutant strain to translation inhibitors points to a role of this protein in translation. (5) Under conditions of high glucose stress the translation recovery is quicker than when high salinity is applied. This recovery shows a certain delay in the Δ*yhr087w* strain. (6) The ratio between the association to polysomal and free and monosomal fractions of mRNAs corresponding to several stress response genes (*HSP104*, *HSP78* and *GPD1*) is reduced in the deletion mutant when compared with the wild type strain after the application of high sugar stress which indicates that the lower levels of some stress proteins in the mutant strain might be due to defects in their translation, and provides a possible explanation for the lower osmotic stress resistance described for this strain. The existence of homologues in other yeasts and fungi reinforces the relevance of this protein.

Materials and methods
Yeast strains, growth conditions and genetic methods
All the *S. cerevisiae* strains used for these experiments are shown in Additional file 1: Table S1. Growth of yeast strains without auxotrophies was carried out in YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose) at 30°C with orbital shaking (200 rpm). Strains with auxotrophies were grown in SC medium (0.17% (w/v) nitrogen base without amino acids and ammonium sulphate, 0.5% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) *Drop out* mix without the selected amino acid or nucleobase, 2% (w/v) glucose). The solid medium also contained 2% (w/v) agar. In most experiments, liquid cultures were kept in the exponential growth phase for

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16-24 h; then cells were treated under the required conditions; in the case of osmotic stress by high glucose concentration, cells were collected and transferred from YPD to the same medium containing 20% (w/v) glucose (YP20).

For the growth assays on resistance to osmotic stress or antibiotics, yeast cultures were diluted to the same OD_{600}, and serial dilutions (1:10) were spotted onto YPD-derived plates, which were incubated at the desired temperatures. For the osmotic stress analyses plates contained 20%, 25% or 30% (w/v) glucose (YP20, YP25 and YP30) or 1 M KCl. YPD plates with cycloheximide (0.1 μg/mL) or hygromycin (10 or 20 μg/mL) were used for antibiotic resistance purposes. In some experiments, for a more accurate determination of the differences between strains, colony forming units were counted after application on the plates of the appropriate volumes and dilutions.

To construct *YHR087W* deletion strains, the wild-type coding sequence was replaced with the *URA3* gene through amplification of a disruption cassette from the YEp352 plasmid with oligonucleotides DELW-A and DELW-B (Additional file 1: Table S2) and yeast transformation [44]. A similar procedure was used for *HOG1* gene deletion, with the utilization of the oligonucleotides DELHOG-A and DEL-HOG-B. The *YHR087W* gene was tagged with GFP in the FY86 strain using the Longtine method [45]; for this purpose, oligonucleotides YHR087W-F2 and YHR087W-R1 (Additional file 1: Table S2) were used to amplify the transformation cassette from the pFA6a-GFP(S65T)-kanMX6 plasmid. To introduce the TAP tag into the same gene in strain FY86, a PCR amplification was carried out on the pBS2623 plasmid with oligonucleotides YHR087W-TAP L and YHR087W-TAP R. In the derived strain and in the corresponding wild type strain, genes *TIF32, CDC33, STI1* and *TIF4631* were independently tagged with HA. For this purpose oligonucleotides TIF32-F2, TIF32-R1, CDC33-F2, CDC33-R1, STI-F2, STI-R1 and TIF4631-F2 and TIF4631-R1 (Additional file 1: Table S2) were used to amplify the transformation cassette from the pFA6a-3HA-HIS3MX6 plasmid [45]. Detection of Hsp104p in Western analyses was possible for the introduction of a HA tag by means of the primers HSP-F2 and HSP-R1. Selection of transformants by antibiotic resistance was achieved in YPD plates containing geneticin at a final concentration of 100 mg/L. Selection of transformants by auxotrophy was followed in SC medium plates without the corresponding amino acid or nucleobase.

Gene expression analyses

For RNA isolation and quantification, a protocol explained elsewhere was followed [46]. cDNA was obtained as described previously [47]. The absence of DNA contamination in these preparations was assessed by analyzing the intron-containing gene *ACT1* by semi-quantitative RT-PCR using the oligonucleotides ACT-1 and ACT-2 shown in Additional file 1: Table S2 [17]. The *ACT1* gene was also used as a reference gene in some experiments given its constitutive expression under the conditions considered in this work [2,15]. cDNA preparations were used for the gene expression analysis by means of semi-quantitative RT-PCR or Real-Time RT-PCR as previously described [15].

Gene expression studies were carried out in some cases by Northern analyses, following the procedure described in Carrasco et al. [46]. Probes were obtained by PCR amplification of genomic DNA with the oligonucleotides described in Additional file 1 Table S2. In this case, normalization was carried out with gene *SCR1*, which is transcribed by the RNA polymerase III.

Chromatin immunoprecipitation analyse

Cells from an exponential growth culture (OD_{600} 1) were incubated for 10 or 20 min (depending on the strain considered) under the stress condition (YP20 or 37°C). A control culture was kept for the same time in YPD. Cells were crosslinked with 1% (v/v) formaldehyde for 15 min at room temperature and were then incubated for 5 min with 125 mM glycine before being collected, washed with TBS buffer and frozen. Cells were resuspended in 300 μL of lysis buffer (50 mM HEPES-KOH pH 7.9, 40 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0,1% (w/v) sodium desoxicholate, 1 mM PMSF, 1 mM benzamidine and the *Complete Mini* protease inhibitor (Roche)) and 300 μL of glass beads were added. Cells were lysed for 30 min at 4°C in *Genie-2* (Scientific Industries). Chromatin was then fragmented by sonication in a *BioRuptor Diagenode* and the sample was centrifuged at 12000 rpm for 15 min.

To analyze polymerase binding to DNA, a cell extract was incubated with *Dynabeads Protein G* (Invitrogen) previously bound to an 8WG16 antibody (anti Rpb1, *Covance*) for 15 min at room temperature with orbital rotation. Beads were then washed three times with 200 μL de PBS 0.02% (v/v) Tween, and then once more with 100 μL of the same buffer. For the determination of Yhr087wp or Hot1p binding, a cell extract was incubated with *Dynabeads IgG Pan Mouse* (Invitrogen) with no antibody for 2 h at 4°C with orbital shaking. Then they were washed three times with 200 μL of PBS/BSA (5 mg/mL) and once more with 100 μL of the same buffer. Elution was carried out twice with 40 μL of 50 mM Tris–HCl pH 8.0, 10 mM EDTA, 1% (w/v) SDS, by heating at 65°C for 2 min at 600 rpm. In all cases, crosslinking was reverted by overnight incubation at 65°C

with shaking. The eluted sample was digested for 90 min at 37°C with proteinase K 0.33 mg/mL and DNA was purified with the *High Pure PCR product purification kit* (Roche).

Co-immunoprecipitated DNA was analyzed in triplicate by Real Time RT-PCR in a *DNA Engine Peltier Termal Cycler* (Bio Rad) using the *Platinum SYBR Green qPCR SuperMix-UDG with ROX* (Invitrogen). Amplifications of two different regions of gene *HSP104* (5' and 3'), and an intergenic region of chromosome V were carried out with combinations of oligonucleotides HSP104 3' R/F, HSP104 5' R/F, and Int A/B (Additional file 1: Table S2). For the experiments of Hot1p binding, a region of the *YHR087W* gene promoter was amplified with the primers YHRPRO-2A and YHRPRO-2B. Data were processed with the $\Delta\Delta CT$ method [48] by comparing the results of the amplified from the immunoprecipitated sample (IP) with those of the whole cell extract (*Input*), and by using the intergenic region for normalization.

Semiquantitative PCR analyses were carried out as described by Alepuz et al. [33].

Polysome analysis and ribosome preparation

Cultures from an overnight incubation in YPD up to an OD_{600} of 0.4 were transferred to YP20. The cells corresponding to 80 mL of the culture were collected before transfer (control condition) and at several times under stress and were incubated with 0.1 mg/ml of cycloheximide. Extracts were prepared according to the protocols described by Swaminathan et al. [49] and Garre et al. [43]. The volume corresponding to 10 units of A_{260} was applied to 10-50% sacarose gradients, containing KCl and $MgCl_2$ concentrations of 140 mM and 5 mM, respectively, prepared with the *Density Gradient Fractionation System* (Teledyne ISCO). Samples were centrifuged at 35000 rpm for 2 h 40 min at 4°C, and were then scanned with the same system. Profiles were processed with the *Photoshop* software and the areas of the fractions were determined with *Image J* (NIH, http://rsb.info.nih.gov/ij/). Gradient fractions were collected and processed for RNA isolation as described by Kuhn et al. [50], but including a precipitation with one volume of lithium chloride 5 M before the final precipitation with sodium acetate. The expression data for selected genes in the polysomal and free plus monosomal fractions were determined after normalization with the cDNA corresponding to *Bacillus subtilis LysA* mRNA transcribed *in vitro*: 96 ng of this mRNA were introduced in the ribosomal fractions used for RNA isolation. Finally, the expression data were relativized to the *ACT1* gene expression.

The ribosome fraction was obtained from yeast protein extracts by ultracentrifugación in a cushion of 25% glycerol in accordance with Meskauskas et al. [51]. For the electrophoretic analysis, the fraction corresponding to the whole cell extract was directly mixed with an equal volume of 2X SDS-PAGE solvent. Proteins of the non-ribosomal fraction were extracted by TCA precipitation and ribosomes were suspended in 50 mM Tris–HCl pH 7.5, 5 mM $Mg(CH_3COO)_2$, 50 mM NH_4Cl, 0.1 mM PMSF, 0.1 mM DTT, 25% glycerol.

Methods of protein manipulation and analysis

Protein extracts for routine analyses were prepared by resuspending cells in 200 μL of NaOH 0.1 M. Then they were kept at room temperature for 5 min and centrifuged for 1 min at 12000 rpm. The pellet was resuspended in 250 mM Tris–HCl pH 6.8, 140 mM SDS, 30 mM bromophenol blue, 27 μM glycerol, 0.1 mM DTT. After incubation at 95°C for 5 min, samples were centrifuged for 10 min at 3000 rpm and the supernatant was applied on the appropriate SDS-PAGE gel. TAP-tagged proteins were detected by Western analysis using the α-PAP antibody (Sigma) (dilution 1:2000 in PBS 0.1% Tween, 5% skim milk). For the HA-tagged proteins anti-HA 3 F10 was used (Roche) (dilution 1:10000 in PBS, 0.5% (v/v) BSA). α-tubulin antibody was obtained from GE Healthcare and was diluted 1:10000 in TBS 0.01% (v/v) Tween 20. The Rpl5 antibody (provided by Dr. J.L. Woolford) was used at the 1:5000 dilution in TBS, 0.01% Tween, 5% skimmed milk.

Fluorescent microscopy was performed in a *Zeiss Axioskop II* instrument using live cells grown in synthetic media with 2% or 20% glucose at up to an OD_{600} of 0.4. Nuclei were identified by a 30-min incubation at 30°C with Hoechst reagent (*Invitrogen*) at a final concentration of 2 μg/mL. Images were obtained with a digital camera *SPOT* (Diagnostic Instruments Inc).

Tandem Affinity Purification (TAP) was carried out from the exponential cultures (6 L) of the strain containing the TAP-tagged version of Yhr087wp in YPD transferred for 90 min to YP20 (final OD_{600} of 2.0). Cells were collected, washed with water and frozen at -80°C. Afterwards they were disrupted with a *RETSCH MM301*, to finally obtain 30.42 g of lysed cells. Purification was carried out following the protocol described by Puig et al. [52], which involved two affinity chromatographies, one with IgG *Sepharose* and another with calmoduline. Finally, 6 fractions of 180 μL were obtained. The proteins associated with Yhr087wp were identified by mass spectrometry in the Proteomic Laboratory of the *Centro de Investigación Príncipe Felipe*. The information was analyzed by *Protein Pilot* (*SwissProt*) and *MASCOT DAEMON* (NCBInr).

For the co-immunoprecipitation experiments, exponentially growing cells (OD_{600} of 1) were transferred for 90 min to YP20. The cells corresponding to 200 mL of these cultures were lysed in 150 μL of lysis buffer (50 mM Tris–HCl pH 8.0, 5 mM EDTA, 250 mM NaCl, 1 mM PMSF, 0.01% Triton X-100 and the *Complete*

protease inhibitor mix (Roche)) in the presence of one volume of glass beads (425-600 μm in diameter). Samples were centrifuged at the maximun speed for 5 min. The supernatant obtained, which corresponds to the total protein extract, was then incubated with *Dynabeads IgG Pan Mouse* -prepared as described by the manufacturer (*Invitrogen*)- for 2 h at 4°C in a rotary shaker to immunoprecipitate the Yhr087wp-TAP fusion protein. After collecting the non retained fraction, *Dynabeads* were washed 3 times with PBS buffer containing 5 mg/mL of BSA. Elution was carried out with 15 μL of 50 mM Tris–HCl pH 8.0, 10 mM EDTA, 1% SDS in a rotary shaker for 5-10 min at RT. The three fractions obtained were incubated at 95°C for 5 min in the presence of a protein solvent before being applied to a polyacrylamide gel at the appropriate concentration. Co-immunoprecipitated proteins were detected by Western with the anti-HA antibody 3 F10.

The BLAST tool of NCBI (http://blast.ncbi.nlm.nih.gov) was employed to search for homologous sequences. The sequences obtained were aligned by ClustalX 1.83 [53]. The alignment obtained was edited with Gblocks 0.91b [54,55].

Additional files

Additional file 1: Table S1. Yeast strains used in this work. Table S2: Oligonucleotides used in this work [7,56-60].

Additional file 2: Figure S1. Defects of the *YHR087W* deletion mutant under osmotic stress conditions. Figure S2: Relative binding of Yhr087wp to the 5' and 3' regions of the *HSP104* gene under conditions of osmotic heat shock stress determined by chromatin inmunoprecipitation experiments. Figure S3: Hsp104 protein levels in wild type and Δ*yhr087w* strain. Figure S4: Sequence alignment between fungal protein homologues to *S. cerevisiae* Yhr087wp.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MGA carried out most of the experiments and edited the manuscript. EJM performed experiments and edited the manuscript. MDO conceived the project, helped in several experiments, supervised experiments and wrote the manuscript. All authors read and approved the final version of the manuscript.

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Material suplementario
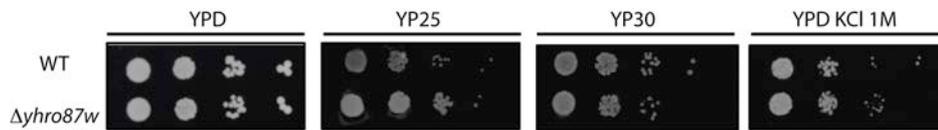
Figure 3.1.S1 Defects of the *YHR087W* deletion mutant under osmotic stress conditions. Growth of the BY4742 and *Δyhr087w* strains under several conditions of osmotic stress (25% glucose, 30% glucose and KCl 1 M). Serial dilutions from the cultures of these strains were spotted and growth was followed for 3 days at 30°C, as described in the *Materials and Methods* section.
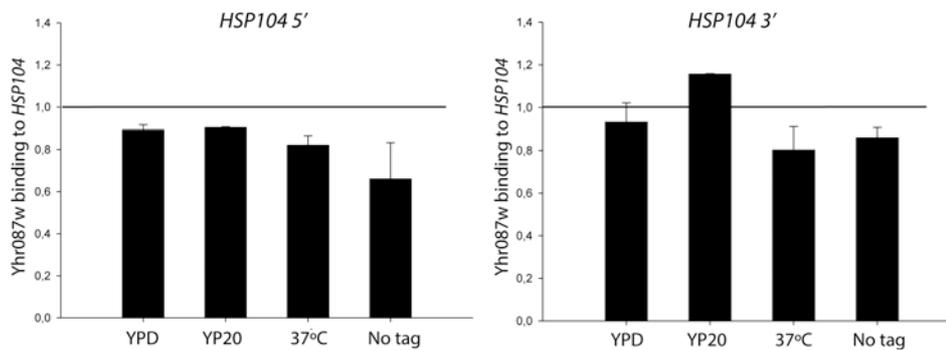
Figure 3.1.S2. Relative binding of Yhr087wp to the 5' and 3' regions of the *HSP104* gene under conditions of osmotic and heat shock stress determined by chromatin immunoprecipitation experiments (ChIP). The cells growing exponentially in YPD medium were incubated for 20 min in the same medium at 37°C or in YP20 at 30°C before collecting samples and carrying out the chromatin immunoprecipitation protocol described in the *Materials and Methods* section. The amount of immunoprecipitate was determined by Real Time RT-PCR.
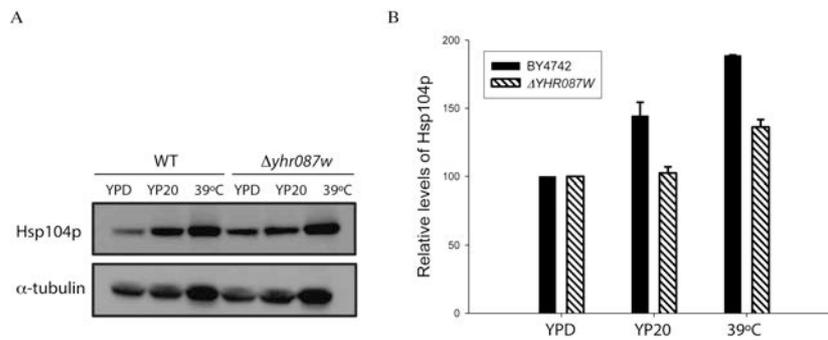
Figure 3.1.S3. Hsp104 protein levels in the wild type and the *Δyhr087w* strains. The yeast cells carrying a Yhr087Wp-TAP tagged version were exponentially grown in YPD before being transferred to YP20 medium or affected by a heat shock of 39°C. At several timepoints after addition of sugar, samples were obtained for the protein studies. Protein levels were analyzed by Western blot using a-PAP antibody. Panel A shows a representative figure of the results of the experiment. In panel B the data obtained from the quantification of three independent experiments are shown, including the average and standard deviation.
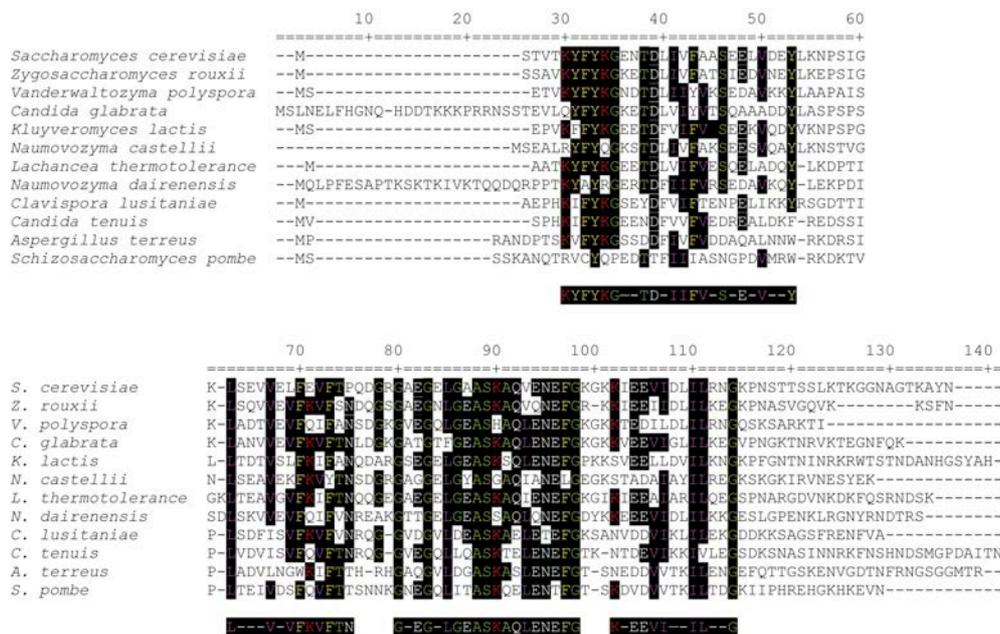
Figure 3.1.S4. Sequence alignment between fungal protein homologues to *S. cerevisiae* Yhr087wp. Alignment was carried out as described in the *Materials and Methods* section. Identical residues are written with the same colour and shadowed in black. The black rectangles below the sequences indicate regions with a high degree of conservation between the proteins considered.

Table 3.1.S1. Yeast strains used in this work.

Strain	Description	Origin
MCY1389	*MATa, ura3-52, leu2::HIS3, SUC2*	F. Estruch (Estruch and Carlson, 1993)
BQS110	MCY1389 *bcy1::LEU2*	F. Estruch (Estruch and Carlson, 1993)
FY86	*MATα, ura3, his3, leu2*	Laboratory stock
BQS2071	FY86 *YHR087W::GFP::kanMX4*	This work
TM141	*MATa, his3Δ1, leu2Δ0, trp1Δ0, ura3Δ0*	F. Posas (Alepuz et al., 2003)
TM233	TM141 *hog1Δ::TRP1*	"
W303-1a	*MATa, ade2-1, trp1-1, can1-100, his3-11,15, leu2-3,112, ura3-1*	Thomas and Rothstein, 1989
W303-1a *Δmsn2Δmsn4*	W303-1a *msn2Δ3::HIS3, msn4-1::TRP1*	F. Estruch (Estruch and Carlson, 1993)
W303-1a *Δhot1*	W303-1a *hot1:: kanMX4*	P. Alepuz (Alepuz et al., 2003)
BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Euroscarf
BY4742	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	Euroscarf
Δyhr087w	BY4742 *yhr087w::kanMX4*	"
PAY575	BY4741 *sko1::kanMX4*	"
Δsti1	BY4741 *yor027w::kanMX4*	"
Δscp160	BY4741 *yjl080c::kanMX4*	"
Δtif4631	BY4741 *ygr162w::kanMX4*	"
YRP840	*MATa leu2-3 112 his4-539 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG*	Hatfield et al., 1996
YRP1321 (cdc33^{ts})	*MATα leu2 trp1 ura3 cdc33::LEU2 cup1::LEU2/PGK1pG/MFA2pG [cdc33-42/TRP1]*	Schwartz and Parker, 1999
Δsti1 Δyhr087w	*Δsti1 yhr087w::URA3*	This work
Δscp160 Δyhr087w	*Δscp160 yhr087w::URA3*	"
Δtif4631 Δyhr087w	*Δtif4631 yhr087w::URA3*	"
cdc33^{ts} Δyhr087w	*cdc33ts yhr087w::URA3*	"
Δbcy1 Δyhr087w	BQS110 *yhr087w::URA3*	"
YHR087W-TAP	FY86 *YHR087W::TAP::kanMX4*	This work
FY86 STI1-HA	FY86 *STI1::HA::HIS6MX*	This work
YHR-TAP STI1-HA	YHR087W-TAP *STI1::HA::HIS6MX*	This work
FY86 TIF4631-HA	FY86 *TIF4631::HA::HIS6MX*	This work
YHR-TAP TIF4631-HA	YHR087W-TAP *TIF4631::HA::HIS6MX*	This work
FY86 TIF32-HA	FY86 *TIF32::HA::HIS6MX*	This work
YHR-TAP TIF32-HA	YHR087W-TAP *TIF32::HA::HIS6MX*	This work
FY86 CDC33-HA	FY86 *CDC33::HA::HIS6MX*	This work
YHR-TAP CDC33-HA	YHR087W-TAP *CDC33::HA::HIS6MX*	This work
HOT1-TAP	BY4741 *HOT1::TAP::HIS3*	Thermo
HOT1-TAP *Δhog1*	HOT1-TAP *hog1::URA3*	This work
SKO1-TAP	BY4741 *SKO1::TAP::HIS3*	Thermo
SKO1-TAP *Δhog1*	SKO1-TAP *hog1::URA3*	This work
Y16036	BY4742 *cna1::kanMX4*	Euroscarf
Δcna1Δyhr087w	Y16036 *yhr087w::URA3*	This work
DAmP* TIF32	BY4741 *tif32-kanR-3'UTR*	Thermo
DAmP TIF32 *Δyhr087W*	DAmP TIF32 *yhr087w::URA3*	This work
tif32^{ts}	YKO^{+} derived *tif32::URA3*	Ben Aroya et al., 2008
tif32^{ts}	*tif32^{ts} yhr087w::kanMX4*	This work

Table 3.1.S2. Oligonucleotides used in this work.

Oligonucleotide	Sequence (5' to 3')	Use
ADH1-A	AACGTTTCCGTTTCCGAAGC	PCR of *ADH1* gene "
ADH1-B	AAACCTCTGGCGAAGAAGTC	"
CLB2-UP	GGAAATAGCCGCCAAAAGAC	PCR for Chromatin inmunoprecipitation experiments
CLB2-DOWN	CTGAAACTCTATGCCCATGC	"
CLN2-F	ATCTTTTTCGTATCCTCCGC	PCR for Chromatin inmunoprecipitation experiments
CLN2-R	AAAGGGCCAACAGTTGTTTC	"
DELHOG-A	ACAAAGGGAAAACAGGGAAAACTACAACTATCGTATATAATAATTCGGTAATCTCCGAGC	*HOG1* disruption
DELHOG-B	AAGAAGTAAGAATGAGTGGTTAGGGACATTAAAAAAACACGTCCCGGGTAATAACTGATA	"
HOG +200	TGACGGTTCTTGGAGTCT	Confirmation of *HOG1* disruption
HOG -200	TGGCAGTGATGGACAGAT	"
URA3 UP	CTTAACTGTGCCCTCCAT	Confirmation of *HOG1* disruption
DELW-A	GATAAAAATAAGAAAACCATCGCATACACAAATAAAATCAAACATTCGGTAATCTCCGAGC	*YHR087W* gene disruption with *URA3*
DELW-B	TATTTCAATGCAGGCTGTCCAGACAGAAGGAAAAGCTACCCCGGGTAATAACTGATA	"
DELYHR-F1	GATAAAAATAAGAAAACCATCGCATACACAAATAAAATCAACCGGATCCCCGGGTTAATTAA	*YHR087W* gene disruption with *KanMX* in *tif32^{ts}*
DELYHR-R1	TATTTCAATGCAGGCTGTCCAGACAGAAGGAAAAGCTACGAATTCGAGCTCGTTTAAAC	"
YHR087W +200	AATCATGTGTGACGAACG	Confirmation of *YHR087W* gene disruption
YHR087W -200	TGTCGAACTCTTCGAAGT	Confirmation of *YHR087W* gene disruption
URA CHECK	GCGAAGAGCGACAAAGA	"
GPD1-1	TTGAATGCTGGTAGAAAG	PCR of *GPD1* gene
GPD1-2	TGACCGAATCTGATGATC	"
HSP78-A	GTTGGGTGATGATGGTAAGA	PCR of *HSP78* gene
HSP78-B	GCCAATCCTTCGCTTCATCA	"
HSP104-1	GCGGTCTTACCGATACCTGG	PCR of *HSP104* gene
HSP104-2	GACTGAGCAGGCTCGTCAAGG	"
HSP104 3' R	GATCCTTAGTGCCAGTTTGTTC	Amplification of a 3' region of *HSP104* gene
HSP104 3' F	GAAATTGAAGAGAGATTCGAGC	"
HSP104 5' R	AGATCATAGTCGTAACGGC	Amplification of a 5' region of *HSP104* gene
HSP104 5' F	ATATGAACGACCAAACGC	"
HSP-F2	ACGATAATGAGGACAGTATGGAAATTGATGATGACCTAGATCGGATCCCCGGGTTAATTAA	HA-tagging of *HSP104* gene
HSP-R1	AAACGTTAAAATGTGAGCTCTTTTGCTCGGGTGTCAAGTTCGAATTCGAGCTCGTTTAAAC	"
CHECK HSP 5'	GAATGTCTGGAAGTTCTACC	Confirmation of HA-tagging of *HSP104*
CHECK HSP 3'	TTATCAACGCCATATGTCCC	Confirmation of HA-tagging of *HSP104*
Int-A	GGCTGTCAGAATATGGGGCCGTAGTA	Amplification of an intergenic region
Int-B	CACCCCGAAGCTGCTTTCACAATAC	"
IPP1-A	AAGAACAAGAAGTACGCTTTG	PCR of *IPP1* gene
IPP1-B	TTGGAGCATCTGCCTTT	"
PDAQ-1	CTCCTCCAGAAGCCAAAT	PCR of *PDA1* gene
PDAQ-2	CCTAGAGGCAAAACCTTG	"

STI-F2	TTCAGACGTTGATCGCTGCTGGTATCATCCGGA CTGGCCGCCGGATCCCCGGGTTAATTAA	HA-tagging of *STI1*gene
STI-R1	TGAGAAGATCATTATATGTACGTATGTATGAAA AAGCAGTAGAATTCGAGCTCGTTTAAAC	"
CHECK STI	GCTGCGATCATGCAAGATCCT	Confirmation of HA-tagging of *STI1* gene
STI +100	GATCCGGAGAACGACAGGAAA	"
TIF4631-F2	ATATGTTCAGTGCATTAATGGGAGAAAGTGATG ACGAAGAGCGGATCCCCGGGTTAATTAA	HA-tagging of *TIF4631* gene
TIF4631-R1	TTAGACTTTCTACCAACATCCTTGTATCCAAGT GACATTTTGAATTCGAGCTCGTTTAAA	"
CHECK TIF4631 5'	AAGAAATAGCCAAAGGGCTCC	Confirmation of HA-tagging of *TIF4631* gene
CHECK TIF4631 3'	GCACAGTAAGAGGATTACCCA	"
TIF32-F2	CTGAAAAGTTGAGAGCCAAGAGATTGGCCAAG GGGGGCAGGCGGATCCCCGGGTTAATTAA	HA-tagging of *TIF32* gene
TIF32-R1	ACTGCCATCTTTACTGCTCTTCTGCTCCTTCCTT TGACATGGAATTCGAGCTCGTTTAAAC	"
CHECK TIF32 5'	ATCGAACAGAGACTAGCC	Confirmation of HA-tagging of *TIF32* gene
CHECK TIF32 3'	AGTGCTCTTCCGCTAACG	"
CDC33-F2	CCAGTGCCAATGGTAGACACCCTAACCATCAAT CACCTTGCGGATCCCCGGGTTAATTAA	HA-tagging of *CDC33* gene
CDC33-R1	TGTTAACATGATGAGTTTATACGTGCATCCTAA CTAGTAGAGAATTCGAGCTCGTTTAAAC	"
CHECK CDC33 5'	TGCCTTATGGACTAAATC	Confirmation of HA-tagging of *CDC33* gene
CHECK CDC33 3'	GTCCTTTTAACGTCATCC	"
YGR052 R	TCTTGAATTAGGCAAGGTTTCTC	PCR of *YGR052W* gene
YGR052 F	GTTTTGGGAACAGTGGTCTG	"
YHR087W-1	GGTAAGCTATCTGAAGTTGTC	PCR of *YHR087W* gene
YHR087W-2	ACTTCTTCGATCTTCTTG	"
YHR087-F2	TCAAAACCAAAGGGGGTAACGCCGGGAACCAA AGCCTACAATCGGATCCCCCGGGTTAATTAA	GFP-tagging of *YHR087W*
YHR087-R1	TTATGACAGGGCAGCACGCCAATTTTAACTTAT TGCATATTTCAAGAATTTGCAGCTCGTTTAAAC	"
YHR check 5'	TGAGTTCGGTAAGGGCAAGAA	Confirmation of GFP-tagging of *YHR087W*
YHR check 3'	TGTGTGACGAACGCGA	"
YHR087-TAP L	AGTCTCAAAACCAAAGGGGGTAACGCCGGAAC CAAAGCCTACAATTCCATGGAAAAGAGAAG	TAP-tagging of *YHR087W*
YHR087-TAP R	TGTTCTTTCTACTACTGTAAAAGAAGAATGCAT GCGTTATGACAGGGCGACTCACTATAGGG	"
YHR CHECK 5'	TGAGTTCGGTAAGGGCAAGAA	Confirmation of TAP-tagging of *YHR087W*
OBS 292	TTGTATTTTCAGGGTGAGCTC	"
YHRPRO-2A	GTCCCTTTCTGACAATAAGACC	Chromatin inmunoprecipitation
YHRPRO-2B	TGTTTCTGGCGATCCCTTCG	"
YJL107C-1	GAAACCTCCACATACGTACC	PCR of *YJL107C* gene
YJL107C-2	TGTTACAGCTTGTGAATCTG	"

3.2. Capítulo 2

Dissection of the elements of osmotic stress response transcription factor Hot1 involved in the interaction with MAPK Hog1 and in the activation of transcription

Dissection of the elements of osmotic stress response transcription factor Hot1 involved in the interaction with MAPK Hog1 and in the activation of transcription

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ARTICLE INFO

ABSTRACT

The response to hyperosmotic stress is mediated by the HOG pathway. The MAP kinase Hog1 activates several transcription factors, regulates chromatin-modifying enzymes and, through its interaction with RNA polymerase II, it directs this enzyme to osmotic stress-controlled genes. For such targeting, this kinase requires the interaction with transcription factors Hot1 and Sko1. However, phosphorylation of these proteins by Hog1 is not required for their functionality. In this study, we aim to identify the Hot1 elements involved in Hog1-binding and in the activation of transcription. Two-hybrid experiments demonstrated that the Hot1 sequence between amino acids 340 and 534 and the CD element of Hog1 are required for the interaction between the two proteins and the Hot1-dependent transcription regulation. Inside this Hot1 region, short sequence KRRRR (KR4, amino acids 381–385) is essential for the kinase binding. Our data show that another element, sequence EDDDDD (ED5, amino acids 541–546), is essential for Hot1 binding to chromatin. Under osmotic stress conditions, both Hot1 elements, Hog1-interaction KR4 and DNA-binding ED5, are involved in the appropriate recruitment of Hog1 and RNA polymerase II to genes controlled by this transcription factor. Moreover, both sequences are required for osmotolerance and KR4 is necessary for the functionality of the HOG pathway. According to several experiments described in this study, the Hot1 protein is capable of forming homodimers.

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1. Introduction

Stress response in the yeast *Saccharomyces cerevisiae* involves the detection of adverse conditions, activation of signal transduction pathways, and transcriptional and posttranscriptional regulation, which result in the accumulation of protective agents and repairing activities [1]. Under several different stress conditions, *S. cerevisiae* displays a common response, the so-called *Environmental Stress Response* (ESR), characterized by changes in the expression of approximately 900 genes [2]. Most of these genes contain the AGGG consensus sequence in their promoter [3], which is recognized by transcription factors Msn2p and Msn4p [4]. The activity of these factors is regulated by two pathways that control cell growth: *Protein Kinase A* (PKA, [5]) and *Target of Rapamycin* (TOR, [6]).

Besides this general response, yeast cells display specific mechanisms of response to particular adverse conditions. In the case of hyperosmotic stress, produced by a high concentration of salt, sorbitol, glucose, or of any other osmolyte, the response is mediated by the *High Osmolarity Glycerol* (HOG) pathway. A genetic screen designed to detect

the genes required for adaptation to hyperosmolar stress has identified the MAP kinase Hog1 [7]. *S. cerevisiae* has two osmosensing mechanisms: the first (SLN1 branch) involves the Sln1–Ydp1–Ssk1 system, which activates the redundant Ssk2 and Ssk22 MAPKKKs [8–10]; the second one (SHO1 branch) is an intracellular signal generated by tetraspan membrane protein Sho1 and membrane glycoproteins Hrk1 and Mbs2, resulting in the activation of STE11 MAPKKK [11–14]. Irrespective of the osmosensing mechanism, MAPKK Pbs2 activates MAPK Hog1p [7,8,11,15]. An addition of 2% glucose to raffinose growing cells induces Hog1 phosphorylation to the same extent as treatment with 110 mM NaCl. Moreover, an addition of higher sugar concentrations has led to higher levels of Hog1p phosphorylation [16,17].

Phosphorylation of Hog1 results in its translocation to the nucleus [18,19] and, once inside this compartment, it activates several transcription factors (Hot1, Msn1, Smp1, Gcn4, Skn7, Sko1p, Msn2 and Msn4) [1,20–23] and regulates chromatin-modifying enzymes [24,25]. Besides, several reports have provided evidence for the interaction between Hog1 and RNA polymerase II [20,26,27]. This response rapidly alters the mRNA levels of more than one third of the genome genes [28]. In the initial osmotic shock response phase, the global transcription rate drops by 50% [29] but an induction in the expression of about 10% of the yeast genes has been detected under conditions of stress caused by high salt or sorbitol concentrations [2,30–34]. Recently, it has been shown that the large-scale redistribution of RNA polymerase II from

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housekeeping genes to osmotic stress-controlled genes requires Hog1 [26,27]. These authors have suggested that RNA polymerase II, either in complex with Hog1 or modified by Hog1 via phosphorylation, reduces the affinity for non-stress responsive genes; the entry of Hog1p in the nucleus upon stress allows transcription factors Sko1 and Hot1 to bind, together with Hog1, at the cis-elements located in regulatory regions of stress responsive genes, thus targeting the Hog1–RNA Pol II complex to these genes.

The molecular response to high osmolarity has been reported to show particular traits when it is caused by high glucose concentrations [17,35,36]. An analysis of gene expression in single and multiple mutant strains carried out by Capaldi et al. [37] has revealed that the HOG pathway activates fewer genes in glucose than in KCl under the same total molar osmolarity. The explanation offered by these authors is that the osmotic stress response in high glucose is modulated by the inhibition of Msn2/4 activity, which leads to an overall decrease in the activation of the general stress response, and shifts the Hog1-dependent expression program toward those genes regulated by Sko1 and Hot1.

Hot1 has been identified in a two-hybrid screen in which HOG1 coding sequences were used as a bait [30]. This transcription factor has shown similarity to Msn1 and Gcr1 S. cerevisiae proteins [30] and, under osmotic stress, it has been seen to be involved in the transcriptional induction of several genes, including STL1, GPD1, GPP2 and HGI1 [30,31,38]. Hot1 binds to the promoter of its regulated genes, but the relationship among this binding, stress conditions and Hog1 depends on each particular gene considered. In the STL1 gene, which is fully regulated by Hot1, and in HGI1, which is partially controlled by this factor, Hot1 binding is dependent on Hog1 [38,39]. In GPD1, Hot1 is bound to its promoter before the application of stress [39]. Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of RNA polymerase II but, surprisingly, this recruitment does not depend on the phosphorylation of Hot1 by the MAPK [20]. According to its role in the response to osmotic stress, cells with deletion of HOT1 are sensitive to severe osmotic stress conditions although in a much lower degree than those in which HOG1 is not expressed. Besides, a hot1 mutation partially suppresses the detrimental cell growth defect caused by the hyperactivation of the HOG pathway through the deletion of the N-terminal negative regulatory domain of MAPKKK Ssk2p [30].

One of the main interests in the analysis of the signal transduction pathways is the understanding of how substrates, activators and regulators become connected. Many studies have indicated that the interactions between these components are achieved through the specific MAPK-docking sites located in kinases, transcription factors and phosphatases. Moreover, comparative sequence analyses have indicated that, in almost all known members of the MAPKK and MAPKAPK (MAP kinase activated protein kinases) families, these docking sites are characterized by a cluster of charged amino acids (mainly positively) outside the catalytic domain [40–46]. For the yeast HOG pathway, two adjacent docking sites, the PBD-2 region and the common docking (CD) domain (with two critical acidic amino acids), have been reported to differentially interact with its activator, the Pbs2 MAPKK, and its major inactivator, the Ptp2 protein tyrosine phosphatase, respectively [47]. Furthermore, the serine/threonine protein kinase Rck2p/Clk1p, a predominantly cytoplasmic substrate of Hog1 [48,49], interacts with this protein through the CD domain of the MAPK [47]. The Rck2p C-terminal region contains two arginine residues that are required for this interaction [50].

This work focuses on the analysis of the Hot1 regions involved in the interaction with Hog1 and in the transcriptional activation of the genes controlled by this transcription factor.

2. Materials and methods

2.1. Yeast strains and growth conditions

All the S. cerevisiae strains used for these experiments are provided in Supplementary Table 1. Growth of yeast strains without auxotrophies

was carried out in YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose) at 30 °C with orbital shaking (200 rpm). The yeast cells carrying plasmids were grown in SC medium (0.17% (w/v) nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) $(NH_4)_2SO_4$, 0.2 % (w/v) Drop out mix without the selected amino acid or nucleobase, 2% (w/v) glucose). In the strains with the ade2 mutation (derived of W303-1a), adenine was added to a final concentrations of 20 μg/mL. The solid medium also contained 2% (w/v) agar.

In most experiments, the strains transformed with plasmids were grown in liquid cultures in a selective SC medium and were kept in the exponential growth phase for 16 h. Then cells were transferred to YPD medium and grown for 3 h before being exposed to the stress conditions considered. For the osmotic stress treatment with a high glucose concentration, the exponential cells grown in YPD were transferred to the same medium containing 20% (w/v) glucose (YP20) and were incubated for 5 min (in the Hog1–Hot1 co-immunoprecipitation experiments), 10 min (in the Hot1–Hot1 co-immunoprecipitation experiments and in the crosslinking studies), or 30 min (in the gene expression experiments). For the osmotic stress treatment with salt, NaCl 5 M was added to the exponential cultures to achieve a final concentration of 0.4 M and cells were incubated for 10 min (in the chromatin immunoprecipitation experiments) or for 30 min (in the one-hybrid and gene expression experiments) more. For viability experiments, the exponentially growing cells were incubated for 23 h in YPD medium containing 1.7 M NaCl, and colony-forming units were counted after plating the appropriate volumes and dilutions on the YPD solid medium.

For the analysis of the suppression of the growth defect caused by a constitutively activated HOG pathway, a N-terminally truncated Ssk2 protein was ectopically expressed under the control of an inducible GAL promoter in the yeast cells (W303-1a strain) transformed with pBTM116- or pRS313- derived plasmids. Exponentially growing yeast cultures were diluted to the same OD_{600}, and serial dilutions (1:10) were spotted onto plates with the selective SC medium containing 2% glucose (w/v) (SC) or 2% galactose (w/v) (SC-GAL). Plates were incubated at 30 °C for 48 h (SC) or between 3 and 7 days (SC-GAL).

2.2. Strains and plasmid constructions

The description of all the plasmids used in this work is included in Supplementary Table 2.

The pBTM116-derived plasmids used for the one- and two-hybrid experiments (pHOT.0 to pHOT.6) were constructed by the insertion of the regions corresponding to the HOT1 coding sequence. They were introduced as SmaI-PstI, SalI-PstI or BamHI-PstI PCR fragments, as indicated in Supplementary Table 2. The exact sequence included in each case is indicated in terms of the codon numbers in that Table and in Fig. 1.

The HOG1 coding region between codons 1 and 422 and the entire coding region (1–435) were cloned as BamHI PCR fragments into the pGAD424 plasmid to obtain pGAD-HOG.0-2.

For the mutagenesis of the KR4 and ED5 elements of Hot1 and the DPTDE sequence of Hog1, the Quik Change Lightning site-directed mutagenesis kit (Stratagene) was used following the manufacturer's instructions. The plasmids obtained were named pHOT.R2/M2, pHOT.R3/M3, pHOT.KR4/M5, pHOT.KR4Δ, pHOT.ED5Δ and pHOG.CDΔ. In some of these plasmids, the amino acid sequence was deleted, while it was substituted for methionine (M) residues in others.

The correct construction of all these plasmids was confirmed by sequencing.

Some of the analyses described in this work were carried out using strains W303-1a, FY86, and their derivatives hog1Δ and hot1Δ. For the gene expression, chromatin immunoprecipitation and phenotypic characterization experiments, the full length HOT1 coding region with its promoter, terminator and 3 HA epitopes before the STOP codon
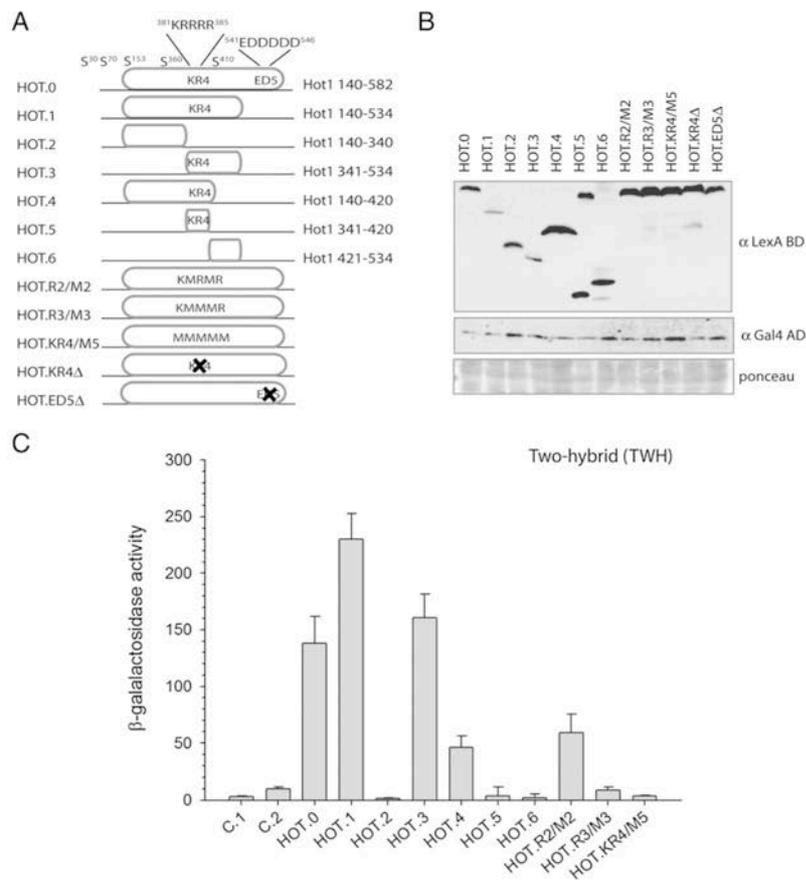
Fig. 1. The Hot1 region between amino acids 340 and 534 is required for the interaction with Hog1 and the KR4 element at positions 381–385 plays an essential role. A. A schematic representation of the regions of the Hot1 protein expressed as the LexA fusions included in the pBTM116-derived plasmids. The numbers on the right refer to the Hot1 amino acids included in each fusion protein. B. Detection of the expression of the corresponding fusion proteins in the strain used for the two-hybrid (TWH) experiments (EGY40/pSH18-34/pGAD424HOG/pBTM116HOT respectively) by Western blot using a α LexA binding domain antibody. The presence of the Hog1 protein fused to the activation domain of Gal4 is also verified with an anti Gal4AD antibody. Ponceau staining is shown as a loading control. C. Determination of the β-galactosidase activity of the EGY40 strain transformed with pSH18-34, pGAD424-HOG1 and the corresponding pBTM116 plasmids containing different regions of the Hot1 protein. In this Figure, C.1 refers to the EGY40 strain carrying plasmids pSH18-34 and HOT.0, and C.2 refers to EGY40 with pSH18-34, HOT.0 and pGAD424. The experiments were carried out in triplicate; the average and standard deviation data are shown.

was introduced into pRS313 as a *Sac*I PCR fragment. This plasmid (pRS313-HOTwt) was used as a substrate for site-directed mutagenesis to obtain the derived Hot1 versions KR4/M5, KR4Δ and ED5Δ (pRS313-HOT.KR4/M5, pRS313-HOT.KR4Δ and pRS313-HOT.ED5Δ, respectively). Plasmid YCplac111-HA-HOGwt (expressing an HA-tagged full length version of Hog1) was used for site-directed mutagenesis to obtain the HOG.CDΔ version.

Yeast transformation with all the described plasmids was carried out following the protocol described by Gietz and Woods [51].

For the detection of proteins in the Western, co-immunoprecipitation, chromatin immunoprecipitation (ChIP) and crosslinking analyses, TAP tags were introduced into Hog1 and Hot1. For this purpose, a transformation cassette was obtained by a PCR amplification carried out on the pBS2623 plasmid with the oligonucleotides described in Supplementary Table 3.

The strains carrying the disruptions of genes *HOT1*, *HOG1* or *HGI1* were previously described [38]. To construct useful *HOT1* or *HOG1* deletion strain for several experiments, the wild-type coding sequence was replaced with the *URA3* gene through an amplification of a disruption cassette from plasmid YEp352 with the oligonucleotides described in Supplementary Table 3. To construct the *STL1* deletion strain, the coding sequence was replaced with the kanamycin resistance gene by the Longtine method [52].

2.3. Genetic methods

The two-hybrid experiments were carried out with yeast strain EGY40 transformed with pSH18-34, a pGAD-Hog1 plasmid (pGAD424-HOG$^{1-422}$, pGAD424-HOG$^{1-422DPTDEΔ}$ or pGAD424-HOG$^{1-435DPTDEΔ}$) and the pBTM116-Hot1 plasmid specified in each case. The assays to determine the β-galactosidase activity in the resulting strains were performed using 0.5 units OD$_{600}$ from exponential cultures by the permeabilized cell method, using ONPG as a substrate, as described by Adam et al. [53]. At least three independent transformants were used for each assay. β-galactosidase

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activity is expressed in arbitrary units using the formula 1000 × $OD_{420}/(OD_{600} \times$ volume assayed (mL) × time of reaction (min)).

For the one-hybrid analyses W303-1a, the W303-1a Δhot1 and W303-1a Δhog1 strains containing plasmid pSH18-34 and the indicated pBTM116-Hot1 plasmid were utilized. Salt osmotic stress was applied to test Hot1p transcription activation ability following the procedure described in the previous section. The β-gal assay was performed as described above but 1 unit OD_{600} was employed.

2.4. Gene expression analyses

For RNA isolation and quantification, a protocol explained elsewhere was followed [54]. cDNA was obtained as described previously [55]. The absence of DNA contamination in these preparations was assessed by analyzing intron-containing gene ACT1 by semi-quantitative RT-PCR [56] using oligonucleotides ACT-1 and ACT-2, shown in Supplementary Table 3. The ACT1 gene was also used as a reference gene. cDNA preparations were utilized for the gene expression analysis by Real-Time RT-PCR as previously described [17].

2.5. Chromatin immunoprecipitation analyses

Cells from an exponential growth culture (OD_{600} 1) were incubated for 5 min under the selected stress condition (0.4 M NaCl). A control culture was kept for the same time in YPD. Cells were crosslinked with 1% (v/v) formaldehyde for 15 min at room temperature and were then incubated for 5 min with 125 mM glycine before being collected, washed with TBS buffer (20 mM Tris–HCl (pH 8.0), 0.5 M NaCl) and frozen. Cells were resuspended in 300 μL of lysis buffer (50 mM HEPES-KOH pH 7.9, 40 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium desoxicholate, 1 mM PMSF, 1 mM benzamidine and the *Complete Mini* protease inhibitor (Roche Diagnostics)), and 300 μL of glass beads were added. Cells were lysed for 30 min at 4 °C in *Genie-2* (Scientific Industries). Chromatin was then fragmented by sonication in a *BioRuptor Diagenode* and the sample was centrifuged at 12,000 rpm for 15 min.

To analyze Hot1p and RNA polymerase II binding to chromatin, the cell extract was incubated with orbital rotation for 15 min at room temperature with *Dynabeads Protein G* (Invitrogen Corporation) previously bound to an HA 3F10 antibody (Roche Diagnostics) or an 8WG16 antibody (anti Rpb1, *Covance*) for Hot1p or RNApolII ChIPs, respectively. Beads were then washed 3 times with 200 μL of PBS (150 mM NaCl, 40 mM Na_2PO_4, 10 mM NaH_2PO_4) containing 0.02% (v/v) Tween 20, and then once more with 100 μL of the same buffer. For the determination of Hog1p binding to chromatin, a cell extract was incubated with *Dynabeads IgG Pan Mouse* (Invitrogen Corporation) with no antibody for 2 h at 4 °C with orbital shaking. Then they were washed 3 times with 200 μL of PBS/BSA (5 mg/mL) and once more with 100 μL of the same buffer. Elution was carried out twice with 40 μL of 50 mM Tris–HCl pH 8.0, 10 mM EDTA, 1% (w/v) SDS, by heating at 65 °C for 2 min at 600 rpm. In all cases, crosslinking was reverted by overnight incubation at 65 °C with shaking. The eluted sample was digested for 90 min at 37 °C with 0.33 mg/mL proteinase K and DNA was purified with the *High Pure PCR Product Purification Kit* (Roche Diagnostics).

Co-immunoprecipitated DNA was analyzed in triplicate by Real Time RT-PCR in a *DNA Engine Peltier Termal Cycler* (Bio-Rad Laboratories, Inc.) using the *SYBR® Premix Ex Taq™ Tli RNase H Plus Green with ROX* (Takara). Amplifications of the promoter region of genes STL1 and HGI1 were carried out with combinations of oligonucleotides STL1 1/2 and YHRPRO-2A/YHRPRO-2B; Int A/B were used for an intergenic region (Supplementary Table 3). Data were processed with the ΔΔCT method [57] by comparing the results of the amplified from the immunoprecipitated sample (IP) with those of the whole cell extract (*Input*), and by using the intergenic region for normalization.

2.6. Methods of protein manipulation and analysis

Protein extracts were prepared for routine analyses by resuspending cells in 200 μL of NaOH 0.1 M and then incubating at room temperature for 5 min. Samples were centrifuged for 1 min at 12,000 rpm and the pellet was resuspended in protein solvent (250 mM Tris–HCl pH 6.8, 140 mM SDS, 30 mM bromophenol blue, 27 μM glycerol, 0.1 mM DTT). After incubation at 95 °C for 5 min, samples were centrifuged for 10 min at 3000 rpm and the supernatant was applied on the appropriate SDS-PAGE gel.

TAP-tagged proteins were detected by Western analysis using the α-PAP antibody (Sigma-Aldrich Corporation) (dilution 1:2000 in PBS 0.1% Tween 20, 5% skimmed milk). For the HA-tagged proteins anti-HA 3 F10 peroxidase was used (Roche Diagnostics) (dilution 1:10,000 in TBS, 0.01 % (w/v) Tween 20). As protein loading control the α-tubulin antibody (GE Healthcare) was utilized (diluted 1:10,000 in TBS 0.01% (v/v) Tween 20). The LexA fusions in the pBTM116-derived plasmids were detected with the LexA antibody (2–12): sc-7544 (Santa Cruz Biotechnology, Inc.) (dilution 1:1000 in TBS, 0.01 % (w/v) Tween 20). Fusions with the Gal4 activation domain were detected with Gal4-TA (C-10): sc-1663 antibody (Santa Cruz Biotechnology, Inc.) under the same dilution conditions used for the LexA antibody. For the detection of phosphorylated Hog1, the phospho-p38 MAPK (Thr180/Tyr182) antibody (Cell Signaling Technology # 9211) was used at the 1:5000 dilution in TBS, 0.01% Tween, 5% skimmed milk.

For the co-immunoprecipitation experiments, glucose was added to 100 mL of exponentially growing yeast cultures (OD_{600} around 1) at a final concentration of 20% (p/v) and cells were incubated for 5–10 min. Then, cells were resuspended in 300 μL of lysis buffer (100 mM NaCl, 20 mM HEPES K-OH pH 7.6, 5 mM $MgCl_2$, 10% glycerol (p/v), 0,1% Triton X-100 (p/v) and the *CompleteMini EDTA free* protease inhibitor mix (Roche Diagnostics)) and 1 volume of glass beads (425–600 μm in diameter) was added. Cells were lysed for 15 min at 4 °C in *Genie-2* (Scientific Industries). Samples were centrifuged at 1000 rpm for 1 min, glass beads were washed once with 400 μL of lysis buffer and the two supernatants obtained were mixed and centrifuged at the maximum speed for 20 min. The total protein concentration in the extracts was quantified by the Bradford method [58] using the Bio-Rad reagent. The same μg of protein of all the samples were incubated with *IgG Shepharose 6 fast flow* (*GE*) beads, which were previously washed 3 times with lysis buffer, for 90 min at 4 °C in a rotary shaker to immunoprecipitate the Hot1-TAP or Hog1-TAP fusion protein. After collecting the non-retained fraction, *IgG Sepharose* beads were washed 3 times with lysis buffer. Elution was carried out with 100 μL of SDS 1% (p/v) at 65 °C for 15 min. The total extract (input), and the non-retained (unbound), and eluted (IP) fractions obtained were incubated at 95 °C for 5 min in the presence of a protein solvent (125 mM Tris–HCl pH 6.8, 70 mM SDS, 15 mM bromophenol blue, 13.5 μM glycerol, 0.1 mM DTT). Then proteins were detected by Western blot with the anti-HA antibody 3 F10 peroxidase (Roche Diagnostics).

Protein crosslinking was achieved by treatment with two different reagents: dimethyl adipimidate (DMA) and ethylene glycol disuccinate bis(sulfo-N-succinimidyl) ester (EGS). For the DMA treatment, the procedure described by Kurdistani and Grunstein [59] was followed. For EGS, the protocol by Peña et al. [60] was used.

3. Results

3.1. The Hot1 region between amino acids 340 and 534 is necessary for the interaction of this transcription factor with Hog1

Rep et al. [30] carried out a two-hybrid screen with HOG1 coding sequences (residues 1 to 343) as a bait (fused to the coding sequence of the LexA activator in the pBTM116 plasmid) to find targets of the

encoded MAPK. In these experiments a clone into plasmid pGAD424, showing the greatest β-galactosidase activity, was shown to include codons 140 to 645 of the uncharacterized open reading frame *YMR172w*, which was named at that time *HOT1* (*H*igh-*O*smolarity-induced *T*ranscription).

In order to delimit the regions of Hot1 required for the interaction with Hog1, different regions of the transcription factor coding sequence between codons 140 and 582 were subcloned in frame to LexA in plasmid pBTM116 and were tested for interaction with Hog1 (codons 1 to 422) subcloned in plasmid pGAD424. Supplementary Table 2 includes a complete description of the resulting pBTM116-derived plasmids and a schematic representation is shown in Fig. 1 panel A. HOT.0 includes the sequence between codons 140 and 582 and several truncated regions of this sequence are located in plasmids HOT.1 to HOT.6. In all the EGY40 derivative strains carrying the pSH18-34 reporter plasmid, the pGAD-HOG.0 plasmid and each one of the pBTM116-Hot plasmids, Gal4AD-Hog1 and LexA-Hot1 fusion proteins of the expected sizes were detected (Fig. 1B).

The two-hybrid results (Fig. 1C) show that a Hot1 region between amino acids 534 and 582 is not relevant for the Hog1–Hot1 interaction (compare the data from both HOT.0 and HOT.1). However, the sequence between 420 and 534 was required for a full interaction with Hog1 (compare HOT.4 and HOT.1), and the region between amino acids 340 and 420 was essential for this interaction (compare HOT.4 and HOT.2 in the same Fig. 1). Besides, HOT.3 shows a similar level of interaction to that displayed by HOT.0, therefore the Hot1 sequence present in this plasmid (from residues 341 to 534) should contain the elements required for the Hog1 docking to Hot1. In order to further delimit this region, two more constructs (HOT.5 and HOT.6) were designed, and each contained one part of this region. None of these plasmids supported the interaction with Hog1, suggesting that the regions included in both are essential for the interaction between the transcription factor and the MAPK. However, it cannot be excluded that the Hot1 regions included in HOT.5 and HOT.6 might be too short to allow an accurate three-dimensional structure for protein interaction.

3.2. A Hot1 basic-rich sequence between amino acids 381–385 (KRRRR, KR4) acts as a docking-site for MAPK Hog1

After identifying the region responsible for the interaction of Hot1 with Hog1, a sequence analysis was carried out to determine the putative sequences that can support the contacts between the two proteins. A region with several basic residues, KRRRR (KR4), was detected at positions 381 to 385. Since sequences of a similar composition in protein targets have been demonstrated to act as docking sites for kinases [43,47] and other references included in the Introduction section, several mutations in this short basic region from HOT.0 were carried out by site-directed mutagenesis (Fig. 1A). In all cases, the expression of the corresponding fusion proteins in strain EGY40/pSH18-34/pGAD424Hog1(1–422) was confirmed by Western blot (Fig. 1B). The mutation of the two arginines (residues 382 and 384) to the methionines in HOT.R2/M2 resulted in a reduced Hot1–Hog1 interaction by 2.3 times, while another mutation of arginine 384 to methionine (HOT.R3/M3) yielded a reduction of 93% (Fig. 1C). When all five basic amino acids of the Hot1 KR4 region were mutated to methionine (HOT.KR4/M5), beta-galactosidase activity was almost abolished (Fig. 1C). Accordingly, the sequence KR4 is absolutely required for the interaction of Hot1 140–582 with MAPK Hog1, although other elements might also be involved in the interaction, according to the result found for HOT.4, in which the interaction between the two proteins is reduced despite the presence of this element.

To further confirm the relevance of the KR4 element for the interaction between Hot1 and Hog1, coinmunoprecipitation experiments were carried out using the strains in which Hog1 was TAP-tagged

and plasmid-borne Hot1 constructs (HOTwt and HOT.KR4Δ) were HA-tagged. As shown in Fig. 2, although the inmunoprecipitation of Hog1 did not result in large amounts of Hot1 protein being coimmunoprecipitated (probably because of the transitory interaction between both proteins), when sequence KR4 was been deleted, these levels were lower than those detected for the wild-type Hot1 construct and were similar to those found in the control experiment.

Hot1 is a direct target of the MAPK Hog1 and alanine substitution of three serine/proline residues that can serve as phosphoacceptor sites reduces Hot1 hyperphosphorylation [39]. As shown in Fig. 3, the stress-induced migration pattern of Hot1 on acrylamide gels reduced when this sequence was substituted by five methionine residues or deleted, resulting in a similar degree of phosphorylation for this protein to that found in a strain carrying a *HOG1* gene disruption. These experiments demonstrate that the Hog1–Hot1 KR4-dependent interaction is involved in the phosphorylation of this transcription factor.

3.3. The common docking (CD) element of Hog1 is responsible for the interaction with Hot1

The CD domain of Hog1 has been reported to be essential for the interaction of this MAPK with its inactivator, phosphatase Ptp2 [47], and with its cytoplasmic substrate Rck2 [48,49]. To determine whether this element is also relevant for the interaction with Hot1, the Hog1 mutant versions were obtained in which the DPTDE residues between amino acids 307 and 311 (including the two relevant acidic residues previously described) were deleted over the sequence of Hog1 included in HOG.0 plasmid, and also over the entire coding sequence of the protein (Fig. 4A, B). The two-hybrid analysis of the interaction between Hot1 and the Hog1 versions containing CD deletions gave a negative result, thus indicating that the Hog1 CD element is necessary for the interaction of the MAPK with the transcription factor (Fig. 4C). This result has been confirmed by the co-immunoprecipitation experiments shown in Fig. 4D.

3.4. Transcriptional activation by LexA-Hot1 fusions requires the KR4 sequence in 381–385, and the ED5 acidic element (541–546)

Next, attempts were made to identify which Hot1 regions are required for its ability to activate transcription. To address this point, one-hybrid experiments were carried out in the strain W303-1a containing reporter plasmid pSH18-34 and also the *HOT1* plasmids
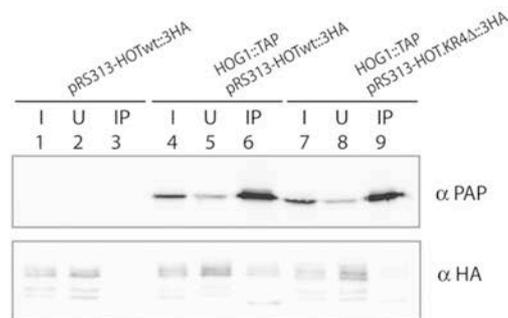
Fig. 2. Relevance of the Hot1 KR4 sequence for its physical interaction with Hog1 by co-immunoprecipitation. The cells of the FY86 *hot1*Δ strain carrying a TAP-tagged version of Hog1 and an HA-tagged version of Hot1, or its mutated form Hot.KR4Δ were exponentially grown in YPD at 30 °C. After a 5-minute incubation in high glucose medium (YP20) at the same temperature, cells were lysed and Hog1 was immunoprecipitated as described in the Materials and methods section. This figure shows the Western analysis carried out with the total protein extracts (I = input), the non-retained fraction (U = unbound) and the immunoprecipitate fraction (IP). Hog1 was detected using the α-PAP antibody, while Hot1 versions were visualized with the α-HA antibody.
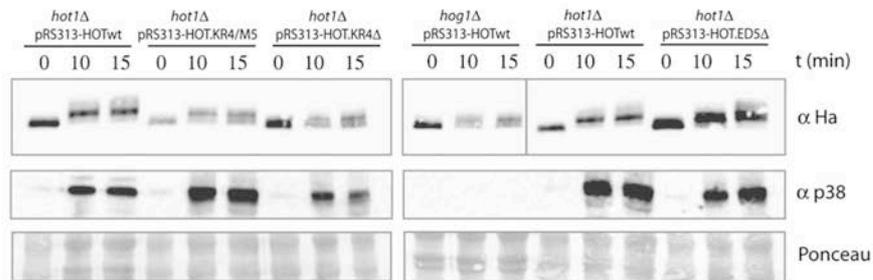
Fig. 3. Stress-induced Hot1 hyperphosphorylation is reduced when its KR4 element is substituted for M5 or is deleted. The Western blot analysis of the Hot1 protein and its derivatives carrying a substitution of the KR4 element for M5 or a deletion of the KR4 or ED5 sequences in the *hot1Δ* (and in some cases *hog1Δ*) strains. The upper panel shows the detection of Hot1-HA expressed from these strains and constructs during the exposure of yeast cells to an osmotic stress caused by high glucose concentrations (20%, w/v). Detection of the phosphorylated form of MAPK Hog1 indicates that the effects in the migration pattern found for Hot1 in the upper panel are not due to an inactive Hog1 protein. Ponceau staining of the filters was carried out as a loading control. This figure shows the result of a representative experiment.

described above (Fig. 1), both under control conditions and after the osmotic stress provoked by the incubation of the cells with 0.4 M NaCl for 30 min. In this sense, the approach is different to that followed in the two-hybrid experiments, carried out under control conditions, when Hot1 is unable to activate transcription and requires interaction with the Hog1–GAD fusion. As shown in Fig. 5A, the LexA-Hot1 fusion containing the Hot1 amino acids from 140 to 420 (HOT.4) was able to induce the transcription from the *lexA* operators at high levels under osmotic stress. However, the further deletion of residues 340 to 420 in the HOT.2 construct yielded no induction. It was also observed that the progressive mutation of the residues of sequence KR4 resulted in a gradual reduction in transcriptional activation compared to the HOT.0 control, and the complete deletion of the sequence KR4 also led to a significant decrease in β-galactosidase activity under stress (Fig. 5A).

Acidic residues have been found in many transcriptional activators. The C-terminus region of Hot1 contains a sequence carrying several residues of this kind at positions 541 to 552. To analyze the functionality of this Hot1 acidic region, a new plasmid (HOT.ED5Δ) was constructed in which amino acids 541 to 546 (ED5) were deleted. This deletion also significantly reduced the ability of Hot1 to induce transcription during osmotic stress (Fig. 5A), similar to the effect found with the modifications in KR4, suggesting that the ED5 element is involved in transcriptional activation.

When comparing the transcription activation results (Fig. 5A) with the two-hybrid experiment data (Fig. 1C) corresponding to the same Hot1 deletions or mutations, it can be concluded that loss of transcriptional induction by Hot1 correlates with loss of its interaction with Hog1. Moreover, in the absence of osmotic stress the transcription activation by Hot1 became almost undetectable (Fig. 5A), which suggests that the recruitment of Hog1 by Hot1 may be required for the transcriptional role of Hot1.

To confirm this hypothesis, the one-hybrid experiments were carried out in the same genetic background (W303-1a strain) but with deletion of *HOT1* or *HOG1* (Fig. 5B). The results indicate that when a functional Hog1 protein is not present in the cells, transcriptional activation is almost completely absent, and no differences are detected depending on whether osmotic stress was applied or not.

On the other hand, in the absence of the *HOT1* chromosomal copy, the transcriptional activation under osmotic stress by the LexA fused to the different Hot1 versions was lower than the induction detected in the corresponding wild-type cells in all cases. However, the differences noted in relation to the deleterious effect of the different Hot1 deletions and mutations were similar, regardless a Hot1 native protein being present not of (Fig. 5B).

All these results together indicate that the Hot1 protein is capable of activating transcription from the *lexA* operators when fused to LexA, but

this activation is completely dependent on Hog1 and requires the sequences located between positions 340–534, particularly the KR4 element in 381–385. Additionally, the acidic ED5 element (541–546) also plays an important role in Hot1-dependent transcription activation. According to the function found for this element, its absence does not affect Hot1 phosphorylation, as shown in Fig. 3.

3.5. The transcriptional activation of Hot1-dependent genes by osmotic stress requires the KR4 and ED5 elements of the transcription factor

In the experiments described so far a reporter system was considered to determine the elements involved in the transcriptional activation conducted by Hot1. In order to check whether the identified Hot1 elements required for the activation of reporter systems and/or the interaction with Hog1 are also functional at chromosomal genes in yeast cells, we carried out analyses of the expression of two Hot1-target genes which show high expression levels under high osmolarity and particular traits in their transcription control. One of them, *STL1*, is completely dependent on Hot1 for its activation under osmotic stress, and it shows transient induction under these conditions [35,39]. The other target, *HGI1*, is partially dependent on Hot1 and displays a more sustained increased expression during incubation with high glucose [35,38]. For these experiments, strain W303-1a *hot1Δ* transformed with the derivatives of plasmid pRS313 was utilized, and stress caused by 0.4 M NaCl or 20% (w/v) glucose was considered. The changes in mRNA levels were followed by Real-Time RT-PCR from the corresponding cDNA samples with the oligonucleotides described in the Material and methods section.

For the analysis of the expression of these genes under osmotic stress due to 0.4 M NaCl, a kinetics was carried out between 0 and 30 min. The results are shown in Fig. 6, panel A. The yeast cells containing a *HOT1* deletion and expressing a wild-type Hot1 protein showed the induction of the *HGI1* gene under osmotic stress treatment (350 times approximately 20–30 min after salt addition). A high expression level (7 times lower) was still found in the absence of Hot1 due to the partial regulation of this gene by Hot1-independent mechanisms [38]. Similar levels of expression were observed in the Hot1 KR4/M5, and ED5Δ mutants as in the *hot1Δ* strain carrying an empty plasmid, indicating that most of the Hot1-dependent induction of *HGI1* is abolished in the mutants. For *STL1*, the increased the expression caused by osmotic stress was completely dependent on Hot1. The presence of this factor resulted in an increase of around 350 times 20 min after salt addition, which quickly decreased, and is in agreement with previous analyses [39]. The activation of this gene significantly reduced when the KR4 sequence of Hot1 was substituted for M5. In a *hot1Δ* strain carrying an empty plasmid, activation by stress was almost undetectable, as occurred in the ED5Δ mutant.
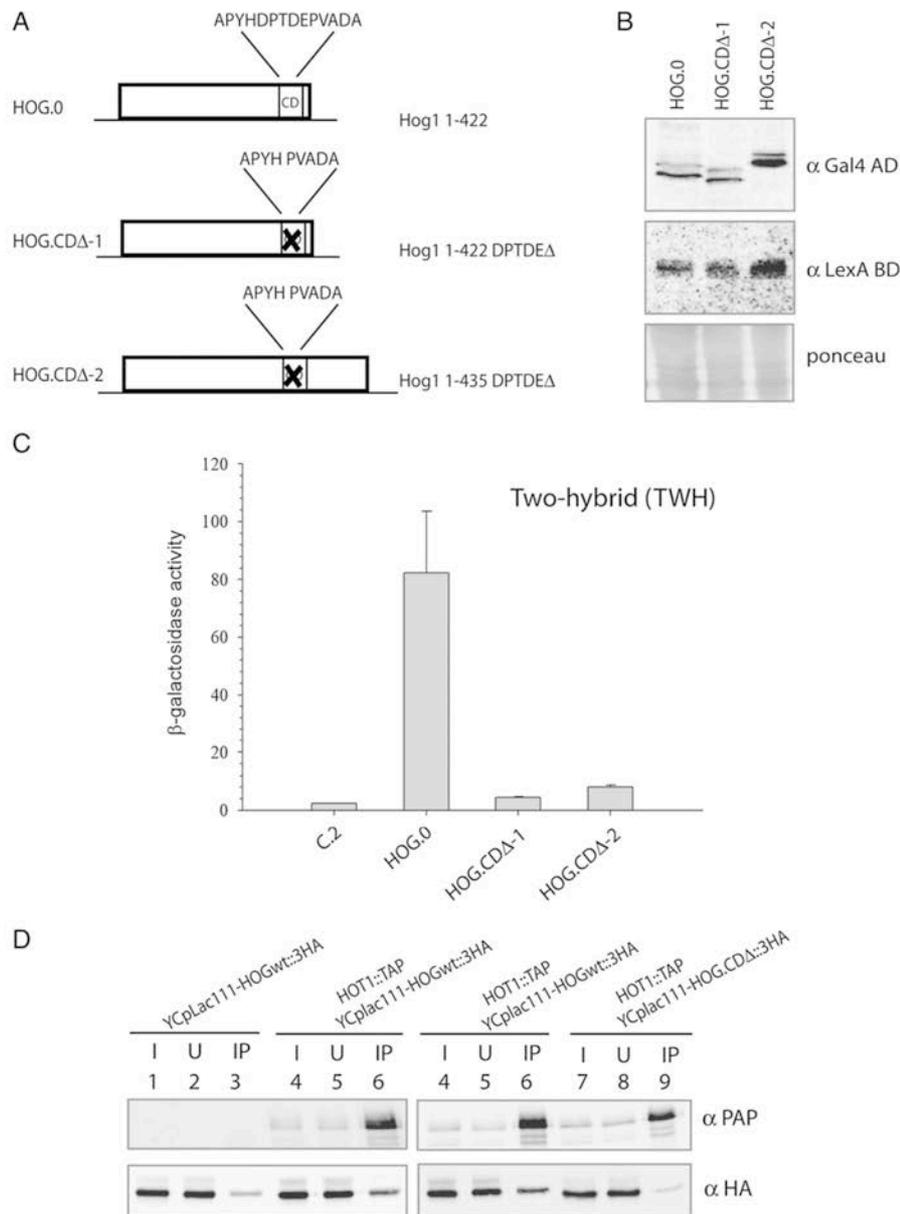
Fig. 4. The CD region of Hog1 is required for its interaction with Hog1. (A) A schematic representation of the regions of the Hog1 protein expressed as the Gal4AD fusions included in the pGAD424-derived plasmids HOG.0, HOG.1 and HOG.2. (B) Detection of the expression of the corresponding fusion proteins in the strain employed for the two-hybrid (TWH) experiments (EGY40/pSH18-34/pGAD424HOG/pBTM116HOT respectively) by Western blot using α LexA binding domain antibody and an anti Gal4AD antibody. Ponceau staining is shown as a loading control. (C) Determination of the β-galactosidase activity of the EGY40 strain transformed with plasmids pSH18-34, HOT.1 and the different pGAD424-HOG plasmids. In this figure, C.2 refers to EGY40 with pSH18-34, HOT.0 and pGAD424. The experiments were carried out in triplicate; the average and standard deviation data are shown. (D) The co-immunoprecipitation experiments using the cells of the FY86 *hog1Δ* strain expressing a TAP-tagged version of Hot1 and an HA-tagged version of Hog1, or its mutated Hog.CDΔ form and carried out and shown as described in the caption of Fig. 2. The results of the control experiments carried out with a FY86 *hog1Δ*-derived strain carrying Hog1-HA-tagged but without Hot1-TAP (lane 3) reveal a low level of background signal for Hog1-HA as compared to the signal for the strain expressing Hot1-TAP (lane 6).

Gene expression analyses were also carried out after a 30-min incubation in 20% (w/v) glucose. As can be seen in Fig. 6, panel B, the wild-type yeast cells showed an induction of the *HGI1* gene of approximately 60 times. A high expression level (3 times lower) was still found in the absence of Hot1. The same level of expression was found in the Hot1 KR4/M5, KR4Δ and ED5Δ mutants as in the *hot1Δ* strain. For *STL1*, the expression of wild-type Hot1 resulted in an increase of around 25 times. This transcriptional activation was reduced by between 6 and 8
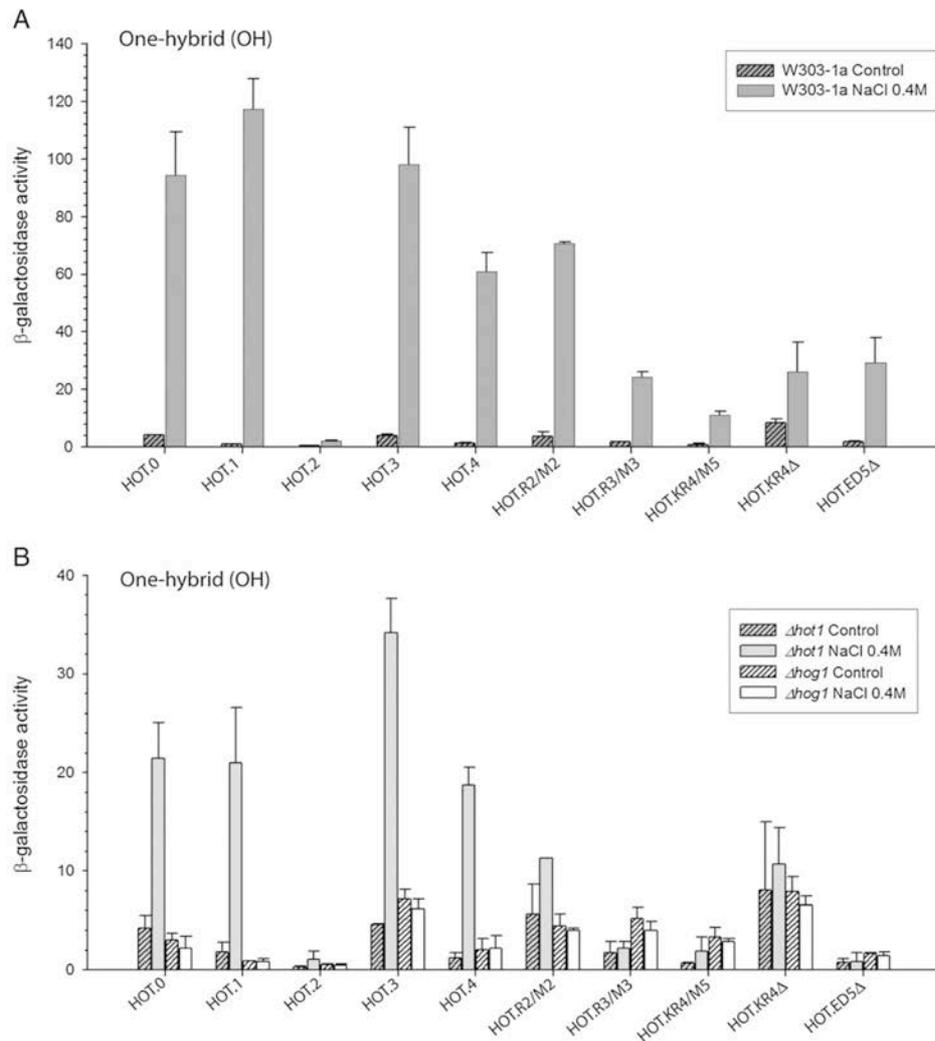
Fig. 5. Determination of the elements required for Hot1-dependent transcriptional activation from *LexA* operators by the one-hybrid (OH) system. Wild-type strain W303-1a (panel A) or the derived *hot1Δ* and *hog1Δ* mutant strains (panel B) transformed with the pSH18-34 reporter plasmid and the pBTM116 plasmids containing different regions of Hot1 were used to determine β-galactosidase activity as described in the Materials and methods section. The experiments were carried out in triplicate; the average and standard deviation data are shown.

times when the KR4 and ED5 elements were deleted or substituted (in the case of KR4) by methione residues.

All these results together indicate that the KR4 and ED5 sequences of Hot1 are required for the in vivo transcription activation of target genes.

3.6. The Hot1 KR4 and ED5 elements are required for the osmotic stress-regulated binding of Hot1 and Hog1 to target genes and to influence the later recruitment of RNA polymerase II

To gain a further understanding about the involvement of the KR4 and ED5 elements in Hot1 binding to its target promoters and about the recruitment of Hog1 and the RNA polymerase to them, chromatin immunoprecipitation experiments were carried out in the *hot1Δ* cells containing plasmids with the wild-type sequence of Hot1, or mutants KR4/M5 and ED5Δ.

An addition of 0.4 M NaCl to exponentially growing cells resulted in an increase of Hot1 binding to *STL1* and, especially, *HGI1* promoters in the cells expressing the wild-type version of Hot1p (Fig. 7A and B). The binding of this transcription factor to *STL1* was also detected under non-stressed conditions (Fig. 7B), while no basal binding of Hot1 occurred in the case of *HGI1* (Fig. 7A). The increase in Hot1 binding during osmotic stress reduced in both genes when the KR4 region was substituted with M5; actually in the case of *STL1*, the cells did not respond to the salt addition in terms of Hot1 location in its promoter. Moreover, when the ED element was removed, the binding of the transcription factor to both promoters was almost completely abolished under both basal and stress conditions, suggesting that the ED sequence is an essential component of the DNA-binding domain of Hot1p.

Next, the binding of Hog1 to these promoters was investigated. As shown in Fig. 7C, salt addition resulted in increased Hog1 binding to the *HGI1* promoter, even in the absence of a functional Hot1, due to
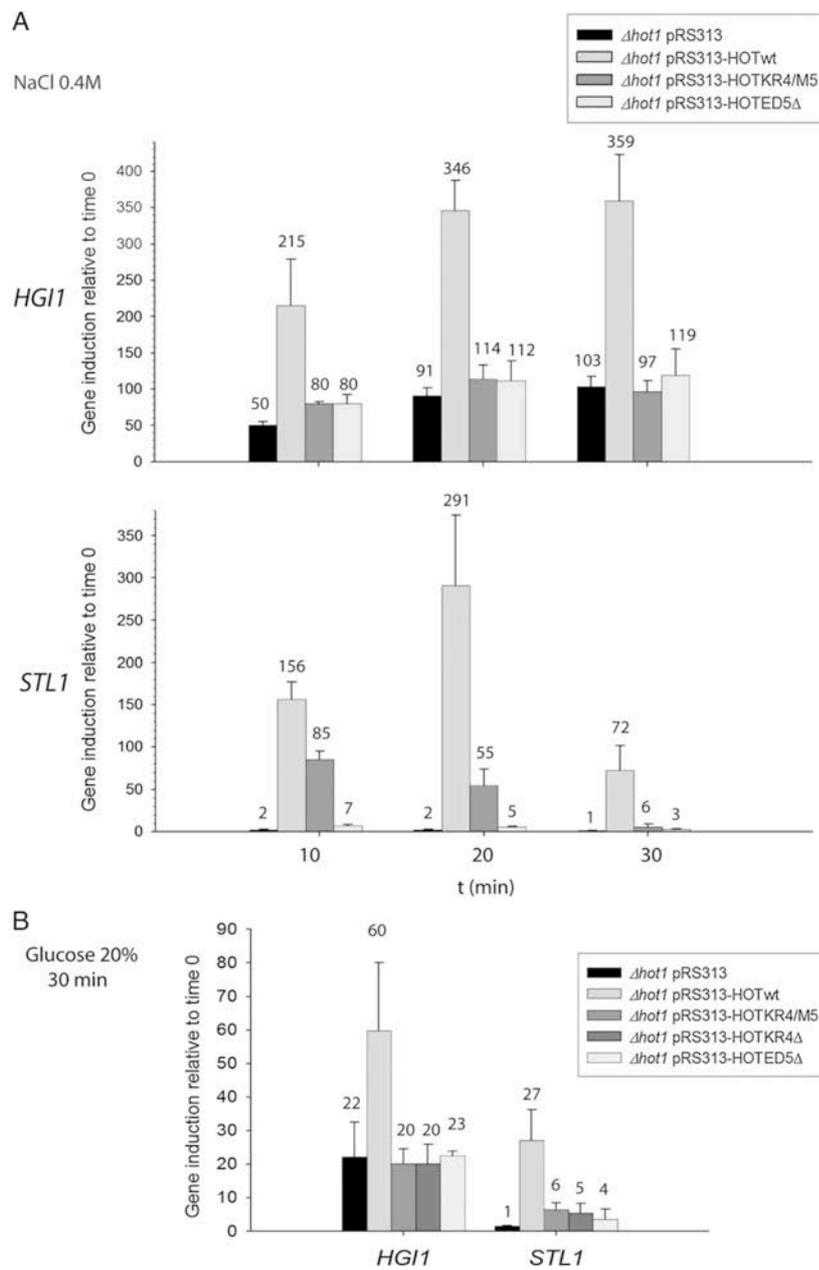
Fig. 6. The Hot1 KR4 and ED5 elements are required for the full activation of its gene targets *HGI1* and *STL1*. (A) RNA was isolated from the exponentially growing W303-1a *hot1Δ* strains carrying different versions of *HOT1* into the pRS313 plasmid at the time of NaCl addition at a final concentration of 0.4 M and at several timepoints later on (10, 20 and 30 min). (B) RNA was isolated from the same strains at the time of glucose addition at a final concentration of 20% (w/v) and 30 min later. Under both conditions the expression of those genes was determined by Real-Time RT-PCR of the cDNA prepared from these samples using the olignoucleotides described in the Materials and methods section. This figure shows the expression levels under high salt or glucose stress normalized to those obtained for the *ACT1* gene, referring to the value found at time 0 in each strain. The experiments were carried out in triplicate. The average and standard deviation data are shown. For clarity the average value is also indicated over the bars.

the particular regulation of the expression of this gene [38]. In the *hot1Δ* cells expressing wild-type Hot1, the Hog1 binding increase noted during stress was approximately 3.5 times for *HGI1* (Fig. 7C) and around 2 times for *STL1* (Fig. 7D) promoters. In both cases, the modification of the KR4 element and, more importantly, the deletion of the ED5 element, resulted in diminished MAPK binding, similar to the levels found in the *hot1Δ* mutant (Fig. 6C and D). These results are in agreement with the effect of the KR4 mutation on the interactions
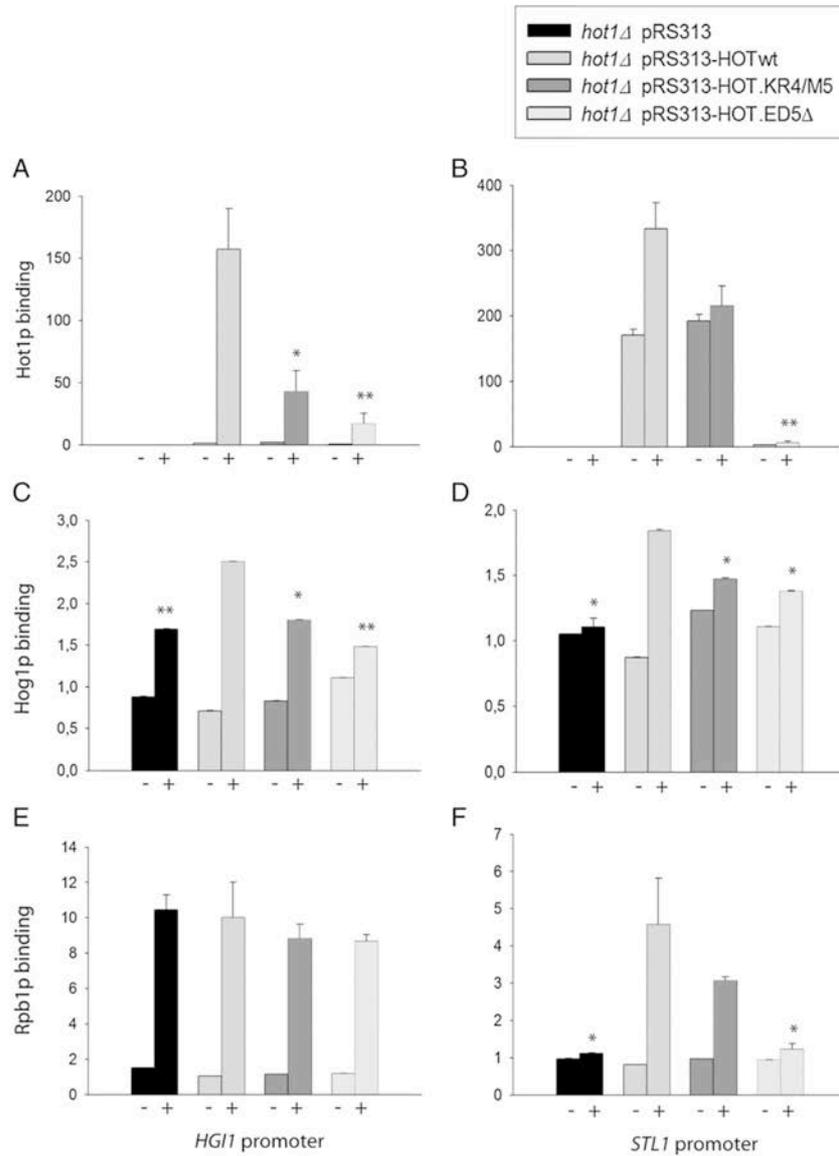
Fig. 7. Role of the Hot1 KR4 and ED5 elements in the binding of Hot1, Hog1 and Rpb1 to promoters *STL1* and *HGI1*. This figure shows the occupation of these proteins over the *HGI1* promoter region between −360 and −181 and that corresponding to promoter *STL1* between −372 and −313, determined by chromatin immunoprecipitation (ChIP) followed by Real-Time RT-PCR. The experiments were carried out with the W303-1a *hot1Δ* strain containing plasmid pRS313 or its derived plasmids containing the wild-type *HOT1* coding sequence or its KR4/M5 or ED5Δ mutations. The cells from the exponentially growing cultures were incubated (+), or not (−), for 10 min in NaCl 0.4 M as described in the Materials and methods section. For immunoprecipitation, Hot1 was tagged with HA, Hog1 with TAP and, in the case of Rpb1, specific antibodies against this subunit were used. The experiments were carried out in triplicate; the average and standard deviation data are indicated. Any statistically significant difference found between the strains containing HOT.KR4/M5 or HOT.ED5D and HOTwt are indicated by * (p-value lower than 0.05) or ** (p-value lower than 0.01).

between Hot1 and Hog1 and to that found for ED5 deletion in the binding to DNA.

Regarding the recruitment of the RNA polymerase II subunit Rpb1 to the *HGI1* promoter, only a very slight and non-significant reduction was observed between the *hot1Δ* strains carrying KR4/M5 and ED5Δ and that expressing the wild-type Hot1 sequences. However, it is worth mentioning that the expression of the *HGI1* gene is only partially controlled by Hot1, as described previously in our laboratory [38]. Finally,

the recruitment of RNA polymerase II to the *STL1* promoter during stress was affected consistently by the relevance of the Hot1 transcription factor in the control of the expression of this gene. In this sense, KR4 and, even to a greater extent, ED5 mutations, resulted in lower levels of polymerase binding, similar to those found in the *hot1Δ* mutant for the latter case. These results are consistent with the data about the expression of these genes under the same stress conditions shown in Fig. 6.

All together, our results demonstrate that the Hot1 KR4 and ED5 elements are required for Hot1 and Hog1 binding of to their target genes under osmotic stress conditions. In addition, they affect the recruitment of RNA polymerase II and, therefore, the transcription activation under these conditions, at least in the case of the *STL1* gene.

3.7. The Hot1 KR4 sequence is relevant to the stress response in yeast

Having determined the role of KR4 and ED5 in Hot1-dependent transcriptional regulation, the next objective was to analyze if the manipulation of these sequences can have some phenotypic effect relating to the stress response. Two alternative strategies were adopted to test this possibility.

The first was based on the use of hyperactive mutants in MAPKK Ssk2. In a wild-type strain, this protein is kept inactive under basal conditions due to the function of an N-terminal negative regulatory domain [10]. Deletion of this domain leads to the constitutive activation of the downstream kinase cascade, which is detrimental for cell growth; this effect has been shown to be suppressed by the deletion of the genes encoding downstream components of the pathway, such as *HOG1*, *HOT1* and *GPD1* [10,11,30]. Panel A in Fig. 8 reproduces the control experiments which confirm the data reported by these authors: the expression of the truncated version of *SSK2* results in the growth inhibition of the wild-type strain, which is supressed by the deletion of *HOG1* or *HOT1*. Then, growth under the glucose and galactose carbon sources of this wild-type and the *hot1Δ* mutant strains carrying *PGAL::SSK2ΔN* and different regions of *HOT1* in the multicopy plasmid pBTM116 was tested. While the *hot1Δ* strain expressing wild-type Hot1 (from the HOT.0 plasmid) is incapable of growing on galactose plates by the recovery of the wild-type phenotype, Hot1 with mutation KR4/M5 allows the suppression of the phenotype. This demonstrates that this sequence is essential for normal Hot1 activity and for HOG pathway functionality (Fig. 8B). For the strain carrying the deletion of the ED5 sequence, the observed phenotype corresponded to a wild-type strain, suggesting that this element is not that essential for the reconstruction of the signaling via this pathway (Fig. 8B). In accordance with this result, the mutants in Hot1-controlled genes *STL1* and *HGI1*, whose expression was significantly affected in strains carrying a HOT.ED5Δ version (Fig. 6), were not capable of suppressing the growth defect (Fig. 8C). To avoid the possibility of effects due to the overexpression of proteins through the use of episomal plasmid pBTM116 under the control of a strong promoter, the same experiments were carried out with the derivatives of the pRS313 plasmid containing the same Hot1 regions. The results shown in panel D of the same figure confirm that the expression at the wild-type level of Hot1 KR4/M5 suppressed the growth defect produced by hyperactive Ssk2. Panel E includes a Western blot analysis which demonstrates that the corresponding Hot1 proteins are expressed in each strain hence a lack of the suppression of the growth defect found in some cases is not due to protein expression problems.

A second strategy was followed to determine the functionality of the KR4 and ED5 elements in osmotolerance. Rep et al. [30] have reported that *hot1Δ* cells are sensitive to severe osmotic stress conditions. The percentage of viable cells after the incubation of exponentially growing cells in media with a concentration of NaCl 1.7 M for 24 h was about 5 times higher in *hot1Δ* cells when expressing a Hot1 wild type (1.5 ± 0.06) than when containing an empty plasmid (0.33 ± 0.09). It is important to note that viability lowered to its normal levels when *hot1Δ* cells expressed KR4/M5 or ED5Δ. These data indicate that both the KR4 and ED5 elements are relevant to acquire osmotolerance in yeast.

3.8. The Hot1 protein is capable of forming stable interactions with itself

The active structure of many transcription factors is a dimer. As a matter of fact, the results obtained in the one-hybrid experiments, in which the transcriptional activation dependent on the Hot1

sequences introduced into vector pBTM116 diminished when the genomic *HOT1* copy was deleted, suggest to this possibility (Fig. 5, compare the fold induction by HOT.0 in A and B). To determine if this were the case, a co-inmunoprecipitation experiment was carried out in a FY86-derived strain containing a chromosomic TAP-tagged version of Hot1 and expressing an HA-tagged version of Hot1 from plasmid pRS313. After the immunoprecipitation of Hot1-TAP by the α-PAP antibody, it was possible to detect Hot1 in the retained fraction using non-stressed cells or cells treated for 10 min with high glucose concentrations (20% w/v) (Fig. 9A). These results indicate that Hot1 is able to interact with itself, therefore suggesting that the formation of Hot1 homodimers can occur. Furthermore, the obtained data reveal that the presence of dimers is not dependent on the phosphorylation by Hog1 since a Hot1-Hot1 interaction is observed under non-stressed conditions.

In order to detect the presence of Hot1 dimers, in vivo crosslinking experiments were carried out. According to the results shown in Fig. 9B, under both the non-stressed exponential growth conditions and the stress conditions caused by high glucose concentrations, the complexes corresponding to the expected size for the Hot1 homodimers were detected after treatment with dimethyl adipimidate (DMA) and after analyzing the extracts by Western blot using antibodies against this protein. Similar results were obtained with another crosslinking reagent (ethylene glycol disuccinate bis(sulfo-N-succinimidyl) ester, EGS) (data not shown).

4. Discussion

In response to environmental perturbations such as osmotic shock, large-scale changes take place in the *S. cerevisiae* transcriptional program [2]. These changes are associated with important modifications of the RNA polymerase II localization toward stress-responsive genes, unlike housekeeping genes which correlate with a global down-regulation of the general transcription, and requires the Hog1 MAPK [26,27]. These authors have reported that this kinase targets the RNA Pol II machinery and induces chromatin remodeling at stress-responsive loci.

Among the several transcription factors found to be activated by Hog1, Sko1 and Hot1 have a major effect on the osmotic stress-dependent gene expression [37]. Although Hog1 lacks a DNA binding domain, it is able to associate with promoters through physical interactions with these transcription factors [20,39,61]. Given the relevance of the Hog1–Hot1 interaction for not only the transcription reprogramming under osmotic stress but also for the ability of yeast cells to cope with this adverse condition, several experiments in this manuscript provide further insights into this topic.

According to the two-hybrid experiments carried out in this work, the Hot1 region between amino acids 340 and 534 is required for the interaction with Hog1, and the KR4 element located in positions 381–385 is absolutely essential (Figs. 1 and 2), and it therefore acts as a docking site for the MAPK. Actually in the absence of this sequence, the pattern of stress-induced hyperphosphorylation of the transcription factor is affected (Fig. 3). This element is composed of basic residues, a common feature for the docking sites previously described for other factors controlled by kinases [40–46]. Hog1 is capable of activating proteins by phosphorylation; however, in the case of Hot1 [20] and also Sko1 [26] this modification is not required for their activity. The fact that the KR4 element is not related to a Hog1 phosphorylation site reinforces the idea suggested by other authors that the activation of Hot1 by the MAPK is related to a sustained physical interaction between both proteins [26] and not to phosphorylation-dependent control. Furthermore, the previously described CD domain of Hog1, involved in the interaction of this MAPK with Ptp2 [47] and Rck2 [48,49] is also required for its interaction with Hot1 (Fig. 4).

The relevance of the interaction between Hog1 and Hot1 mediated by the KR4 element described above can explain some results of the
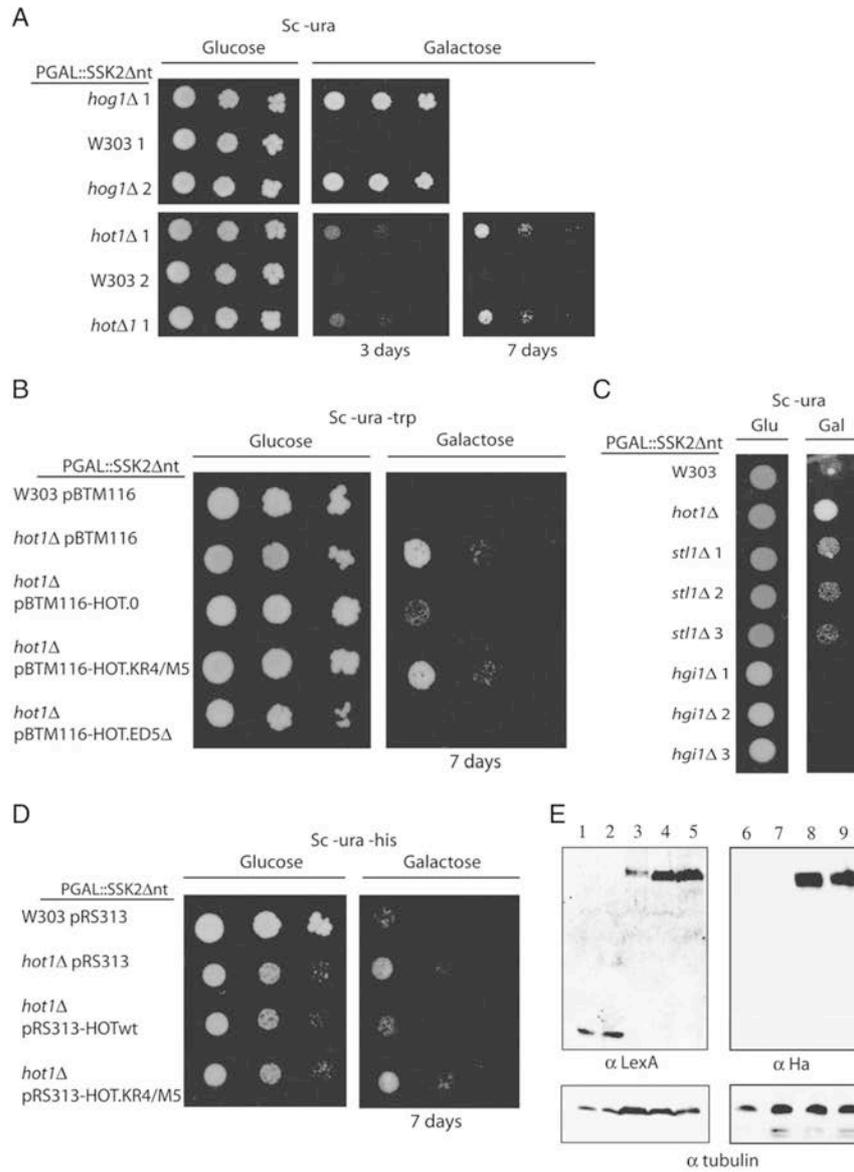
Fig. 8. Mutation of the KR4 element suppresses the growth defect caused by a constitutively activated HOG pathway. Strains W303-1a wild-type, *hog1Δ* and *hot1Δ* without (A) or with several *HOT1* versions in plasmids pBTM116 (B) or pRS313 (D) were grown in the adequate solid minimal medium with glucose or galactose. C. The results for the mutants in *STL1* and *HGI1*. E. Detection of the expression of the Hot1 derivatives considered by Western blot using α LexA BD (for the expression from vector pBTM116) or the α HA antibodies (in the case of the use of pRS313). All the strains also contained the plasmid carrying *PGAL::SSK2Δnt*. Lanes 1 and 6 correspond to the wild-type strain, while the others are *hot1Δ*. Besides, those shown in lanes 1 and 2 include plasmid pBTM116, line 3 corresponds to HOT.0, line 4 to HOT.KR4/M5 and line 5 to HOT.ED5Δ. The strains in lines 6 and 7 include pRS313, line 8 includes its derivative HOT.0 and line 9 its corresponding HOT.ED5Δ. Each panel of this figure shows the result of a representative experiment. In some cases, several transformants (indicated by numbers) have been included to demonstrate that the effect observed is consistent.

one-hybrid experiments shown in Fig. 5. According to them, Hot1 behaves as an unusual transcription factor as it is unable to sustain high levels of induction (Fig. 5) in the absence of the genome-encoded Hot1 or Hog1 proteins or when KR4 is deleted/mutated. These experiments support the data described by other authors about the relevance of the Hog1–Hot1 interaction for targeting MAPK and RNA Pol II to target genes during osmotic stress. Furthermore, the results obtained

in these analyses have allowed the identification of another Hot1 region, the ED5 element at positions 541–546, as an essential component in the Hot1 function, for transcriptional activation in this case. The role of the KR4 and ED5 elements to induce the expression of Hot1 targets, such as genes *STL1* and *HGI1* (Fig. 6), confirms their relevance for the function of this transcription factor. Moreover, the chromatin immunoprecipitation experiment results displayed in Fig. 7 demonstrate that KR4 is
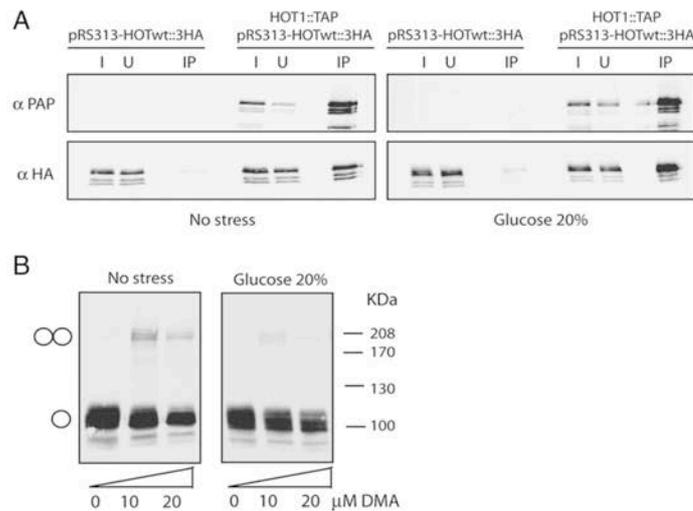
Fig. 9. The Hot1 protein is capable of interacting with itself and of forming dimers under exponential growth and in the presence of stress caused by high glucose concentrations. A. The co-immunoprecipitation experiments carried out in an FY86-derived strain expressing a chromosomic TAP-tagged version of Hot1 and a HA-tagged version from a pRS313-derived plasmid. The α-PAP antibody was used for the precipitation of Hot1-TAP and the α HA antibody was employed to detect the presence of Hot1 in the retained fraction. I refers to total protein extracts (input), U to the unbound fraction and IP to the eluted fraction. B. The in vivo crosslinking experiments carried out with the DMA reagent in the cells of the strain W303-1a transformed with plasmid pRS313-HOTwt::3HA. The procedure described in the Materials and methods section was followed, then extracts were analyzed by Western blotting using the *a* HA antibodies. This figure shows the result of a representative experiment in each panel.

important and that ED5 is essential for the Hot1 binding to DNA, and that both are also required for the normal binding of Hog1 and RNA Pol II to the promoter of gene *STL1*. Similar data have been obtained for the gene *HGI1*. However in this case, polymerase binding is not affected to the same extent, probably because this gene is controlled by not only Hot1 under osmotic stress conditions [38]. These results are consistent with the interrelationship described between the binding of Hot1 and Hog1 to the promoters of the genes controlled by this transcription factor. In this sense, Hog1 recruitment to these regions depends on the presence of Sko1 and Hot1, and it is not recruited to promoters, ORFs or downstream regions when these two factors are absent [20,26]. Cook et al. [26] have proposed that Hog1 is loaded to the set of genes with the nearby Sko1 and Hot1 binding sites along with RNA Pol II during transcription initiation. According to the data obtained from the one-hybrid and the chromatin immunoprecipitation experiments, a dual function of the ED5 element, as both a DNA-binding element and a transcription activator, cannot be ruled out.

The relevance of KR4 in the functionality of Hot1 and the HOG pathway is demonstrated by not only the analysis of the suppression of the growth defect provoked by the expression of a hyperactive Ssk2, but also by the results of the viability of cells against severe osmotic stress (Fig. 8 and other data reported in the Results section). A version of Hot1 in which the KR4 element has been deleted behaves as a *hot1Δ* mutant in terms of resistance to osmotic stress and the suppression of the constitutive activation of the HOG pathway [10]. The ED5 element appears to be relevant for osmotolerance but not for the integrity of the HOG pathway. It is likely that the phenotype relating to the constitutive expression of the HOG pathway is more difficult to understand because, although the mutants in *HOT1*, *HOG1* and *GPD1* suppress the deleterious defect of hyperactive Ssk2 [30], the mutants in *STL1*, *HGI1* (in this work), and also in the transcription factors encoding the genes *MSN1*, *MSN2* and *MSN4* [30], do not support the same effect, despite their relevance in the response to osmotic stress [37]. The relevance of the KR4 and ED5 elements in the Hot1p function is also reinforced by an analysis carried out with the *Fungal Sequence Alignment* of the *Saccharomyces Genome Database*, which reveals that both are conserved

in the Hot1p homologs found in yeasts *Saccharomyces bayanus*, *Saccharomyces mikatae* and *Saccharomyces paradoxus* (data not shown).

Finally, this work provides considerable evidence for the ability of Hot1 to form dimers. The LexA-Hot fusions in the one-hybrid experiments show lower transcriptional activation in the cells in which native *HOT1* was deleted. Besides, the co-inmunoprecipitation experiments reveal an interaction between the TAP-tagged and the HA-tagged versions of the Hot1 protein expressed in the same cells. Finally, the in vivo crosslinking experiments and the Western blot analysis demonstrate the presence of a complex with a molecular size corresponding to a homodimeric form of Hot1. Further experiments are required to understand the relevance of dimer formation for the function of this transcription factor.

5. Conclusions

This is the first study about the sequences involved in the interaction between the MAP kinase Hog1 and the transcription factor Hot1, and their relevance in the response of yeast cells to hyperosmotic stress. The two-hybrid experiments described in this work demonstrate that the Hot1 sequence between amino acids 340 and 534 and the CD element of Hog1 are required for this interaction. Particularly, the sequence KR4 (381–385) of Hot1 is essential for kinase binding. Besides the ED5 element (amino acids 541–546) is necessary for Hot1 binding to chromatin. Both KR4 and ED5 sequences are required for osmotolerance. Finally, KR4 plays an essential role in the functionality of the HOG pathway.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2013.07.009.

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Material suplementario

Table 3.2.S1. Yeast strains used in this work

Strain	Description	Origin
EGY40	*MATa, ura3-52, his3, trp1, leu2*	M. del Olmo
EGY40 pSH18-34	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34]	This work
TWH C1	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.0]	This work
TWH C2	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.0] [pGAD424]	This work
TWH HOT.0	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.0] [pGAD.HOG.0]	This work
TWH HOT.1	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.1] [pGAD.HOG.0]	This work
TWH HOT.2	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.2] [pGAD.HOG.0]	This work
TWH HOT.3	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.3] [pGAD.HOG.0]	This work
TWH HOT.4	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.4] [pGAD.HOG.0]	This work
TWH HOT.5	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.5] [pGAD.HOG.0]	This work
TWH HOT.6	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.6] [pGAD.HOG.0]	This work
TWH HOT.R2/M2	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.R2/M2] [pGAD.HOG.0]	This work
TWH HOT.R3/M3	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.R3/M3] [pGAD.HOG.0]	This work
TWH HOT.KR4/M5	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.KR4/M5] [pGAD.HOG.0]	This work
TWH HOT.KR4Δ	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.KR4Δ] [pGAD.HOG.0]	This work
TWH HOT.ED5Δ	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.ED5Δ] [pGAD.HOG.0]	This work
TWH HOG.1	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.0] [pGAD.HOG.1]	This work
TWH HOG.2	*MATa, ura3-52, his3, trp1, leu2* [pSH18-34] [pHOT.0] [pGAD.HOG.2]	This work
FY86	*MATα leu2Δ1 ura3-52 his3Δ200*	Lab Stock
FY86 HOT::TAP	*MATα leu2Δ1 ura3-52 his3Δ200* HOT1-TAP::kanMX6	This work
FY86 HOG::TAP	*MATα leu2Δ1 ura3-52 his3Δ200* HOG1-TAP::kanMX6	This work
FY86 pRS313-HOTwt::3HA	*MATα leu2Δ1 ura3-52 his3Δ200* [pRS313-Hotwt]	This work
FY86 HOT1::TAP pRS313-HOTwt::3HA	*MATα leu2Δ1 ura3-52 his3Δ200* HOT1-TAP::kanMX6 [pRS313-Hotwt]	This work
W303	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1*	Lab Stock
W303 OH HOT.0	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.0]	This work
W303 OH HOT.1	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.1]	This work
W303 OH HOT.2	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.2]	This work
W303 OH HOT.3	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.3]	This work
W303 OH HOT.4	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.4]	This work

W303 OH HOT.R2/M2	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.R2/M2]	This work
W303 OH HOT.R3/M3	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.R3/M3]	This work
W303 OH HOT.KR4/ED5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.KR4/M5]	This work
W303 OH HOT.KR4Δ	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.KR4Δ]	This work
W303 OH HOT.ED5Δ	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [pSH18-34] [pHOT.EDΔ]	This work
W303 *hot1Δ*	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4*	P.M Alepuz
W303 *hot1Δ* OH HOT.0	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.0]	This work
W303 *hot1Δ* OH HOT.1	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.1]	This work
W303 *hot1Δ* OH HOT.2	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.2]	This work
W303 *hot1Δ* OH HOT.3	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.3]	This work
W303 *hot1Δ* OH HOT.4	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.4]	This work
W303 *hot1Δ* OH HOT.R2/M2	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.R2/M2]	This work
W303 *hot1Δ* OH HOT.R3/M3	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.R3/M3]	This work
W303 *hot1Δ* OH HOT.KR4/M5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.KR4/M5]	This work
W303 *hot1Δ* OH HOT.KR4Δ	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.KR4Δ]	This work
W303 *hot1Δ* OH HOT.ED5Δ	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pSH18-34] [pHOT.ED5Δ]	This work
W303 *hog1Δ*	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4*	P.M Alepuz
W303 *hog1Δ* OH HOT.0	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.0]	This work
W303 *hog1Δ* OH HOT.1	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.1]	This work
W303 *hog1Δ* OH HOT.2	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.2]	This work
W303 *hog1Δ* OH HOT.3	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.3]	This work
W303 *hog1Δ* OH HOT.4	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.4]	This work
W303 *hog1Δ* OH HOT.R2/M2	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.R2/M2]	This work
W303 *hog1Δ* OH HOT.R3/M3	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.R3/M3]	This work
W303 *hog1Δ* OH HOT.KR4/M5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.KR4/M5]	This work
W303 *hog1Δ* OH HOT.KR4Δ	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.KR4Δ]	This work
W303 *hog1Δ* OH HOT.ED5Δ	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pSH18-34] [pHOT.ED5Δ]	This work

W303 *hot1Δ* pRS313::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pRS313]	This work
W303 *hot1Δ* pRS313-HOTwt::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pRS313-hotwt]	This work
W303 *hot1Δ* pRS313-HOTKR4/M5::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pRS313-HotKR4/M5]	This work
W303 *hot1Δ* pRS313-HOTkr4Δ::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pRS313-HotKR4Δ]	This work
W303 *hot1Δ* pRS313-HOTED5Δ::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [pRS313-HotED5Δ]	This work
W303 *hog1Δ* pRS313-HOTwt::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [pRS313-hotwt]	This work
W303 *hot1Δ (URA3)*	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::URA3*	This work
W303 *hot1Δ (URA3)* HOG::TAP	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::URA3 HOG1-TAP::kanMX6*	This work
W303 *hot1Δ (URA3)* HOG::TAP pRS313::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::URA3 HOG1-TAP::kanMX6* [pRS313]	This work
W303 *hot1Δ (URA3)* HOG::TAP pRS313-HOTwt::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::URA3 HOG1-TAP::kanMX6* [pRS313-hotwt]	This work
W303 *hot1Δ (URA3)* HOG::TAP pRS313-HOTKR4/M5::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::URA3 HOG1-TAP::kanMX6* [pRS313-HotKR4/M5]	This work
W303 *hot1Δ* (URA3) HOG::TAP pRS313-HOTED5Δ::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::URA3 HOG1-TAP::kanMX6* [pRS313-HotED5Δ]	This work
W303 *hot1Δ* (URA3) HOG::TAP pRS313-HOTEKR4Δ::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::URA3 HOG1-TAP::kanMX6* [pRS313-HotKR4Δ]	This work
W303 *hog1Δ (URA3)* YCplac111-HOGwt::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::URA3* [YCplac111-HOGwt]	This work
W303 *hog1Δ (URA3)* HOT::TAP YCplac111-HOGwt::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::URA3 HOT1-TAP::HIS3* [YCplac111-hogwt]	This work
W303 *hog1Δ (URA3)* HOT::TAP YCplac111-HOGCDΔ::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::URA3 HOT1-TAP::HIS3* [YCplac111-HogCDΔ]	This work
W303 pGAL::SSK2Δnt	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [PGAL1::SSK2Δnt]	This work
W303 *stl1Δ*	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 stl1::kanMX4*	P.M Alepuz
W303 *hgi1Δ*	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hgi1::kanMX4*	This work
W303 *hot1Δ* pGAL::SSK2Δnt	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [PGAL1::SSK2Δnt]	This work
W303 *hog1Δ* pGAL::SSK2Δnt	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hog1::kanMX4* [PGAL1::SSK2Δnt]	This work

W303 *stl1Δ* pGAL::SSK2Δnt	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 stl1::kanMX4* [PGAL1::SSK2Δnt]	This work
W303 *hgi1Δ* pGAL::SSK2Δnt	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hgi1::kanMX4* [PGAL1::SSK2Δnt]	This work
W303 pGAL::SSK2Δnt pRS313::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [PGAL1::SSK2Δnt] [pRS313]	This work
W303 *hot1Δ* pGAL::SSK2Δnt pRS313::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [PGAL1::SSK2Δnt] [pRS313]	This work
W303 *hot1Δ* pGAL::SSK2Δnt pRS313-HOTwt::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [PGAL1::SSK2Δnt] [pRS313-hotwt]	This work
W303 *hot1Δ* pGAL::SSK2Δnt pRS313-HOTmet5::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [PGAL1::SSK2Δnt] [pRS313-HotKR4/M5]	This work
W303 *hot1Δ* pGAL::SSK2Δnt pRS313-HOTED5D::3HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [PGAL1::SSK2Δnt] [pRS313-HotED5Δ]	This work
W303 pGAL::SSK2Δnt pBTM116	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1* [PGAL1::SSK2Δnt] [pBTM116]	This work
W303 *hot1Δ* pGAL::SSK2Δnt pBTM116	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [PGAL1::SSK2Δnt] [pBTM116]	This work
W303 *hot1Δ* pGAL::SSK2Δnt pBTM116-HOT.0	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [PGAL1::SSK2Δnt] [pHOT.0]	This work
W303 *hot1Δ* pGAL::SSK2Δnt pBTM116-HOT.KR4/M5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [PGAL1::SSK2Δnt] [pHOT.KR4/M5]	This work
W303 *hot1Δ* pGAL::SSK2Δnt pBTM116-HOT.ED5D	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [PGAL1::SSK2Δnt] [pHOT.ED5Δ]	This work

Table 3.2.S2. Plasmids used in this work

Plasmid	Description	Origin
pBS2623	contains TAP tag with kanMX6 marker	M Pamblanco
YEp352	multicopy shuttle vector with *URA3* marker	Lab stock
pRS313	centromeric yeast vector with *HIS3* marker	Lab stock
YCplac111	centromeric yeast vector with *LEU2* marker	Lab stock
pSH18-34	*URA3* yeast plasmid with LexA operatos upstream of the gen GAL4-LacZ	R. Brent
pBTM116	*TRP1* 2 micron yeast plasmid with the LexA DNA Binding domain	R. Brent
pGAD424	*LEU 2* micron yeast plasmid with GAL4 DNA activation domain	P.M Alepuz
pUG603	The Hot1$^{140\text{-}645}$ ORF described in Rep et al., 1999	G. Ammerer
pHOT.0	The Hot1$^{140\text{-}582}$ ORF obtained from pUG603 by cutting with *Sma*I and *Pst*I and cloned in frame with the LexA B.D at these sites in pBTM116	This work
pHOT.1	The Hot1$^{140\text{-}554}$ ORF obtained from pHOT.0 by cutting with *Sal*I and *Pst*I and cloned in frame with the LexA B.D at the these sites in pBTM116	This work
pHOT.2	The Hot1$^{140\text{-}340}$ ORF cloned in frame with the LexA B.D at the *Bam*HI *Pst*I sites in pBTM116	This work
pHOT.3	The Hot1$^{341\text{-}554}$ ORF cloned in frame with the LexA B.D at the *Bam*HI *Pst*I sites in pBTM116	This work
pHOT.4	The Hot1$^{140\text{-}420}$ ORF cloned in frame with the LexA B.D at the *Bam*HI *Pst*I sites in pBTM116	This work
pHOT.5	The Hot1$^{341\text{-}420}$ ORF cloned in frame with the LexA B.D at the *Bam*HI *Pst*I sites in pBTM116	This work
pHOT.6	The Hot1$^{421\text{-}554}$ ORF cloned in frame with the LexA B.D at the *Bam*HI *Pst*I sites in pBTM116	This work
pHOT.R2/M2	Hot1 R382M and R384M mutated from HOT.0	This work
pHOT.R3/M3	Hot1 R382M, R383M and R384M mutated from HOT.0	This work
pHOT.KR4/M5	Hot1 K381M , R382M, R383M, R384M and R385M mutated from HOT.0	This work
pHOT.KR4Δ	Hot1 K381 , R382 R383, R384 and R385 deleted from HOT.0	This work
pHOT.ED5Δ	HOT1 E541 , D542, D543, D544, D545, D546 deleted from HOT.0	This work
pGAD-HOG.0	The Hog1$^{1\text{-}422}$ ORF cloned in frame with the Gal4 A.D at the *Bam*HI site in pGAD424	This work
pGAD-HOG.1	The Hog1$^{1\text{-}422}$ ORF with CD domain mutated by deletion of D307, P308, T309, D310, E311 in pGAD-HOG.0	This work
pGAD-HOG.2	The Hog1 ORF with CD domain mutated by deletion of D307, P308, T309, D310, E311 cloned in frame with the GAL4 A.D at the *Bam*HI site in pGAD424	This work
pRS313-HA	*HIS3* centromeric yeast plasmid modified with 3XHA	This work
pRS313-Hotwt	The entire Hot1 ORF with its endogenous promoter fused to a 3xHA tag before the stop codon cloned in pRS313	This work
pRS313-HotKR4/M5	Hot1 K381M , R382M, R383M, R384M and R385M mutated from pRS313-Hotwt	This work
pRS313-HotKR4Δ	Hot1 K381 , R382 R383, R384 and R385 deleted from pRS313-Hotwt	This work
pRS313-HotED5Δ	Hot1 E541 , D542, D543, D544, D545, D546 deleted from pRS313-Hotwt	This work
YCplac111-Hogwt	The entire Hog1 ORF with its endogenous promoter fused to a 3xHA tag before the stop codon cloned in YCplac111	This work
YCplac111-HogCDΔ	Hog1 D307, P308, T309, D310, E311 deleted from YCPlac111-Hogwt	This work
pPGAL::SSK2Δnt	contains a truncated form of Ssk2 (with its inhibitory domain deleted) under control of an inducible *GAL* promoter	G. Ammerer

Table 3.2.S3. Oligonucleotides used in this work.

Oligonucleotide	Sequence (5' to 3')	Use
HOT-TAP L	AAAAGAACCATTGGCTGGTTGCAAGAGAGCCTTGCTG GAATAACCATGGAAAAGAGAAG	TAP-tagging of *HOT1*
HOT-TAP R	AAAACAGCACTAACGATTTTTGTAGATTTACAGAATGT ATTTATGGACTCACTATAGGG	"
HOT-200	GTATGGTAACAAGTGGAG	Confirmation of TAP-tagging of *HOT1*
HOT+200	CTAAGGGGCGATTTGTCA	"
HOG-TAP L	TACGGTAACCAGGCCATACAGTACGCTAATGAGTTCCA ACAGTCCATGGAAAAGAGAAG	TAP-tagging of HOG*1*
HOG-TAP R	TGTTTCCTCTATACAACTATATACGTAAATACTTTTATG AGTACCGACTCACTATAGGG	"
HOG-200	TGGCAGTGATGGACAGAT	Confirmation of TAP-tagging of *HOG1*
HOG+200	TGACGGTTCTTGGAGTCT	"
OBS 292	TTGTATTTTCAGGGTGAGCTC	Confirmation of TAP-taggings
DELHOTURA A	TACAAAAAAGATTATATTTAGGGTACATATGGCTGGA GCATAATTCGGTAATCTCCGAGC	*HOT1* gene disruption with *URA3*
DELHOTURA B	TCCTTCCTATGATTGTAAACGATTATTTACTATCGTACG TGCCCCGGGTAATAACTGATA	"
DELHOGURA A	ACAAAGGGAAAACAGGGAAAACTACAACTATCGTATA TAATAATTCGGTAATCTCCGAGC	*HOG1* gene disruption with *URA3*
DELHOGURAB	AAGAAGTAAGAATGAGTGGTTAGGGACATTAAAAAAA CACGTCCCGGGTAATAACTGATA	"
URA3 UP	CTTAACTGTGCCCTCCAT	Confirmation of *HOT1* disruption
URA3 CHECK	GCGAAGAGCGACAAAGA	"
YHRPRO-2A	GTCCCTTTCTGACAATAAGACC	Chromatin inmunoprecipitation. PCR of *HGI1* promoter
YHRPRO-2B	TGTTTCTGGCGATCCCTTCG	"
STL1PRO-A	TTGGTTAATCCTCGCCAGGT	Chromatin inmunoprecipitation. PCR of *STL1* promoter
STL1PRO-B	TATGAGTGTGACTACTCCTG	"
INT-A	GGCTGTCAGAATATGGGGCCGTAGTA	Chromatin inmunoprecipitation. PCR of an intergenic region
INT-B	CACCCCGAAGCTGCTTTCACAATAC	"
YHR087W-1	GGTAAGCTATCTGAAGTTGTC	PCR of *HGI1* gene
YHR087W-2	ACTTCTTCGATCTTCTTG	"
STL1-1	CGGAAGAAGTTTGGAGGAAA	PCR of *STL1* gene
STL1-2	GGCATGATCTTCGACTTCTT	"
ACT-1	GGATCTTCTACTACATCAGC	PCR of *ACT1* gene
ACT-2	CACATACCAGAACCGTTATC	"
HOTKR4DEL 1	GAACCAATGGGATTAATACGCAAAG	*HOT1* KR4 mutagenesis (deleted)
HOTKR4DEL 2	GTTACTTTGCGTATTAATCCCATTTGG	"

HOTED5DEL 1	GATGATGGCTATCAAGGTGATGACGAAGG	*HOT1* ED5 mutagenesis (deleted)
HOTED5DEL 2	CATCACCTTCGTCATCACCTTGATAGCCATC	"
HOT1R381M-1	AAGTTGAACCCAAATGGGATTATGATGATGATGAGAAATACGCAA	*HOT1* KR4 mutagenesis (replaced)
HOT1R381M-2	TTGCGTATTTCTCATCATCATCATAATCCCATTTGGGTTCAACTT	"
HOT1R382M-1	TTGAACCCAAATGGGATTAAAATGCGCAGAAGAAATACGCAAAGT	"
HOT1R382M-2	ACTTTGCGTATTTCTTCTGCGCATTTTAATCCCATTTGGGTTCAA	"
HOT1R383M-3	CCCAAATGGGATTAAAATGATGATGAGAAATACGCAAAGTAACAACAATGC	"
HOT1R383M-4	GCATTGTTGTTACTTTGCGTATTTCTCATCATCATTTTAATCCCATTTGGG	"
HOT1R384M-1	CCAAATGGGATTAAAATGCGCATGAGAAATACGCAAAGTAACAAC	"
HOT1R384M-2	GTTGTTACTTTGCGTATTTCTCATGCGCATTTTAATCCCATTTGG	"
HOT1R385M-1	CCAAATGGGATTATGATGATGATGATGAATACGCAAAGTAACAACAATGCG	"
HOT1R385M-2	CGCATTGTTGTTACTTTGCGTATTCATCATCATCATCATAATCCCATTTGG	"
HOGCDDEL-1	CGGCTCCTTACCACCCAGTAGCCG	*HOG1* CD mutagenesis (deleted)
HOGCDDEL-2	GGCATCGGCTACTGGGAGGTCAGGAGC	"
HOT341Bam	AGAGATCGAATTCCCGGGGATCCGTCAGAACCAGTCGCAGGTTTTA	*HOT1* truncated constructions for one hybrid and two hybrid experiments
HOT534Pst	TCTACGATTCATAGATCTCTGCAGGATTACTTTTCACTGTCATTGG	"
HOT140Bam	AGAGATCGAATTCCCGGGGATCCGTGGTACAGGGCGTATGTTAAG	"
HOT340Pst	TCTACGATTCATAGATCTCTGCAGGATTGGCTTGTGTGTTTATAAC	"
HOT420Pst	TCTACGATTCATAGATCTCTGCAGGAGTTGTAGAATTATGAGAATT	"
HOT5-1	CGCGGATCCATCAGAACCAGTCGCAGGTT	"
HOT5-2	TGCACTGCAGAGTTGTAGAATTATGAGA	"
HOT6-1	CGCGGATCCCTAGCATGAATTATACCAAT	"
HOT6-2	TGCACTGCAGATTACTTTTCACTGTCAT	"
HOT-6	CTAGAACTAGTGGATCCCCCTCTCTTACTTCATCAGCATCGTCAAGATTGTTGGGCCACC	*HOT1* entire ORF clonation in pRS313
HOT-7	ATATCGAATTCCTGCAGCCCCCACGCCAAAACTGTGGTGGGCCAGGCCAGCACCGGAAAC	"

3.3. Capítulo 3

The C-terminal región of the Hot1 transcription factor binds GGGACAAA-related sequences in the promoter of its target genes

The C-terminal region of the Hot1 transcription factor binds GGGACAAA-related sequences in the promoter of its target genes

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The response to hyperosmotic stress in the yeast *Saccharomyces cerevisiae* involves the participation of the general stress response mediated by the Msn2/4 transcription factors and the HOG pathway. One of the transcription factors activated through this pathway is Hot1, which contributes to the control of the expression of several genes involved in glycerol synthesis and flux or in other functions related to the adaptation to adverse conditions. In this work new data is provided about the interaction mechanism of this transcription factor with the DNA. By means of one-hybrid and electrophoretic mobility assays we demonstrate that the C-terminal region corresponding to amino acids 610 to 719, is the DNA-binding domain of Hot1. Besides we describe how this domain recognizes the sequence 5'-GGGACAAA-3' located in the promoter of the *STL1* gene. *In silico* analysis carried out during this work allowed the identification of identical or similar sequences (with up to two mismatches) in the promoter of other Hot1 targets. Finally, we found that the similarity of the particular sequence located in each target of the transcription factor influences its ability to be recognized *in vivo*.

Keywords: Hot1, DNA binding domain, Upstream Activation Sequences, Shift assay, *Saccharomyces cerevisiae*

INTRODUCTION

The yeast *Saccharomyces cerevisiae* can be affected during its growth by several adverse conditions. Once being detected, yeast cells activate signal transduction pathways which result in important transcriptional changes (reviewed in Hohmann and Mager, 2003). Under several stress situations, *S. cerevisiae* displays the *Environmental Stress Response* (ESR) which requires the activity of the transcription factors Msn2 and Msn4 (Martínez-Pastor *et al.*, 1996; Gasch *et al.*, 2000).

One of the well-studied stress conditions is the hyperosmolarity due to high concentrations of salt, sorbitol or glucose (reviewed in Hohmann and Mager, 2003; Martínez-Montañés *et al.*, 2010; Saito and Posas, 2012). The specific response to this adverse condition is mediated by the *High Osmolarity Glycerol* (HOG) pathway. Two osmosensing mechanisms (the SLN1 and the SHO1 branches) provoke the activation by phosphorylation of the MAPK Hog1 (Boguslawski *et al.*, 1992; Brewster *et al.*, 1993; Maeda *et al.*, 1994, 1995; Posas *et al.*, 1996; Posas and Saito, 1998; Raitt *et al.*, 2000; Tatebayashi *et al.*, 2006, 2007), which results in its nuclear translocation. Hog1 regulates several unrelated transcription factors (Hot1, Msn1, Smp1, Gcn4, Skn7, Sko1, Msn2/4 and Rtg1/3), each of which is responsible for controlling the expression of a subset of osmoresponsive genes (Rep *et al.*, 1999a, 1999b, 2000; Posas *et al.*, 2000; de Nadal and Posas, 2008; Ni *et al.*, 2009). Besides, Hog1 regulates chromatin-modifying enzymes (Da Nadal *et al.*, 2004; Mas *et al.*, 2009) and interacts with RNA polymerase II (Alepuz et al., 2003; Cook and O'Shea, 2012; Nadal-Ribelles *et al.*, 2012).

Hot1 was identified in a two-hybrid screen using *HOG1* coding sequences as a bait (Rep *et al.*, 1999b). This protein binds to the promoter of several genes, including *STL1*, *GPD1*, *GPP2* and *HGI1* (Rep *et al.*, 1999b, 2000; Gomar-Alba *et al.*, 2012) and is involved in their transcriptional induction under osmotic stress. In the *HGI1*, which is partially controlled by this factor, binding occurs only under stress conditions, while for *STL1* gene Hot1 interacts with its promoter also under non-stress conditions (Alepuz *et al.*, 2001; Gomar-Alba *et al.*, 2013).

Studies carried out by Capaldi *et al.* (2008) suggest that, due to the inhibition of Msn2/4 under high glucose concentrations, Hot1, together with Sko1, are more relevant for the response to this osmostress condition. This could explain differential traits described previously in the presence of this osmolyte (Kaeberlein *et al.*, 2002; Erasmus *et al.*, 2003; Jiménez-Martí *et al.*, 2011).

According to this, the osmostress response transcription factor Hot1 has, at least, three well-known features (Krantz *et al.*, 2006): (1) phosphorylation by Hog1, (2) Hog1 binding and (3) DNA binding at the promoter of its target genes. It was recently described that the Hot1 basic sequence between amino-acids 381-385 (KRRRR, KR4) is the docking site for the Hog1 MAPK and is required for the functionality of the HOG pathway (Gomar-Alba *et al.*, 2013). This region does not contain Hog1 phosphorylation sites and actually this post-translational modification is not necessary for the activation of Hot1 target genes (Alepuz *et al.*, 2003). All this data together suggests that the interaction itself between Hot1 and the MAPK is needed for the transcription of the Hot1-regulated genes (Gomar-Alba *et al.*, 2013). Once activated by Hog1, the recruitment of this transcription factor at the regulatory regions of stress responsive genes, targets the Hog1-RNA PolII complex to them (Cook and O'Shea, 2012; Nadal-Ribelles *et al.*, 2012).

Due to the relevance of the Hot1 transcription factor in the osmostress response it is interesting to understand how it interacts with the DNA. According to the sequence similarity

with the DNA-binding region of Gcr1 and Msn1, it has been suggested that its C-terminus region would carry out this function (Rep *et al*., 1999b; Krantz *et al*., 2006). It has also been described that the deletion of the region between the amino-acids 541-546 (EDDDD, ED5) prevents the binding of Hot1 to the promoter of target genes (Gomar-Alba *et al*., 2013). On the other hand, Cook and O'Shea (2012) by means of a bioinformatics search of 11 high confidence Hot1 binding peaks proposed as a candidate motif for Hot1 binding to its target genes the sequence TGGG(A)CAA(T)G. Recently, Bai *et al*. (2015) have identified a sequence in the promoter of *STL1* gene (positions -654 and -626) composed of two CATTTGGC repeats and a third similar repeat, which has been called HoRE (Hog1-Responsive Element). This sequence is essential for maximal Hog1-dependent and osmostress-dependent induction of the transcription of this gene and these authors found that recombinant Hot1 protein binds to it *in vitro*. This sequence, however, is not present in other promoters recognized by this transcription factor.

In this manuscript we demonstrate that the Hot1 DNA-binding domain is located in the C-terminal region of the protein, between positions 610-719. Besides we show *in vitro* and *in vivo* that it recognizes in its target genes variations of the sequence GGGACAAA. We describe how the coincidence between sequences found in the target promoters is related to the binding of this transcription factor in ChIP analyses.

MATERIALS AND METHODS

Yeast strains, plasmid constructions and growth conditions

Yeast strains used in this work are described in Supplementary Table 1. Growth of yeast strains was carried out in YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose) at 30°C with orbital shaking (200 rpm). The yeast cells carrying plasmids were grown in SC medium (0.17% (w/v) nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) *Drop out* mix without the selected amino acid or nucleobase, 2% (w/v) glucose). For strains with the *ade2* mutation (derived of W303-1a), adenine was added at a final concentration of 20 mg/mL. The solid medium also contained 2% (w/v) agar.

For most experiments, yeast cultures were kept in the exponential growth phase for 16 h. Then cells were exposed to the considered osmotic stress conditions, these being 0.4 M NaCl, 1 M sorbitol or 1.11 M (20% (w/v)) glucose. For this purpose, they were transferred to the corresponding YPD or SC-derived media containing the osmolyte and incubation continued for the time indicated in each experiment. YP20/SC20, YPS/SCS and YPNaCl/SCNaCl refer to media containing 20% (w/v) glucose, 1M sorbitol or 0.4M NaCl respectively.

The description of all the plasmids used in this work is included in Supplementary Table 2. pGEX-3X was used for the introduction of Hot1 or its truncated versions in frame with the Glutathione-S-transferase coding sequence. When required, site-directed mutagenesis was carried out using the "QuikChange Lightning Site-Directed Mutagenesis Kit" (Stratagene, La Jolla, USA), whose instructions were followed. The introduction of the desired sequences and their location were confirmed in all cases by sequencing. The oligonucleotides used for all the plasmid construction and confirmation steps are described in Supplementary Table 3.

Two different One-Hybrid assay systems were performed. The first one was carried out in W303-1a *hot1Δ* strain transformed with pGADT7-Rec (*Clontech*) derivatives carrying the Hot1 regions of interest in frame with the GAL4AD in N-terminal position under the control of the *ADH1* promoter. The same strain was transformed with a YEp356-derived plasmid containing the 600 pb of the *HGI1* promoter plus 72 pb downstream +1 before the *LacZ* reporter gene. Spotting β-galactosidase assays were carried out by plating appropriate dilutions in minimal medium containing X-Gal.

The second system was performed using *HIS3* as a reporter gene. The assay was done again in a *hot1Δ* mutant, and the Hot1 regions considered were cloned in frame with the Gal4AD in the pGADT7-Rec vector as described above. Putative sequence recognized by the transcription factor and flanking nucleotides up to 20 bp and the corresponding controls were cloned in the pFL98 plasmid to control the expression of the *HIS3* reporter gene, as described in Fernandes *et al*. (1997) and Amaral *et al*. (2013). *STL1* and *HGI1* sequences were chosen to complete these sequences to their final lengths. Transcription activation ability was determined in this case by dilution plating assays on SC+ his, SC-his and SC-his containing 1 mM 3-amino-1,2,4-triazole (3AT) plates. For this purpose, 5 μL of the 10-fold serial dilutions of cultures (10^6 cells/mL starting concentration) were spotted onto these plates and incubation was carried out at 30°C for 3 days. In some experiments NaCl 0.4 M was added to the plates to test Hot1p transcription activation ability under osmostress conditions.

Microarray experiments

To determine genes regulated by the Hot1 transcription factor, microarray analysis were carried out with the W303-1a wild-type strain and its corresponding *hot1Δ* deletion mutant. For this purpose cells of both strains were maintained in exponential phase in YPD medium for 16 h. Afterwards cells were transferred to YP20 or YPS media. 30 min later cells were collected, washed and frozen at -80°C. These samples were used for RNA isolation, quantification and cDNA preparation, following the protocols explained elsewhere (Jiménez-Martí *et al*. 2011).

For the microarray experiments the strategy described in Jiménez-Martí *et al*. (2011) was adopted. In this case, the Cy3-Cy5 combinations for hybridization were: YPD.2-YPS.2, YPS.3-YPD.3, YPD.2-YP20.2, YP20.3-YPD.3, YPD.Δ2-YPS.Δ2, YPS.Δ3-YPD.Δ3, YPD.Δ2-YP20.Δ2 and YP20.Δ3, YPD.Δ3. Reproducibility of replicates was tested by the ARRAYSTAT software (Imaging Research Inc., Ontario, Canada). The entire set of supporting microarray data has been deposited in the GEO (Gene Expression Omnibus) database (http://www.ncbi.nlm.nih.gov/projects/geo/) with accession numbers GSE59659 and GSE68816.

ChIP analyses

Cells from an exponential growth culture (OD_{600} 1) expressing HA-Hot1 or its truncated versions were incubated for 10 min under the selected stress condition (0.4 M NaCl). A control culture was kept for the same time in SC. Cells were cross-linked with 1% (v/v) formaldehyde for 15 min at room temperature and were then incubated for 5 min with 125 mM glycine before being collected, washed with TBS buffer (20 mM Tris-HCl (pH 8.0), 0.5 M NaCl) and frozen. Cells were

resuspended in 300 μL of lysis buffer (50 mM HEPES-KOH pH 7.9, 40 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0,1% (w/v) sodium desoxicholate, 1 mM PMSF, 1 mM benzamidine and the *Complete Mini* protease inhibitor (Roche)), and 300 μL of glass beads were added. Cells were lysed for 30 min at 4°C in *Genie-2* (Scientific Industries). Chromatin was then fragmented by sonication in a *BioRuptor Diagenode* and the sample was centrifuged at 12000 rpm for 15 min.

To analyze Hot1 binding to chromatin, the cell extract was incubated with orbital rotation for 2 h at 4° with *Dynabeads Protein G* (Invitrogen) previously bound to an HA 3F10 antibody (*Roche*). Beads were then washed 3 times with 200 μL of PBS (150 mM NaCl, 40 mM Na$_2$PO$_4$, 10 mM NaH$_2$PO$_4$) containing 0.02% (v/v) Tween 20, and then once more with 100 μL of the same buffer. Elution was carried out twice with 40 μL of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% (w/v) SDS, by heating at 65°C for 2 min at 600 rpm. Crosslinking was reverted by overnight incubation at 65°C with shaking. The eluted sample was digested for 90 min at 37°C with 0.33 mg/mL proteinase K and DNA was purified with the *High Pure PCR product purification kit* (*Roche*).

Co-immunoprecipitated DNA was analyzed in triplicate by Real Time RT-PCR in a *DNA Engine Peltier Termal Cycler* (Bio Rad) using the *SYBR® Premix Ex Taq^{TM} Tli RNase H Plus Green with ROX* (Takara). Amplifications of the promoter region of the genes considered were carried out with the combinations of oligonucleotides described in Supplementary Table 3, which also includes Int A/B, which were used for an intergenic region. Data were processed with the ΔΔCT method (Schmittgen and Livak, 2008) by comparing the results of the amplified from the immunoprecipitated sample (IP) with those of the whole cell extract (*Input*), and by using the intergenic region for normalization.

Preparation of protein extracts and purification of GST-fusion proteins

Protein extracts were prepared for routine analyses according to the procedure described in Gomar-Alba et al. (2013). For the detection of HA-tagged proteins by Western analysis anti-HA 3F10 peroxidase was used (Roche) (dilution 1:10000 in TBS, 0.01 % (w/v) Tween 20).

Cells from the *E. coli* BL21(DE3) strain transformed with the pGEX-3X derived plasmids were used for the purification of selected GST-fusion proteins. The expression of these proteins was induced by treating the transformed cells with 0.1 mM isopropyl-β-D-thiogalactoside overnight at 16°C. The GST recombinant proteins were purified by glutathione-Sepharose column chromatography according to the manufacturer's instructions. Elution was carried out with 30 mM reduced glutathione after a 20-min incubation at room temperature.

Electrophoretic mobility shift assays

EMSA assays were carried out as described in Amaral *et al.* (2013) using total protein extracts or GST-purified proteins and 10% non-denaturing polyacrylamide gels in TBE 0.5X run at 100V. The only modification was the use of fluorescein-labelled oligonucleotides. Detection of the bands was done using blue light excitation from STORM860 (Molecular Dynamics).

In silico approaches

1000 bp of the promoter region of genes regulated by Hot1 were considered in order to carry out an analysis of putative sequences that could be target of this transcription factor. For this purpose we looked for "words" of 4 to 8 letters which appear in the genes described above with at least one repeat (to consider the possibility of the transcription factor recognizing two binding sites).

A well-documented and curated database of models of *S. cerevisiae* transcription factors binding site was used (http://stormo.wustl.edu/ScerTF/). This database contains 1226 matrices from eleven different sources, covering 196 different transcription factors for this yeast. Each candidate word was aligned against all the models in the database to search for that showing the highest score. Each position weight matrix represents a model or family of sequences. A score is obtained when a query sequence is aligned against each model using a function of the R package Biostrings (H. Pages, P. Aboyoun, R. Gentleman and S. DebRoy, Biostrings: String objects representing biological sequences, and matching algorithms. R package version 2.30.1.). A high score indicates that the sequence is a good match to the position weight matrix. However, since transcription factors can bind degenerate sequences with a range of scores, a recommended optimal cutoff is used when scoring a sequence to discriminate potential regulatory sequences from the background genomic sequences.

Those recommended optimal cutoffs were identified for each position weight matrix providing the greatest discriminative power to distinguish between bound and unbound sequences in available ChIP experiments. For each weight matrix, a search was done over a range of cutoff values to use in predicting whether a given probe would be bound by a transcription factor or not, and selected the cutoff that gave the most significant *p*-value in a Fisher's exact test. Each candidate word was finally associated to a "final score" which corresponds to the difference between the alignment initial score between the word and the model and the recommended score cutoff associated to the model.

RESULTS

The region between the amino acids 610-719 is the Hot1 DNA-binding domain

As a first approach for the determination of the DNA-binding domain of Hot1, chromatin immunoprecipitation (ChIP) assays were carried out. For this purpose, a *hot1Δ* strain transformed with centromeric plasmids containing the wild-type sequence of Hot1 with or without the ED5 domain and the putative DNA-binding domain (DBD) fused to HA epitope were used. The region chosen for the DBD corresponds to the C-terminus of Hot1p (between positions 610-719) that shows homology with the DNA-binding domain of the Gcr1 and Msn1 transcription factors (Fig 1A; Rep et al., 1999b; Krantz et al., 2006). For these experiments binding to the promoter (between positions -360 and -181) of *HGI1*, one of the genes activated by Hot1 (Gomar-Alba et al., 2012), was considered. As binding of Hot1 to this region is only detected under stress conditions, exponentially growing cells were exposed for 10 min to salt osmostress (NaCl 0.4 M). The ChIP results (Fig 1B) demonstrate that the ED5 and the putative DNA-binding domain are each one essential for the binding of the transcription factor to DNA.

4

A

B C D
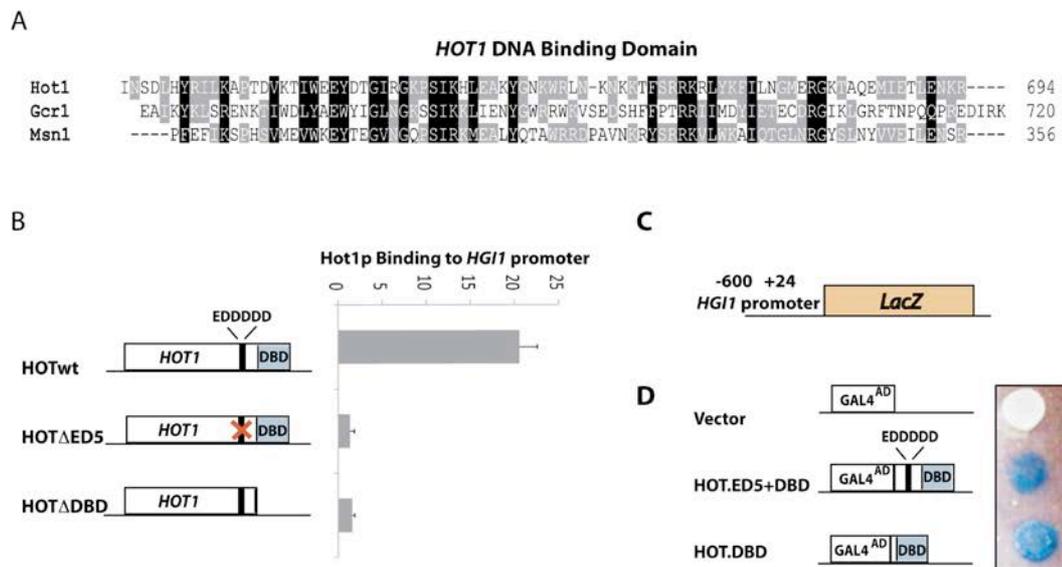
Figure 1. The DNA binding domain of the transcription factor Hot1 is located between positions 610-719. Panel A: sequence alignment between the C-terminal regions of Hot1, Gcr1 and Msn1 proteins. Panel B: ChIP analysis showing the effect of the deletion of ED5 region or the sequence between 610 and 719 (DBD) of Hot1 on its binding to *HGI1* promoter. HA antibody was used for the immunoprecipitation. For PCR, oligonucleotides described in Supplementary Table 3 were used. Experiments were carried out in triplicate; average and standard deviation are shown. Panel C: scheme showing the Yep356-derived construct made for the analysis of the involvement of these elements on the ability to activate transcription by a one-hybrid assay. D: result of the spotting β-galactosidase assay in the *hot1Δ* strain transformed with the plasmid shown in panel C and pGADT7-Rec derived plasmids containing the regions of interest of Hot1.

To further determine the involvement of the ED5 and DBD elements for the interaction with DNA, One-Hybrid assay was performed in a *hot1Δ* strain with two regions of the C-terminus of the protein that were subcloned in the pGADT7-Rec vector, in frame with the GAL4AD. The first one, from the amino acids 534 to 719, includes de acidic region ED5. The second one has only the putative DNA Binding Domain (residues 610 to 719) and hence it does not include the ED5 element. For this assay the same strain was transformed with a YEp356-derived plasmid containing the whole promoter of the *HGI1* gene before the *LacZ* reporter gene (Fig1C). According to the ChIP results described above (Fig1B) all the elements required for Hot1 binding to this promoter are present in this region. The results of the β-gal spot assays made in X-Gal plates (Fig1D) clearly demonstrate, for the first time, that the Hot1 DNA-binding domain comprise the region between amino acids 610-710. As the β-galactosidase activity was indistinguishable between the strain carrying the plasmids with the sequences 534-719 and 610-719, the ED5 element or any sequence inside the region 534-610 is not required for Hot1 binding to the DNA, although considering the results obtained in the ChIP analyses (Figure 1 Panel B) it cannot be ruled out that ED5 may be required for binding efficiency or affinity.

In silico analysis for the determination of the Hot1 target sequences in the DNA

In this work we started our approach for the determination of the Hot1p-binding site by the identification of genes whose expression is regulated by this transcription factor. For this purpose microarray analyses were carried out with both wild type and *hot1Δ* strains under unstressed exponential cell conditions and after a 30 minute-exposure to 1 M sorbitol or 1.11 M (20 % (w/v)) glucose. Table 1 shows the genes for which two criteria can be applied: i) those genes upregulated under these osmostress conditions and ii) where upregulation is reduced more than 2-fold in the *hot1Δ* strain under at least one of these conditions. In the case of *GPD1, DIA3, STL1, NQM1* and *HGI1* the effect of the deletion of *HOT1* gene was found under both sorbitol and glucose stress. It is worth mentioning that other genes were identified in this analysis but have not been further considered because they have functions unrelated with stress response or are putative proteins of unknown function (*ECM12, FMP45* and *FMP48*).

For further analyses, *HSP12* gene has also been considered because results from Capaldi et al. (2008) indicate that its expression is affected in a high extent by this transcription factor under salt stress (0.27 of expression value in the mutant strain compared with the wild type). It is worth mentioning that these authors found results consistent with ours for several of the genes included in Table 1: *GPP2* (ratio of 0.17), *SPI1* (0.24), and *GRE1* (0.33). *CTT1* has also been included because binding of Hot1 to its promoter has already been demonstrated by other authors (Alepuz et al. 2001).

Previous data have demonstrated binding of Hot1p to the promoter of *GPD1, HSP12, STL1* (Alepuz et al., 2001) and *HGI1* genes (Gomar-Alba et al., 2012).

Table 1. Genes induced under osmotic stress conditions that are regulated by Hot1 transcription factor according to our microarray analyses

Gene	ORF	$hot1\Delta$/WT 1.11 M glucose (1)	$hot1\Delta$/WT 1 M sorbitol (2)	Function (3)
GPD1	YDL022W	0.23	0.18	NAD-dependent glycerol-3-phosphate dehydrogenase
DIA3	YDL024C	0.25	0.40	Protein of unknown function; involved in invasive and pseudohyphal growth
STL1	YDR536W	0.03	0.11	Glycerol proton symporter of the plasma membrane
GPP2	YER062C	n.d	0.42	DL-glycerol-3-phosphate phosphatase involved in glycerol biosynthesis
SPI1	YER150W	2.10	0.43	GPI-anchored cell wall protein involved in weak acid resistance
NQM1	YGR043C	0.13	0.28	Transaldolase of unknown function; transcription is repressed by Mot1p
HGI1	YHR087W	0.39	0.39	Stress response protein involved in RNA metabolism and translation
GRE1	YPL223C	0.78	0.42	Hydrophilin essential in desiccation-rehydration process

(1) Ratio between average value of the induced expression in 1.11 M glucose for the $hot1\Delta$ and wild type strains
(2) Ratio between average value of the induced expression in 1M sorbitol for the $hot1\Delta$ and wild type strains
(3) According to the information available in *Saccharomyces Genome Database* (SGD, http://www.yeastgenome.org/)

To determine under the same experimental conditions the binding of Hot1 to the promoter of these genes and to those identified in the microarray experiments, ChIP analyses were carried out. For this purpose, the wild type strain FY86 was transformed with a centromeric plasmid that contains an HA-tagged version of Hot1 expressed under the control of its own promoter (pRS313-Hotwt-HA). Exponentially growing cells were left untreated (control) or exposed to salt osmostress (NaCl 0.4M) for 10 minutes. After cell fixation with formaldehid 3%, the ChIP assay was performed as described in Materials and methods. As shown in Figure 2, binding was detected in all cases although with important differences.

1000 bp of the promoter region of all these genes were considered in order to carry out an analysis of putative sequences that could be target of these transcription factors, using the bioinformatic approach described in the Materials and methods section. To punctuate the words found, two features were considered. By one hand the similarity to the recognition sequences of other yeast transcription factors and by the other hand the similarity to the consensus sequence proposed by Cook and O'Shea (2012): TGGG(A)CAA(T)G. Table 2 shows the list of sequences found in this analysis with final pwm score value higher than 0.8, and complete data is included in Supplementary Table 4. This value was obtained by aligning each candidate word to a particular PWM (Position Weight Matrix) model specially designed to capture the features of that proposed by Cook and O'Shea (2012); with this model the maximum value would be 1 and a value greater or equal to 0.8 would mean that the word is very similar to the consensus sequence proposed by these authors. As expected, hence, this list includes several sequences containing part of the consensus proposed by Cook and O'Shea (2012).

According to the strategy used to punctuate the sequences, some of the sequences display a high degree of similarity to others that appear in databases as binding sites of other transcription factors. For instance, the first two words of the table (GGGACAAC and GGGACAAA) and a few others with lower pwm score resemble the motifs found for Msn1 by Fordyce *et al.* (2010) by means of microfluidic affinity analysis. It is worth mentioning that GAGACAA appeared in all the gene promoters considered.

This list provides, hence, several sequences that could be tested for putative binding sites for Hot1 in order to carry out further experiments.
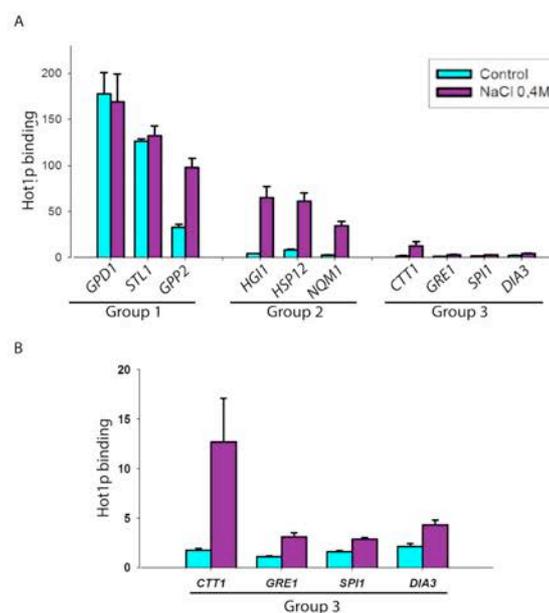
Figure 2. Binding of Hot1 to genes controlled by this transcription factor under non-stress and osmostress conditions (NaCl 0.4 M) depends on the particular genes considered. ChIp analyses were carried out using HA antibody and in the PCR analysis oligonucleotides described in Supplementary Table 3 were used. The experiments were carried out in triplicate; the average and standard deviation data are shown. Panel A shows the results obtained for all the genes considered in the same Y scale. In Panel B the results for Group 3 genes are again included with the scale enhanced for a better observation of the data.

Table 2. Putative DNA-binding sequences according to the bioinformatics analyses presented in this work

word	% seqs (1)	Median (2)	Model (3)	Score. ScerT (4)	rec.score. ScerTF (5)	final.ScerTF (6)	pwm.score (7)
GGGACAAC	36.36	319.50	fordyce.*MSN1*	5.46	6.03	-0.57	0.9990
GGGACAAA	45.45	80.00	fordyce.*MSN1*	6.05	6.03	0.02	0.9970
GGGCCAAA	36.36	200.50	macisaac.*PDR1*	5.22	10.64	-5.42	0.9846
GAGACAAG	36.36	142.50	morozov.*CHA4*	3.79	8.98	-5.19	0.9376
GAGCCAAA	27.27	31.00	macisaac.*PDR1*	3.47	10.64	-7.17	0.9222
GGGACAA	90.91	56.50	fordyce.*MSN1*	6.04	6.03	0.01	0.8987
TGGGACAA	45.45	146.00	fordyce.*MSN1*	6.04	6.03	0.01	0.8987
GGGGACAA	45.45	179.00	zhao.*YGR067C*	6.05	4.68	1.37	0.8987
GGGCCAA	45.45	57.00	fordyce.*NRG2*	5.99	7.04	-1.05	0.8863
GCGGAAAG	9.09	195.00	zhu.*RDR1*	8.43	9.05	-0.62	0.8783
GGACAAG	63.64	37.00	fordyce.*MSN1*	5.67	6.03	-0.36	0.8649
GAGGAAAA	72.73	86.50	badis.*EDS1*	6.81	7.79	-0.98	0.8645
GGACAAA	90.91	64.00	fordyce.*MSN1*	5.85	6.03	-0.18	0.8619
GGAAAAAG	72.73	101.00	macisaac.*RGT1*	8.74	10.89	-2.15	0.8598
GGCCAAA	63.64	32.00	macisaac.*PDR1*	6.27	10.64	-4.37	0.8495
CGAGACAA	18.18	20.00	zhao.*RDS2*	4.14	4.51	-0.37	0.8363
GAGACAA	100.00	153.00	harbison.*MOT2*	4.78	8.05	-3.27	0.8363
AGGACAAA	45.45	254.00	fordyce.*MSN1*	5.97	6.03	-0.06	0.8325
GGGTCAG	36.36	69.00	macisaac.*ASH1*	5.66	9.29	-3.63	0.8228
GGGTAAA	90.91	87.50	macisaac.*STB2*	7.91	6.01	1.90	0.8189
CGTCAAG	72.73	317.00	macisaac.*SKO1*	6.47	8.58	-2.11	0.8036
GCAACGAA	18.18	23.00	harbison.*MOT2*	7.15	8.05	-0.90	0.8008

(1) "pct,of,seqs,with,at,least,one,pair" refers to the percentage of promoter sequences considered in this work that contain at least two repeats of the sequence in any strand and orientation.

(2) "median.dist.between.one.pàir" is the median (for all the sequences in which it is observed) of minimal separation between the two repeats of a given word.

(3) "model" indicates the model of S. cerevisiae transcription factors showing the highest score. The author and the gene showing a good match is indicated.

(4) "score.ScerTF" is the score provided by aligning the input sequence against that position weight matrix model showing the best match. A high score indicates that the sequence is a good match to the position weight matrix.

(5) "recommended.score.ScerTF" contains the list of recommended optimal cutoffs to use when searching for potential regulatory sequences.

(6) "Final.ScerTR" shows the value corresponding to the difference between the initial alignment score between the word and the optimal model, and the "recommended score cutoff" associated to that model. A negative value indicates that the word is not similar to the model or the similarity is not believable. A positive value, however, is not sufficient for consider similarity and it is also needed a high alignment score between the word and the model.

(7) "pmw.score" refers to the punctuation resulting of the alignment between the word and the model proposed by Cook and O'Shea (2012).

The GGGACAAA sequence is the Hot1 UAS

According to the *in silico* results, two of the sequences shown in Table 2 were chosen to initially test both *in vivo* and *in vitro* if they are Hot1 real targets. Besides the pwm.score obtained we considered interesting to focus in sequences that are located in the promoter of the genes used in previous studies in our research group. In this sense, second sequence in the list (GGGACAAA) is present, for instance, in the *STL1* promoter in the position -173 to -180, while the sixth one (GGGACAA) is located in *HGI1* in the positions -318 to -323. Another sequence not found in the list was also considered (AGAATAAA), which can be found also in *HGI1* gene (between -272 to -280).

In order to test if Hot1 is able to bind these sequences *in vivo*, one-hybrid assay was performed, using *HIS3* as a reporter gene. In this system, each one of these three sequences and flanking nucleotides up to 20 bp were cloned in the pYAP-site plasmid to control the expression of the *HIS3* reporter gene.

STL1 and *HGI1* sequences were chosen to complete these sequences to their final lenghts. The assay was done in a *hot1Δ* mutant, and the same truncates described above (Fig 1D) were used, after being cloned in frame with the Gal4AD.

As a control in the one-hybrid assay the DNA-binding domain of the transcription factor Yap8 and its target in gene promoters (Y8RE) were considered. The Yap8 Binding domain has been recently described (Amaral *et al.*, 2013) and Ilina *et al.* (2008) identified the Yap8 Response Element (Y8RE) in the DNA, TGATTAATAATCA, present in *ACR2* and *ACR3*, the only genes regulated by this transcription factor. Since Yap8 has a very restricted DNA binding specificity, the corresponding domain of this protein was also cloned in frame with the Gal4AD in pGADT7-Rec and the Y8RE and flanking sequences as the only regulatory sequences in the *HIS3* plasmid, as described in Amaral *et al.* (2013).

The *HIS3* reporter gene under the control of each one of the putative consensus sequences for Hot1 binding was integrated

by recombination in the *hot1Δ* strain genome and the resulting strains were transformed with the pGAD vectors. Supplementary Figure 1 describes several control analyses carried out with these strains. First of all it was confirmed that all the strains expressed the corresponding proteins (panel A) and were capable to grow, and in a very similar degree, in SC plates (panel B). According to this, none of the pGAD constructs, in which expression is controlled by the *ADH1* promoter to allow a stable level of transcription, is toxic for the yeast cells. On the other hand, the positive control gives the expected result, as the DNA-binding domain of Yap8 binds the *ACR3* sequence TGATTAATAATCA (panel C in

Supplementary Figure 1 and panel A in Figure 3) allowing growth in SC-his medium. Finally, none of the Hot1 constructs was able to recognize this sequence (panel C) in the Supplementary Figure 1.

The one-hybrid spot assay with the putative Hot1 target sequences (Fig 3A) indicated that the strains expressing Hot1 DNA-binding domain fused to GAL4AD and containing the plasmid in which the sequence GGGACAAA controls *HIS3* expression are able to grow in SC- his plates. This result demonstrates that the second sequence on Table 1, GGGACAAA, that is present in *STL1*, is recognized by Hot1 DNA-binding domain, with or without ED5 element, and
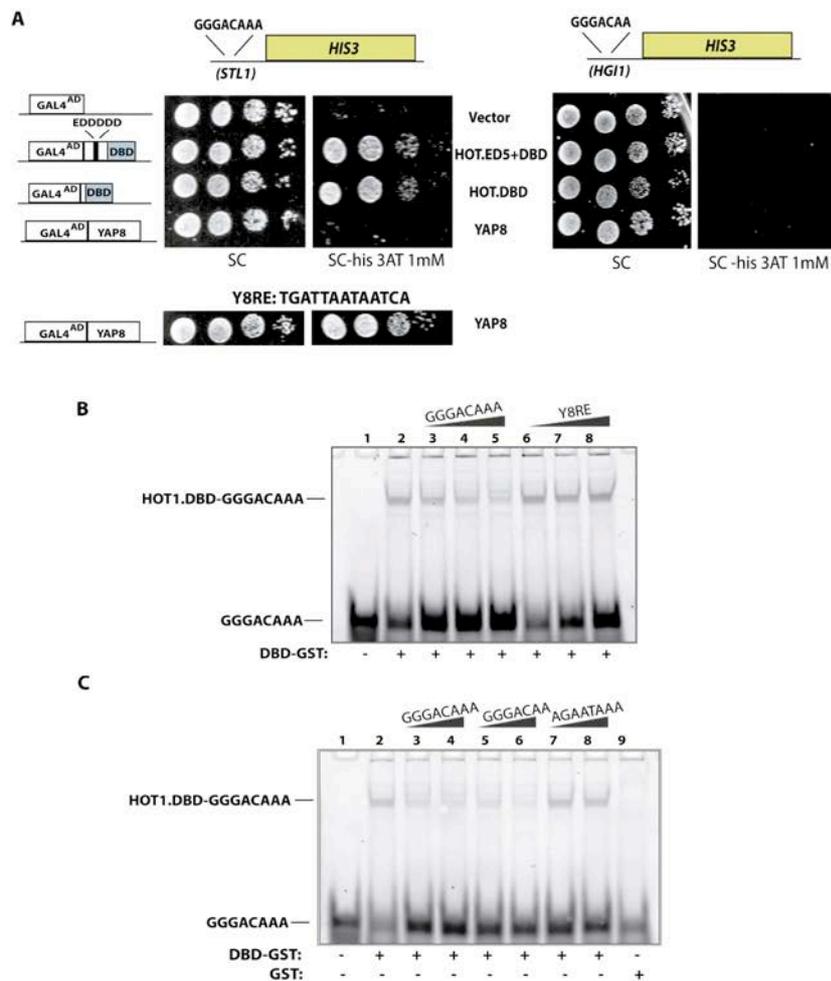
Figure 3. Hot1 binds both *in vitro* and *in vivo* to the sequence GGGACAAA of the *STL1* gene promoter. Panel A: one-hybrid assays using *HIS3* as a reporter gene. Analysis was carried out in a *hot1Δ* strain carrying the Hot1 regions indicated in frame with Gal4AD in pGADT7-Rec vectors and sequences GGGACAAA (in *STL1* promoter), or GGGACAA (in *HGI1* promoter) in pFL98 plasmid. As a positive control, Yap8 DNA-binding sequence and its target (Y8RE element) were included in the assay. Ability to activate transcription was determined by dilution plating assays in SC-his 3AT 1 mM plates. Panel B: Demonstration of the binding to GGGACAAA by EMSA analysis. The Hot1 DNA-binding domain, expressed in *E. coli* as a GST fusion protein and purified by affinity chromatography was incubated with the fluorescence labeled oligonucleotides indicated in the Figure and increasing amounts of non-labelled oligonucleotides GGGACAAA and TGATTAATAATCA (YRE) were added. The protein-DNA complexes were separated from free DNA probe by polyacrylamide gel electrophoresis and a representative result of the gels obtained is shown. Panel C: EMSA analysis of the ability or not of GGGACAA and AGAATAA sequences to compete with GGGACAAA for Hot1 binding. Experiments were carried out as in panel B but increasing amounts of the non-labelled oligonucleotides indicated were added.

corresponds, hence to the Hot1 Upstream Activation Sequence (UAS) for the binding of this transcription factor. These results reinforce that ED5 element is not essential for Hot1 to bind to its target sequence. In this assay, Hot1 was not able to bind any of the two *HGI1* sequences considered, GGGACAA or AGAATAAA, even in the absence of 3AT (Supplementary Figure 1D). The same growing pattern was obtained in plates with and without NaCl 0.4M (data not shown).

In order to determine if Hot1 is capable to bind the sequences analyzed above *in vitro*, Electrophoretic Mobility Shift Assays (EMSA) were carried out with the GST-tagged Hot1 DNA-binding domain (amino acids 610 to 719) expressed and purified from *E. coli* BL21 strain, as described in the Materials and Methods section. The GGGACAAA sequence, with the *STL1* flanking nucleotides up to 20 bp was labeled with fluorescein. The shift in the DNA mobility observed in Figure 3B lane 2, when compared to lane 1 demonstrate that *in vitro* the Hot1 DBD without ED5 is again enough to bind DNA. Moreover, when the same non-labelled sequence is added in increasing concentrations to the reaction mix (lines 3 to 5) as a competing DNA, the shift progressively reduces, while when a non specific DNA (20 bp of the *ACR3* sequence, containing the Y8RE element, lanes 6 to 8) is added at the same concentrations, it does not compete and the shift observed is the same than in lane 2.

It has been previously demonstrated that Hot1 regulates the stress response gene *HGI1* and binds its promoter (Gomar-Alba *et al.*, 2012, 2013). As commented above, according to the bioinformatic analysis carried out in this work, *HGI1* contains in the promoter two possible sequences for Hot1 binding, GGGACAA and AGAATAAA. Interestingly, none of them is recognized by Hot1 in the one hybrid assay used in this report. These two sequences were used as competing with GGGACAAA in the EMSA assays (Figure 3, panel C). Binding of Hot1 to this sequence was progressively decreased when GGGACAA was included (lanes 5 and 6), while it was unaffected by the addition of AGAATAAA (lanes 7 and 8). According to these results, at least *in vitro*, Hot1 can also recognize this sequence, identical to the previously found Hot1 UAS except for the absence of the last adenine nucleotide, which in this gene is substituted by thymine.

The sequence recognized by Hot1 defines the particularities of binding

The results described so far in this work indicate that the DNA-binding domain of Hot1 recognizes the sequence GGGACAAA both *in vivo* and *in vitro* and also the sequence GGGACAA *in vitro*. However these sequences are not present in all the genes regulated by this transcription factor. Figure 4 shows the mapping of the sequences GGGACAAA and GGGACAA with a maximum of one mismatch not corresponding to insertion or deletion in the promoter region of the genes considered in this work. Table 3 summarizes the data about location of GGGACAAA or similar Hot1 target sequences with up to two mismatches without considering insertions and deletions.

A close inspection to this information and to the result of the experiment shown in Figure 2 (data of the ChIP analyses carried out to demonstrate Hot1 binding to their promoters) indicates that the genes considered can be distributed into the three groups indicated in Table 3. Genes in group 1 contain the sequence GGGACAAA in their promoter, Hot1 recognizes it both under stress and non-stress conditions and the binding of
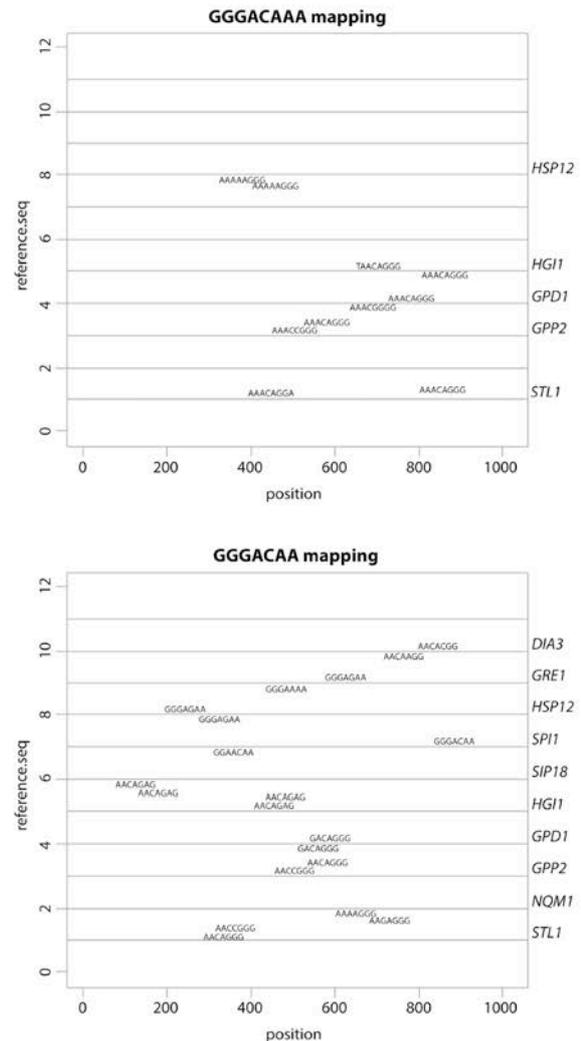
Figure 4. GGGACAAA and GGGACAA mapping in the promoter of the genes considered in this work. No indels and at most one mismatch have been allowed.

Table 3. Hot1 target sequences in the promoters of genes regulated by this transcription factor

	Gene	Secuence	Position
Group 1	*GPD1*	GGGACAAA	-247
	STL1	GGGACAAA	-173
	GPP2	GGGACAAA	-450
Group 2	*HGI1*	GGGACAAT	-318
	HSP12	GGGACAAG	-514
	NQM1	GGACAAAT	-350
Group 3	*CTT1*	Several putative sequences with two mismatches	
	GRE1		
	SPI1		
	DIA1		

this transcription factor is high. For genes in group 2 the sequence resulting from one mismatch in the last position of this sequence (GGGACAA) can be detected, and Hot1 recognizes them only under osmotic stress, being the binding ability lower. Finally, in the case of group 3 genes the promoter contains sequences for the interaction with the transcription factor that show two mismatches when compared to GGGACAAA and the binding of Hot1 is very low (a relative value around 10 or lower) and only occurs under osmostress conditions.

To determine if binding to the GGGACAA sequence found in the *HGI1* promoter could be increased by its conversion to the *STL1*-like sequence GGGACAAA, an A nucleotide was introduced by site-directed mutagenesis after the element GGGACAA in the 600 pb of the *HGI1* promoter sequence cloned into Yep356R plasmid to control *lacZ* expression. As shown in Figure 5, ChIP analysis demonstrate that the binding of Hot1 to this promoter importantly increased both under stress and unstressed conditions, while no changes were found in the interaction of this transcription factor to natural *HSP12* and *NQM1* promoters.

According to these results, the affinity of Hot1 to bind to the promoter of its target genes depends on the similarity of the sequences to the consensus GGGACAAA and in those cases in which one or two mismatches are found, osmostress is required for this interaction.
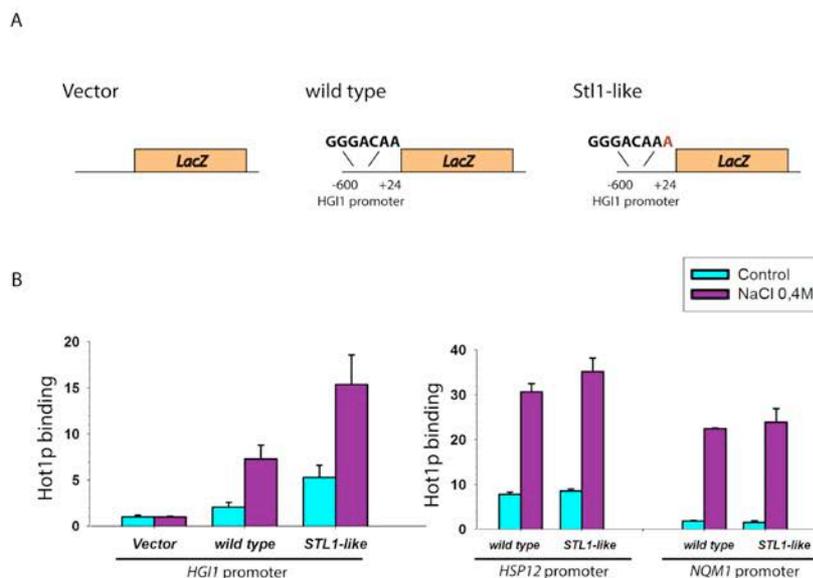
DISCUSSION

The response to osmotic stress in yeast is mediated by the Environmental Stress Response, in which transcription factors Msn2 and Msn4 are involved (Gasch et al., 2000; Martínez-Pastor *et al.*, 1996), and the HOG pathway (Hohman and Mager, 2003; Martínez-Montañés *et al.*, 2010; Saito and Posas, 2012). In this pathway the MAPK Hog1 regulates the transcription factors Hot1, Msn1, Smp1, Gcn4, Skn7, Sko1, Msn2/4 and Rtg1/3. Although the sequence recognized by

Msn2/4 proteins in their target promoters, the STRE sequence 5'-CCCCT-3' was identified a long time ago (Martínez-Pastor et al., 1996), the information about how the other above-mentioned factors interact with the DNA is incomplete or based on indirect evidences.

In this manuscript we provide new information about how Hot1 interacts with the DNA. First of all we demonstrate that the C-terminal region, between amino acids 610 and 719 is the DNA-binding domain of this transcription factor by means of one-hybrid (Figure 1) and electrophoretic mobility shift assays (Figure 3). *S. cerevisiae* transcription factors Msn1 and Gcr1, involved in stress response and activation of glycolytic enzyme genes, showed a pronounced similarity within a stretch of 64 residues, in which Hot1 and Msn1 exhibit 42% identity and 64% similarity, and Hot1 and Gcr1p 27% identity and 59% similarity (Rep et al., 1999b). Besides, the C-terminal region of Gcr1, between amino acids 690 and 844 constitutes the DNA binding of this transcription factors and recognizes the sequence TTTCAGCTTCCTCTAT in the promoter of glycolytic genes controlled by this transcription factor (Huie et al., 1992; Huie and Baker, 1996).

Gcr1 DNA-binding domain contains a possible helix-turn-helix motif between amino acids 784-803 (Baker, 1986). A more detailed analysis of the Hot1 DNA-binding domain (Krantz et al., 2006) have revealed that it may fold into four helices of nine residues arranged in a configuration of two helix-turn-helix motifs separated by a stretch rich in basic residues.

Results described in this manuscript also demonstrate that Hot1 binds to the 5'-GGGACAAA-3' sequence located in the promoter of the *STL1* gene in positions -173 to -180. Evidences for this conclusion have been obtained by one-hybrid and competing EMSA assays (Figure 3) and are in accordance with ChIp data (Figure 2), in which a larger region of the promoter of this gene including this sequence was considered. This element is consistent with that proposed by Cook and O'Shea (2012) for Hot1-binding, as expected according to the

Figure 5. Conversion of the sequence GGGACAA in the *HGI1* promoter to GGGACAAA increases Hot1 binding to the promoter of this gene both under control and stress conditions. ChIP experiments were carried out as described in Figures 1 and 2.

conditions established during the bioinformatic approach followed in this work to punctuate the sequences identified. Fordyce et al. (2010) presented a microfluidics-based approach for *de novo* discovery and quantitative biophysical characterization of DNA target sequences. One of the TFs considered to validate their technique by measuring sequence preference was Msn1p. Actually they identified an element for the binding of this transcription factor showing some similarity to the GGGACAAA sequence found in this work. It is worth to mention that Rep et al. (1999b) described some redundant or overlapping function between Hot1 and Msn1 that could be related to the similarities in the DNA-binding properties of both transcription factors.

Bai *et al.* (2015) have recently reported the identification of a new sequence element (the Hog1-Responsive Element, HoRE) in the promoter of the *STL1* gene, between positions -654 and -626. This element contains two 5'-CATTTGGC-3'

repeats and a third similar repeat. According to the data provided by these authors, it is required for the osmotic stress activation of this gene in a way Hog1 and Hot1-dependent and is recognized by the recombinant Hot1 protein *in vitro*. Curiously, the authors could not find this element in any of the other gene promoters to which this transcription factor binds or that are, at least partially, under its control, according to this work (Table 1 and Figure 2) and other data available in the literature (Alepuz *et al.*, 2001; Capaldi *et al.*, 2008; Cook and O'Shea, 2012). On the contrary, the bioinformatic analyses carried out in this work has allowed the identification of the sequence GGGACAAA in other two genes regulated by this transcription factor and involved in glycerol biosynthesis: *GPD1* and *GPP2*. In other target genes of this transcription factor (those included in Table 1 together to *HSP12* and *CTT1*) and considered in this work, similar sequences with one or two mismatches could be detected (Figure 4). Actually, one of

Figure 6. Hierarchical clustering based on Levenshtein distance applied to those candidate words with a pwm.score greater or equal than 0.65, a pct.of.seqs.with.at.least.one.pair greater or equal than 80%, a final.ScerTF lower than 1 and a median.dist.between.one.pair lower than 60. Both forward and reverse directions have been included in the analysis. An horizontal red line defines eight clusters at height 4.5. For each defined cluster are highlighted in color those words closer to height zero which can be interpreted as the representative words that better distinguish a cluster from each other. The closer are two given cluster in the figure the more similar they are in terms of indels and mismatches.

these sequences (GGGACAA), located in *HGI1* promoter has been shown to be able to compete with GGGACAAA for Hot1 binding *in vitro* in EMSA analyses (Figure 3). It is worth mentioning that the oligonucleotide used by Bai *et al.* (2015) for the EMSA analyses is very long and contains also the sequence TTTGGCCC, whose complementary is very similar (with the only difference of one mismatch) to the GGGACAAA found in this work, and was identified in our *in silico* analysis (in third position in the list shown in Table 2).

In many cases there are repeats of the sequences described here in the promoter of Hot1 target genes (Figure 4), which could be recognized by the two proposed helix-turn-helix motifs in this protein or by a dimeric form of the transcription factor, although further evidences would be needed to support this hypothesis.

A hierarchical clustering applied to representative words of Table 2 (Figure 6) revealed on the one hand that the central core of the model proposed by Cook and O'Shea (2012) GGACA remains unaltered in clusters 2, 5, 6 and 8 (numbered from left to right) in forward, forward, reverse and reverse senses respectively. It means that words in clusters 6 and 8 differ from the proposed model mainly by sense not by real nucleotide changes. On the other hand, the remaining clusters 1, 3, 4 and 7 preserve more the firsts and lasts positions than the core of the proposed model.

The ChIP analyses described in this work with Hot1-target genes show important differences in the binding particularities of this transcription factors. As shown in Figure 2, and in accordance with some previous data found on the literature (Alepuz et al., 2001), Hot1 binds to *STL1*, *GPD1* and *GPP2* promoters both under unstressed and osmotic stress conditions and at high levels. However, in the case of the other targets of this transcription factor binding occurs under hyperosmolarity although differences in the level of interaction can be found. The comparison between the similarity of the sequences identified in the promoters of these genes to that in *STL1* and the ChIP results has allowed us to take some conclusions. Hot1 was capable to bind *in vivo* strongly and under any condition when the sequence GGGACAAA is found (group 1 genes in Figure 2 and Table 3). However, when one or two changes in the sequence are present binding only occurred under hyperosmotic stress but with different intensities (groups 2 and 3). As a confirmation of this hypothesis, the addition of an A nucleotide at the 3' end of the sequence GGGACAA of the *HGI1* gene, to make it identical to that found in *STL1*, increased binding of the transcription factor, with or without the application of osmotic stress (Figure 5). Although previous studies carried out in other transcription factors, such as Haa1, has revealed differences in the *in vitro* affinity for the transcription factor to bind sequences differing in the nucleotide located in certain positions (Mira *et al.*, 2011) in our study we indicate that small sequence changes can affect the particularities of binding *in vivo*.

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Material suplementario
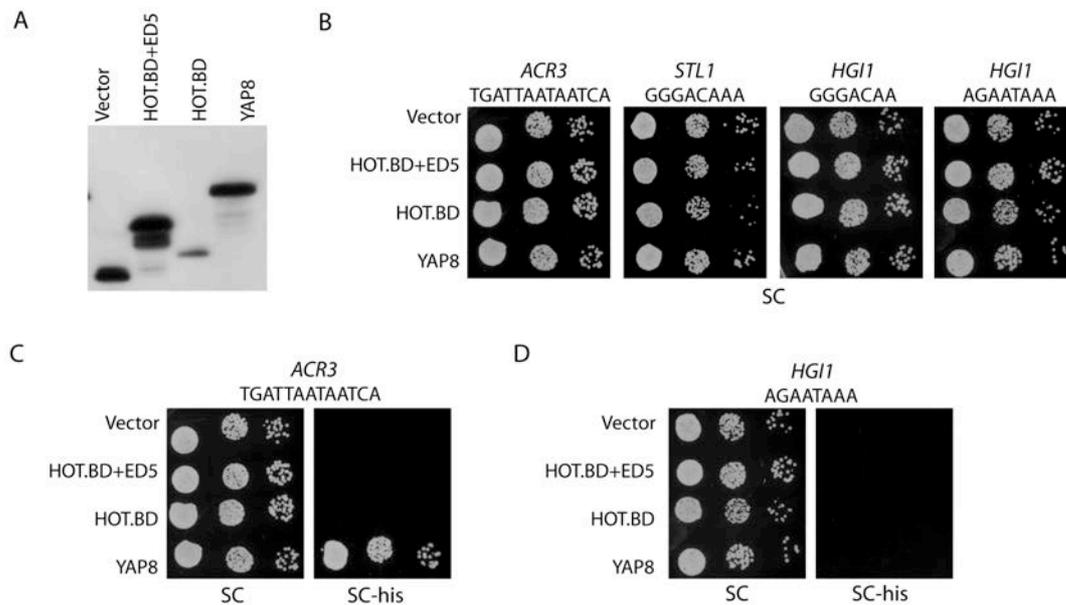
Figure 3.3.S1. Controls and additional analyses about the detection of the DNA binding sequence of Hot1. Panel A: all the fusion proteins made for this analysis are correctly expressed from yeast cells. Figure shows a Western blot analysis of whole cell extracts prepared from all the yeast strains considered. HA antibodies were used for the detection. Panel B: Strains used for one-hybrid assays with the *HIS3* gene as reporter do not show any growth defect in SC medium. Figure shows the result of a representative dilution plate assay in which 10-fold serial dilutions were applied on plates containing this medium. Panel C: Any of the Hot1 constructs is capable to bind the DNA binding domain of Yap8 in these one-hybrid assays. Panel D: Hot1 does not recognize the *HGI1* sequence AGAATAAA according to the results of these one-hybrid assays. Experiments were carried out as described in Figure 3 and the result of a representative experiment is shown
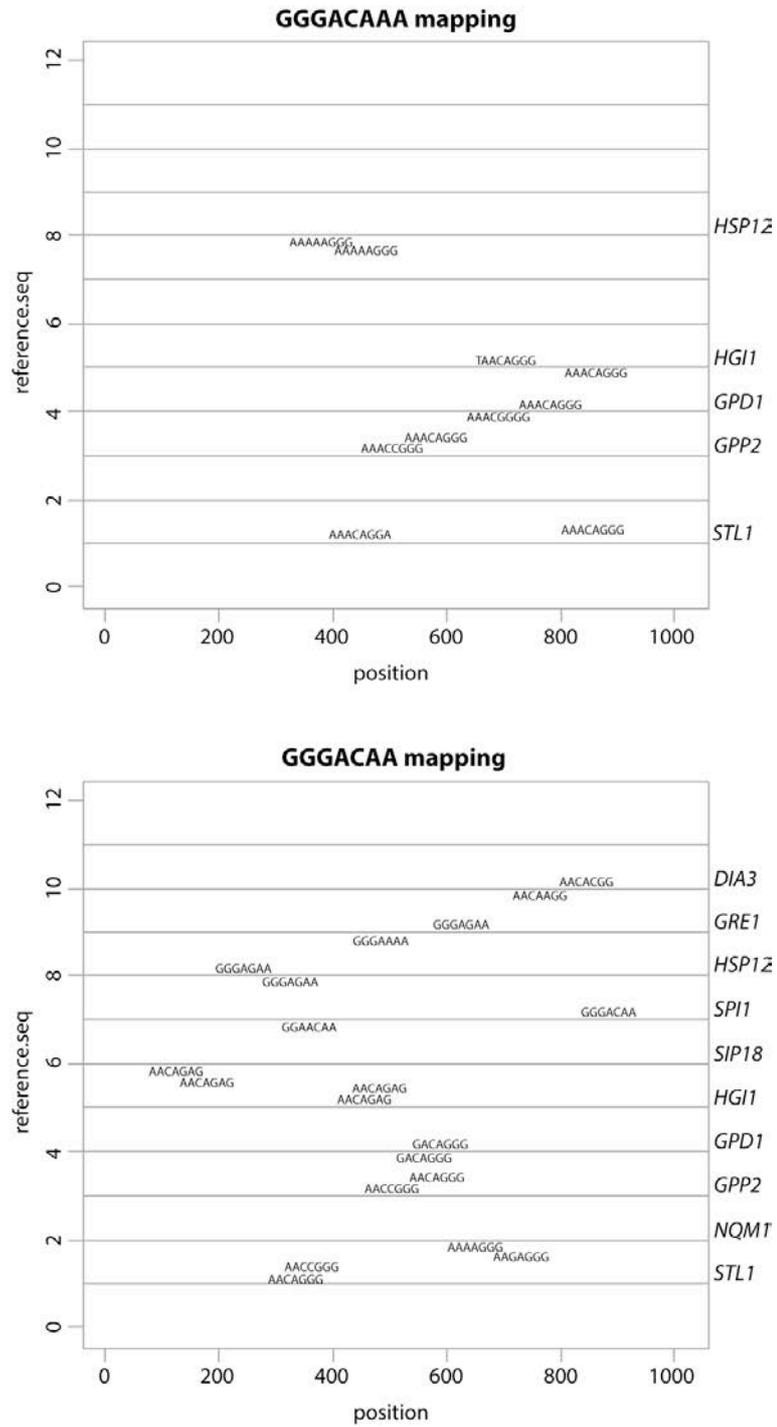
Figure 3.3.4[1]

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[1] Se muestra de nuevo ampliada la Figura para una mejor visualización. Por problemas de formato no se podía mostrar en un tamaño mayor dentro de la publicación.

Table 3.3.S1. Yeast strains used in this work

Strain	Description	Origin
FY86	*MATα leu2Δ1 ura3-52 his3Δ200*	Lab Stock
FY86 pRS313-HOTwt	*MATα leu2Δ1 ura3-52 his3Δ200* [pRS313-*HOT1*wt]	This work
FY86 pRS313-HOTBDΔ	*MATα leu2Δ1 ura3-52 his3Δ200* [pRS313-*HOT1*BDΔ]	This work
FY86 pRS313-HOTED5Δ	*MATα leu2Δ1 ura3-52 his3Δ200* [pRS313-*HOT1*ED5Δ]	This work
W303	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1*	Lab Stock
W303 *hot1Δ*	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4*	P.M Alepuz
W303 *hot1Δ* YEp356R.HGI1wt	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [YEp356R.*HGI1*wt]	This work
W303 *hot1Δ* YEp356R.HGI1wt pGADT7-Rec	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [YEp356R.*HGI1*wt] [pGADT7-Rec]	This work
W303 *hot1Δ* YEp356R.HGI1wt pGADT7-Rec HOT1.BD	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [YEp356R.*HGI1*wt] [pGADT7-Rec *HOT1*.BD]	This work
W303 *hot1Δ* YEp356R.HGI1wt pGADT7-Rec HOT1.BD+ED5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4* [YEp356R.*HGI1*wt] [pGADT7-Rec *HOT1*.BD+ED5]	This work
W303 *hot1Δ* pSTL1	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::stl1-his3*	This work
W303 *hot1Δ* pSTL1 pGADT7-Rec	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::stl1-his3* [pGADT7-Rec]	This work
W303 *hot1Δ* pSTL1 pGADT7-Rec HOTBD	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::stl1-his3* [pGADT7-Rec *HOT1*.BD]	This work
W303 *hot1Δ* pSTL1 pGADT7-Rec HOTBD+ED5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::stl1-his3* [pGADT7-Rec *HOT1*.BD+ED5]	This work
W303 *hot1Δ* pSTL1 pGADT7-Rec YAP8	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::stl1-his3* [pGADT7-*YAP8*]	This work
W303 *hot1Δ* pHGI1.1	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.1-his3*	This work
W303 *hot1Δ* pHGI1.1 pGADT7-Rec	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.1-his3* [pGADT7-Rec]	This work
W303 *hot1Δ* pHGI1.1 pGADT7-Rec HOTBD	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.1-his3* [pGADT7-Rec *HOT1*.BD]	This work
W303 *hot1Δ* pHGI1.1 pGADT7-Rec HOTBD+ED5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.1-his3* [pGADT7-Rec *HOT1*.BD+ED5]	This work
W303 *hot1Δ* pHGI1.1 pGADT7-Rec YAP8	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.1-his3* [pGADT7-Rec YAP8]	This work
W303 *hot1Δ* pHGI1.2	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.2-his3*	This work
W303 *hot1Δ* pHGI1.2 pGADT7-Rec	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.2-his3* [pGADT7-Rec]	This work
W303 *hot1Δ* pHGI1.2 pGADT7-Rec HOTBD	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.2-his3* [pGADT7-Rec *HOT1*.BD]	This work
W303 *hot1Δ* pHGI1.2 pGADT7-Rec HOTBD+ED5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.2-his3* [pGADT7-Rec *HOT1*.BD+ED5]	This work
W303 *hot1Δ* pHGI1.2 pGADT7-Rec HOTBD+ED5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::hgi1.2-his3* [pGADT7-Rec *YAP8*]	This work
W303 *hot1Δ* pACR3	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::acr3-his3*	This work
W303 *hot1Δ* pACR3 pGADT7-Rec	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::acr3-his3* [pGADT7-Rec]	This work
W303 *hot1Δ* pACR3 pGADT7-Rec HOTBD	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::acr3-his3* [pGADT7-Rec *HOT1*.BD]	This work
W303 *hot1Δ* pACR3 pGADT7-Rec HOTBD+ED5	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::acr3-his3* [pGADT7-Rec *HOT1*.BD+ED5]	This work

W303 *hot1Δ* pACR3 pGADT7-Rec YAP8	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4 XbaI::acr3-his3* [pGADT7-Rec *YAP8*]	This work
FY86 $P_{HGI1}::P_{SPI1}$	*MATα leu2Δ1 ura3-52 his3Δ200 PHGI1::PSPI1kanMX4*	This work
FY86 $P_{HGI1}::P_{SPI1}$ YEp356R pRS313-Hotwt	*MATα leu2Δ1 ura3-52 his3Δ200 PHGI1::PSPI1kanMX4* [YEp356R][pRS313-*HOT1*wt]	This work
FY86 $P_{HGI1}::P_{SPI1}$ YEp356R.HGI1wt pRS313-Hotwt	*MATα leu2Δ1 ura3-52 his3Δ200 PHGI1::PSPI1kanMX4* [YEp356R.*HGI1*wt][pRS313-*HOT1*wt]	This work
FY86 $P_{HGI1}::P_{SPI1}$ YEp356R.HGI1.STL1-like pRS313-Hotwt	*MATα leu2Δ1 ura3-52 his3Δ200 PHGI1::PSPI1:kanMX4* [YEp356R.*HGI1* STL1-like][pRS313-*HOT1*wt]	This work

Table 3.3.S2. Plasmids used in this work

Plasmid	Description	Origin
pYapsite	*URA3* integrative vector (*XbaI*) with *HIS3* as a reporter gene (*his3-Δ101,189* with Yap site inserted between *Eco*RI and *Bam*HI site as the only UAS of the *HIS3)*	Fernandes 1997
pHGI.1	*HIS3* reporter gene under the control of the *HGI1* GGGACAA promoter sequence cloned at the *BamHI EcoRI* sites in pYAPsite	This work
pHGI.2	*HIS3* reporter gene under the control of the *HGI1* AGAATAAA promoter sequence cloned at the *BamHI EcoRI* sites in pYapsite	This work
pSTL1	*HIS3* reporter gene under the control of the *STL1* GGGACAAA promoter sequence cloned at the *BamHI EcoRI* sites in pYapsite	This work
pACR3	*HIS3* reporter gene under the control of the *ACR3* TGATTAATAATCA promoter sequence cloned at the *BamHI EcoRI* sites in pYapsite	C. Amaral
pGADT7-Rec	*LEU2* micron yeast plasmid with *GAL4* activation domain (AD) under control of ADH promoter	Clontech
pGADT7-Rec HOT1.BD	Hot1$^{610-719}$ ORF cloned in frame with the *GAL4*^{AD} at the *BamHI NdeI* sites in pGADT7-Rec	This work
pGADT7-Rec HOT1.BD+ED5	Hot1$^{534-719}$ ORF cloned in frame with the *GAL4*^{AD} at the *BamHI NdeI* sites in pGADT7-Rec	This work
pGADT7-Rec YAP8	The Yap8 entire ORF cloned in frame with the *GAL4*^{AD}	C. Amaral
YEp356R	*URA3* multicopy shuttle vector modified with the *LacZ* reporter gene	Myers, Tzagoloff, Klinney, Lusty(1986)
YEp365R HGI1 wt	*LacZ* reporter gene under the control of the *HGI1* promoter cloned at the *Xba1 KpnI* sites in YEp356R	This work
YEp356R HGI1 STL1-like	YEp356R HGI1wt derivative obtained by an A addition after the GGGACAA sequence of *HGI1* promoter	This work
pGEX3X	Plasmid vector for the expression of proteins fused with GST	GE Healthcare
pGEX-3X HOT1.BD	Hot1$^{610-719}$ ORF cloned in frame with the GST tag at the *SmaI-BamHI* sites in pGEX3X	This work
pRS313-HA	*HIS3* centromeric yeast plasmid modified with 3XHA	M. Gomar-Alba
pRS313-Hotwt	The entire Hot1 ORF with its endogenous promoter fused to a 3xHA tag before the stop codon cloned in pRS313	M. Gomar-Alba
pRS315	*LEU2* centromeric yeast plasmid	Lab stock
pRS315-Hotwt	The entire Hot1 ORF with its endogenous promoter fused to a 3xHA tag before the stop codon cloned in pRS315	This work
pRS315-HotED5Δ	Hot1 E541 , D542, D543, D544, D545, D546 deleted from pRS315-Hotwt	This work
pRS315-HotBDΔ	The Hot1$^{1-610}$ ORF with its endogenous promoter fused to 3xHA tag before the codon stop cloned in pRS315	This work
pRS315-HOTprom	The HOT1 complete promoter cloned at the *SacI-XbaI* sites in pRS315	M. Gomar-Alba
pRS315-HOT$^{1-610}$	The Hot1$^{1-610}$ ORF cloned at the *BamHI-PstI* sites in pRS315-HOTprom	M. Gomar-Alba

Table 3.3.S3. Oligonucleotides used in this work

Oligonucleotide	Sequence (5' to 3')	Use
HGI1.2 Fw	GAT CCA TGT TTT TAT TCT CTA AGC TG	Annealing for *BamHI-EcoRI* pYapsite constructions
HGI1.2 Rv	AAT TCA GCT TAG AGA ATAAAA ACA TG	"
HGI1.1 Fw	GAT CCG CAA TTG TCC CTT TGG	"
HGI1.1 Rv	AAT TCG AAA GGG ACA ATT GCG	"
STL1 Fw	GAT CCC AAT TTT GTC CCC TTC AAG	"
STL1 Rv	AAT TCT TGA AGG GGA CAA AAT TGG	"
STL1-fluorescein Fw	CAA TTT TGT CCC CTT CAA	EMSA fluorescein assay, anneal with *STL1* Rv
Xba-HGI Fw	GCT CTA GAG AAT TCC TAC TCC GTG T	Cloning *HGI1* promoter in YEp356R at the *XbaI-KpnI* sites
Kpn-HGI Rv	GGG GTA CCA TTT TCA CCC TTG TAA A	"
HGImut Fw	GCC CAC GCA ATT TGT CCC TTT	*HGI1 STL1*-like construction by direct mutagenesis from YEp356R *HGI1*wt
HGImut Rv	AAA GGG ACA AAT TGC GTG GGG	"
Nde-HOT.1 Fw	GCC CCA TAT GTC TGG AAT GGG TAT TGC	Hot1 constructions in pGADT7-Rec at the *NdeI-BamHI* sites
Nde-HOT.534 Fw	GCC CCA TAT GGT CGA CGA TGA TGG GTA T	"
Nde-HOT.610 Fw	GCC CCA TAT GCA TTA TAG GAT ATT G	"
Bam-HOT.719 Rv	GGT GGA TCC CTA TAT TCC AGC AAG GCT CT	"
Bam-HotBD Fw	CGC GGA TCC ATT ATA GGA TAT TG	HotBD (610-719) construction in pGEX3x at the *BamHI-SmaI* sites
Sma-HotBD Rv	TCC CCC GGG TAT TCC AGC AAG GCT	"
HOTED5DEL 1	GATGATGGCTATCAAGGTGATGACGAAGG	*HOT1* ED5 mutagenesis (deletion) from pRS315-*HOT1*wt
HOTED5DEL 2	CATCACCTTCGTCATCACCTTGATAGCCATC	"
HOT-HA Sma Fw	TCC CCC GGG TCT GGA ATG GGT ATT GC	*HOT1* entire ORF with 3xHA amplified from pRS313-HOTwt for its cloning in pRS315-HOTprom at the *SmaI-PstI* sites
HOT-HA Pst Rv	AAA CTG CAG TCA ACC ACG CCA AAA CT	"
Hot HA TER Fw	AAA CTG CAG GGG ATA GGC GGC CGC	HOT 3'UTR with 3xHA amplified from pRS313-HOTwt for its cloning in pRS315-HOT[1-610] at the *PstI* site
Hot HA TER Rv	AAA CTG CAG ACC GCG GTG GAG CTC	"
GPD1 Fw	TTGCTTCTCTCCCCTTCCTT	PCR of *GPD1* promoter
GPD1 Rv	CAGCAGCAGCAGCACATCTTT	"
CTT1 Fw	AGGCACATGGGGATAGAACC	PCR of *CTT1* promoter
CTT1 Rv	GGGAGTCGGGGGCATTATCG	"
HSP12 Fw	GGGCGGCACAAAATAACATAG	PCR of *HSP12* promoter
HSP12 Rv	CGCAATTGAGGACGCAATTGAGGA	"
NMQ1 Fw	AGGGACTGGACTGTTAAC	PCR of *NMQ1* promoter
NMQ1 Rv	GTCCACCCCATTCTAGAT	"
GPP2 Fw	CTCAAGTATTTTGGCACC	PCR of *GPP2* promoter
GPP2 Rv	GCTTGTGTATTTAAGCGC	"
SIP18 Fw	GTGGACGGCTCATAGTGA	PCR of *SIP18* promoter
SIP18 Rv	CATGTTAGACATCTTGAC	"

SPI1 Fw	GCAAGTTACCTAATCTGG	PCR of *SPI1* promoter
SPI1 Rv	ACCTGGCAAAAGTAGGCC	"
GRE1 Fw	GGTAATTCTAGGACCACG	PCR of *GRE1* promoter
GRE1 Rv	AAGCACGTAACGGAGACC	"
DIA3 Fw	CCATCCTGAGTTTTTACC	PCR of *DIA3* promoter
DIA3 Rv	CATTGTTCCTGTATGGTG	"
HGItoSPI promoter Fw	CAGGAGGAAAAGAAAGCGCAGCTTGAAACTCCG TTCAGAAGATCGTACGCTGCAGGTCGAC	
HGItoSPI promoter Rv	GTATTTTCACCCTTGTAAAAGTATTTGGTTACAG TAGACATGATTAGTAATAGTACTG	
YHRPRO-2A	GTCCCTTTCTGACAATAAGACC	PCR of *HGI1* promoter
YHRPRO-2B	TGTTTCTGGCGATCCCTTCG	"
STL1PRO-A	TTGGTTAATCCTCGCCAGGT	PCR of *STL1* promoter
STL1PRO-B	TATGAGTGTGACTACTCCTG	"
INT-A	GGCTGTCAGAATATGGGGCCGTAGTA	PCR of an intergenic region
INT-B	CACCCCGAAGCTGCTTTCACAATAC	"

Table 3.3.S4. Complete data of the bioinformatic analyses presented in this work to determine the putative Hot1 DNA-binding sequences. File: "Supplementary Table 3.3.S4.xls" in the electronic version.

3.4. Capítulo 4

The Hot1 transcription factor physically binds the transcription co-activator Sub1 and is genetically linked with the elongation complex Spt4/5

In final phase of preparation

The Hot1 transcription factor physically binds the transcription co-activator Sub1 and is genetically linked with the elongation complex Spt4/5

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Hyperosmotic stress response involves the transcription activation of several stress response genes. The osmo-stress transcription factor Hot1 regulates the expression of several genes involved in glycerol biosinthesis, and the presence of this transcription factor at their promoter is essential for the recruitment of the RNApolII. The MAPK Hog1 activates Hot1 and directs the RNApolII localization at these promoters. But the phosphorylation of Hot1 by Hog1 is not essential for Hot1 functionality, and other kinases, such as CK2, also regulate the transcription factor, although their implications in stress response are still unclear. In this work we provide new data about the physical interactions in wich Hot1 is involved after its activation. By *Tandem Affinity Purification* and CoIP we demonstrate that Hot1 interacts with the transcription co-activator Sub1 and, moreover, that the recruitment of this protein to the promoter is strongly dependent on the presence of the Hot1 previously bound. Since more physical interactions have been reported in this work between Hot1 and proteins involved in transcription elongation and post-transcriptional control, and genetic interactions have been observed between Hot1 and Spt4/Spt5 complex, we propose that Hot1 could have a role further than transcription initiation.

Keywords: Hot1, Sub1, transcription elongation, *Tandem Affinity Purification, Saccharomyces cerevisiae*

INTRODUCTION

The response of yeast cells to increases in external osmolarity is mediated by the *High Osmolarity Glycerol* (HOG) pathway. The stress-activated Hog1 SAPK is essential for the induction of diverse osmoadaptive responses (Brewster et al., 1993; Hohmann, 2002; O'Rourke et al., 2002; Westfall et al., 2004; Hohmann et al., 2007; Brewster and Gustin, 2014). Irrespectively of the initial osmosensor, involving the Sln1-Ydp1-Ssk1 system or the tetraspan membrane protein Sho1, the anchor Opy2 and the glycoproteins Hkr1 and Msb2, MAPKK Pbs2 phosphorylates Hog1 in the cytosol and promotes its rapid nuclear accumulation (Ferrigno et al. 1998; Reiser et al. 1999; Ruiz-Roig et al., 2012; Tanaka et al. 2014). Hog1 regulates several unrelated transcription factors (Hot1, Msn1, Smp1, Gcn4, Skn7, Sko1, Msn2/4 and Rtg1/3), each of which is responsible for controlling the expression of a subset of osmoresponsive genes (Rep et al. 1999a, 1999b, 2000; Posas et al. 2000; de Nadal and Posas 2008; Ni et al. 2009).

This response rapidly induces the expression of about 10% of the yeast genes, which results in the synthesis of proteins implicated in carbohydrate metabolism, general stress protection, protein production, mitochondrial function and signal transduction (Posas et al., 2000; Rep et al., 2000; Causton et al., 2001; Capaldi et al., 2008; Miller et al., 2011; Ruiz-Roig et al., 2012). The MAPK Hog1 also regulates chromatin-modifying enzymes (de Nadal et al., 2004; Mas et al., 2009). Besides, it interacts with RNA polymerase II and is required for the large-scale redistribution of this enzyme from housekeeping genes to osmotic stress-controlled genes (Alepuz et al., 2003; Cook, E.K. O'Shea, 2012; Nadal-Ribelles et al., 2012). RNA polymerase II, either in complex with Hog1 or modified by this kinase via phosphorylation, reduces the affinity for non stress responsive genes; the entry of Hog1p in the nucleus upon stress allows transcription factors such as Sko1 and Hot1 to bind, together with Hog1, at the *cis*-elements located in regulatory regions of stress responsive genes, thus targeting the Hog1-RNA Pol II complex to these genes (Cook, E.K. O'Shea, 2012; Nadal-Ribelles et al., 2012).

The mechanisms by which Hog1 regulates the transcription factors above-mentioned differ from one to another and in some cases are not completely understood. Hog1 activates Sko1 by phosphorylation, switching its activity from a repressing to an activating state (Proft et al., 2001; Proft and Struhl, 2002; Guha et al., 2007). Msn2/4 and Sko1 transcription factors are not only controlled by Hog1 but also by PKA (Rep et al., 2000; Alepuz et al., 2001). Smp1 is also activated by Hog1-dependent phosphorylation (de Nadal et al., 2003). Under osmostress Hog1 interacts with Rtg1/3 *in vivo* and phosphorylates them; the MAPK is required for the nuclear accumulation of this complex, its recruitment at the promoters controlled by these factors and their proper transcriptional activation (Ruiz-Roig et al., 2012).

Osmostress-induced transcription by Hot1 requires a Hog1-mediated recruitment of RNA polymerase II but, surprisingly, this recruitment does not depend on the phosphorylation of Hot1 by the MAPK (Alepuz et al., 2003). Actually, the relationship among the binding of this transcription factor, stress conditions and Hog1 depends on each particular gene considered. In *HGI1*, which is partially controlled by Hot1, its binding is dependent on Hog1 (Alepuz et al., 2001; Gomar-Alba et al., 2012). In *STL1*, which is fully regulated by this factor, and in *GPD1*, Hot1 is bound to its promoter before the application of stress (Alepuz et al., 2001, Alepuz et al., 2003; Gomar-Alba et al., 2012). Recently we have demonstrated that the Hot1 sequence between amino acids 340 and 534 and the CD element of Hog1 are required for the interaction between the two proteins and the Hot1-dependent transcription regulation (Gomar-Alba et al., 2013). Inside this Hot1 region, short sequence KRRRR (KR4, amino acids 381-385) is essential for the kinase binding. Another element, sequence EDDDDD (ED5, amino acids 541-546), is essential for Hot1 binding to chromatin. Under osmotic stress conditions, both KR4 and ED5 elements, are involved in the appropriate

recruitment of Hog1 and RNA polymerase II to genes controlled by this transcription factor and are required for osmotolerance. Besides, KR4 is necessary for the functionality of the HOG pathway. Unlike Msn2/4 and Sko1, there is no changes in Hot1 localization upon salt stress (Gomar-Alba *et al.*, 2015) or evidences of PKA regulation of Hot1.

The activity of this transcription factor is also regulated by the kinase CK2. Several autors have described the interactions between different subunits of this kinase and Hot1 (Collins *et al.*, 2007; Breitkreutz *et al.*, 2010, Ho *et al.*, 2002; Gavin *et al.*, 2006; Krogan *et al.*, 2006). CK2 directly phosphorylates and inhibits Hot1 during salt stress (Burns and Wente, 2014); these authors have proposed that this mechanism might serve to dampen the Hog1-induced transcriptional response, which is toxic to cells when constitutively activated (Wurgler-Murphy *et al.*, 1997).

Hot1 is particularly relevant for the transcriptional response to high osmolarity caused by high glucose concentrations. An analysis of gene expression in single and multiple mutant strains carried out by Capaldi *et al.* (2008) has revealed that the HOG pathway activates fewer genes in glucose than in KCl under the same total molar osmolarity. The explanation offered by these authors is that the osmotic stress response in high glucose is modulated by the inhibition of Msn2/4 activity, which leads to an overall decrease in the activation of the general stress response, and shifts the Hog1-dependent expression program toward those genes regulated by Sko1 and Hot1. Recent data obtained by us confirms this proposal, but it also indicates that Msn2/4 still control the expression of several genes under high sugar osmostress (Gomar-Alba *et al.*, 2015).

Besides to the above-mentioned interactions with kinases Hog1 and CK2, several analyses have reported physical and/or genetic interactions between Hot1 and other proteins. Although some of them participate in processes apparently unrelated with osmotic stress response, several transcription factors (Msn2/4p for instance) involved in stress response that could have common targets with Hot1 have been detected (Thorne *et al.*, 2011). It is worth mentioning the presence among the group of Hot1-interacting proteins of the TATA-binding protein Spt15 (Sanders *et al.*, 2002), the highest subunit of the RNA polymerase II (Costanzo *et al.*, 2010), and the Gim5 protein, that facilitates protein elongation (Tong *et al.*, 2004).

Most of these interactions have not been demonstrated by several approaches and their relevance under osmotic stress conditions has not been determined. This work focuses on the analysis of the proteins with which Hot1 interacts under these adverse conditions in order to get further insights about the activity and regulation of this transcription factor.

MATERIALS AND METHODS

Yeast strains and growth conditions

All the yeast strains used in this work are described in Supplementary Table 1. Growth of yeast was carried out in YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose) at 30°C with orbital shaking (200 rpm). The yeast cells carrying plasmids were grown in SC medium (0.17% (w/v) nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) *Drop out* mix without the selected amino acid or nucleobase, 2% (w/v) glucose). For most experiments, yeast cultures were kept in the exponential growth phase for 16 h. Then cells were exposed to the considered osmotic stress conditions, these

being 0.4-0.9 M NaCl, 1 M sorbitol or 1.11 M (20% (w/v)) glucose. YPD media supplemented with these osmolytes are respectively named YPNaCl, YPS and YP20. For the growth assays on resistance to osmotic stress or transcription elongation inhibition, yeast cultures were diluted to the same OD_{600} (0.1) and serial dilutions (1:10) were spotted onto SC or YPD plates, which were incubated at 30°C or 33°C when required. For the transcription elongation inhibition, plates with 25, 50 or 75 μM of 6-AU were used.

Chromatin Immunoprecipitatioon Analyses

Cells from exponential growth cultures (OD_{600} 1) of strains expressing HA-Hot1, HA-Sub1 or HA-Yra1 were incubated for 10 min under 20% (w/v) glucose stress condition. A control culture was kept for the same time in YPD. Cells were cross-linked with 1% (v/v) formaldehyde for 15 min at room temperature and were then incubated for 5 min with 125 mM glycine before being collected, washed with TBS buffer (20 mM Tris-HCl (pH 8.0), 0.5 M NaCl) and frozen. Cells were resuspended in 300 μL of lysis buffer (50 mM HEPES-KOH pH 7.9, 40 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0,1% (w/v) sodium desoxicholate, 1 mM PMSF, 1 mM benzamidine and the *Complete Mini* protease inhibitor (Roche)), and 300 μL of glass beads were added. Cells were lysed for 30 min at 4°C in *Genie-2* (Scientific Industries). Chromatin was then fragmented by sonication in a *BioRuptor Diagenode* and the sample was centrifuged at 12000 rpm for 15 min.

To analyze Hot1, Sub1 or Yra1 binding to chromatin, the cell extract was incubated with orbital rotation for 2 h at 4° with *Dynabeads Protein G* (Invitrogen) previously bound to an HA 3F10 antibody (*Roche*). In the case of polimerase binding to DNA, the cell extract was incubated during 15 min at room temperature with the same *Dynabeads* but previously bound to an 8WG16 antibody (anti Rpb1, *Covance*). Beads were then washed 3 times with 200 μL of PBS (150 mM NaCl, 40 mM Na_2PO_4, 10 mM NaH_2PO_4) containing 0.02% (v/v) Tween 20, and then once more with 100 μL of the same buffer. Elution was carried out twice with 40 μL of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% (w/v) SDS, by heating at 65°C for 2 min at 600 rpm. Cross-linking was reverted by overnight incubation at 65°C with shaking. The eluted sample was digested for 90 min at 37°C with 0.33 mg/mL proteinase K and DNA was purified with the *High Pure PCR product purification kit* (Roche).

Co-immunoprecipitated DNA was analyzed in triplicate by Real Time RT-PCR in a *DNA Engine Peltier Termal Cycler* (Bio Rad) using the *SYBR® Premix Ex Taq™ Tli RNase H Plus Green with ROX* (Takara). Amplifications of the promoter region of the genes considered were carried out with the combinations of oligonucleotides described in Supplementary Table 3, which also includes Int A/B, which were used for an intergenic region. Data were processed with the ΔΔCT method (Schmittgen and Livak, 2008) by comparing the results of the amplified from the immunoprecipitated sample (IP) with those of the whole cell extract (*Input*), and by using the intergenic region for normalization.

Methods of protein manipulation and analysis

Protein extracts were prepared for routine analyses according to the procedure described in Gomar-Alba *et al.* 2013. For the dephosphorylation assays, extracts were treated with λ phosphatase as described in Soriano-Carot *et al.* (2014).

For the detection of HA-tagged proteins by Western analysis anti-HA 3F10 peroxidase was used (Roche) (dilution 1:10000 in TBS, 0.01 % (w/v) Tween 20).

Tandem Affinity Purification (TAP) was carried out from the exponential cultures (30 L) of the strain containing the TAP-tagged version of Hot1p in YPD transferred for 10 min to YP20 (final OD_{600} of 2.0). Purification was carried out as described in Gomar-Alba *et al.* (2012). The proteins associated with Hot1p were identified by mass spectrometry in the Proteomics Facility of the SCSIE of the Universitat de Valencia. The information was analyzed by Protein Pilot (SwissProt) and MASCOT DAEMON (NCBInr). TAP analysis was also carried out from the exponential cultures in the absence of osmotic stress.

For the coimmunoprecipitation experiments, exponentially growing cells (OD_{600} of 1) were transferred for 10 min to YP20. The cells corresponding to 100 mL of these cultures were lysed in 600 μL of lysis buffer (50 mM Tris–HCl pH 8.0, 5 mM EDTA, 250 mM NaCl, 1 mM PMSF, 0.01% Triton X-100 and the Complete protease inhibitor mix (Roche)) in the presence of one volume of glass beads (425-600 μm in diameter). Samples were centrifuged at the maximun speed for 5 min. The supernatant obtained, which corresponds to the total protein extract, was then incubated with Dynabeads IgG Pan Mouse -prepared as described by the manufacturer (Invitrogen)- for 2 h at 4°C in a rotary shaker to immunoprecipitate the Hot1p-TAP fusion protein. After collecting the non retained fraction, Dynabeads were washed 3 times with PBS buffer containing 5 mg/mL of BSA. Elution was carried out with 100 μL of 1% SDS for 5-10 min at 65ºC. The three fractions obtained were incubated at 95°C for 5 min in the presence of protein solvent (250 mM Tris-HCl pH 6.8, 140 mM SDS, 30 mM bromophenol blue, 27 μM glycerol, 0.1 mM DTT) before being applied to a polyacrylamide gel of the appropriate concentration. Coimmunoprecipitated proteins were detected by Western with the anti-HA 3F10 peroxydase antibody.

RESULTS

Hot1 interacts with proteins involved in protein phosphorylation control, transcription elongation and post-transcriptional processes under osmotic stress

In order to gain more information about putative interactions in which Hot1p could be involved, *Tandem Affinity Purification (TAP)* analyses were carried out under two different conditions: exponential growth phase (non-stress condition) and after a 10 min-incubation in the presence of 20% (w/v) glucose (hyperosmotic stress condition). Among the different osmolytes that can determine a hyperosmotic stress, high sugar concentrations were considered due to the relevance of Hot1 under this particular condition (Capaldi *et al.*, 2008; Gomar-Alba *et al.*, 2015).

Some proteins appear to be interacting with Hot1 independently of the presence of stress. These proteins are included in Table 1, which also shows their known function. This is the case of the enzymes Gpp1/2, involved in glycerol biosynthesis, the transcription factor Yap1, the mRNA export factor Yra1, the poly(A) binding protein Pab1p and Hot1 itself.

However, according to our experiments, some proteins only interact with Hot1 under osmotic stress. This occurs for subunits of the RNA polymerases, factors involved in transcription elongation and/or mRNA processing and nuclear

export, components of the nuclear pore complex and some cytoplasmic proteins related to ribosome biogenesis and post-transcriptional processes.

To further confirm these interactions by an alternative approach, coimmunoprecipitation experiments were carried out with strains expressing a TAP-tagged version of Hot1 and HA-tagged versions of some of the putative interacting proteins involved in different cellular functions: Pab1, Pbp1, Rat8, Sub1, Yra1, Rpb6 and Yrb1. As shown in Figure 1, in extracts obtained from cells growing in exponential growth affected by the same stress condition used for the TAP analyses, it was possible to confirm only the interaction Hot1-Pab, Hot1-Sub1 and Hot1-Rpb6. The physical interaction found between Hot1 and Spt5 in TAP analysis has not been confirmed by coimmunoprecipitation because it is reinforced by the recently demonstrated interaction between Sub1 and Spt5 (García *et al.*, 2012), suggesting that the three proteins, Sub1-Hot1-Spt5, can interact between them.

In order to confirm the possibility of other interactions between Hot1p and subunits of the RNA polymerase II, as suggested by this analysis and by others (Costanzo *et al.*, 2010), immunoprecipitation of a Hot1-tagged protein was carried out and Rpb1 was searched by means of an antibody against this protein. No interactions were found (data not shown).
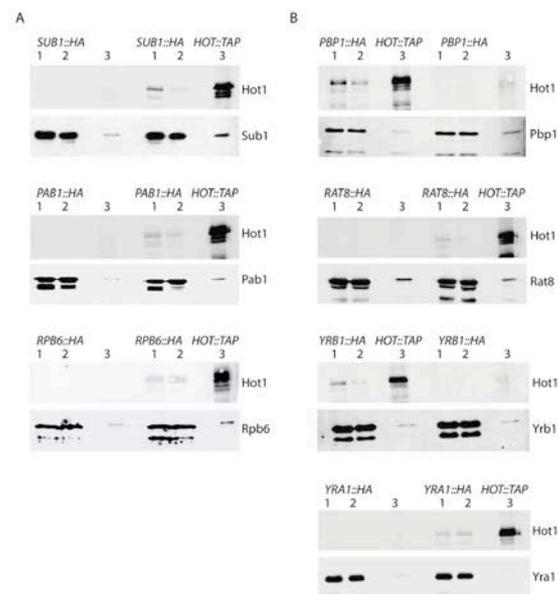
Figure 1. Physical interaction between Sub1, Pab1 and Rpb6 with Hot1 by Coimmunoprecipitation Analysis. Cells of the strains carrying a TAP-tagged version of Hot1 and an HA-tagged version of Sub1, Pab1, Pbp1, Rat8, Yrb1, Yra1 or Rpb6 proteins were exponentially grown in YPD at 30°C. After a 10-minute incubation in YP20 at the same temperature, cells were lysed and Hot1 was immunoprecipitated as described in the *Materials and Methods* section. This Figure shows the Western analysis carried out with the total protein samples (input, 1), the non-retained fraction (unbound, 2) and the retained fraction (immunoprecipitate, 3). Hot1 is detected with α-PAP antibody, while the other proteins were visualized with α-HA antibody. The results of the control experiments carried out with a FY86-derived strain carrying HA-tagged versions of the Sub1, Pab1, Pbp1, Rat8, Yrb1,Yra1 and Rpb6 proteins are also shown.

TABLE1. Proteins identified in the TAP experiment described in this work.

Protein	Stress (1)	% coberture	Num peptides	Function (2)
Def1	Yes	38.89	12	RNAPII degradation factor, forms a complex with Rad26 in chromatin, enables ubiquitination and proteolysis of RNAPII present in an elongation complex
Nsr1	Yes	43.72	10	Nuclear localization sequence-binding protein
Stm1	Yes	50.55	3	Protein required for optimal translation under nutrient stress; involved in TOR signaling
Sub2	Yes	32.09	1	Component of the TREX complex required for nuclear mRNA export; member of the DEAD-box RNA helicase superfamily and involved in early and late steps of spliceosome assembly
Dbp5	Yes	8.90	3	Cytoplasmic ATP-dependent RNA helicase of the DEAD-box family; involved in mRNA export from the nucleus
Nop3	Yes	3.10	1	RNA-binding protein; promotes elongation, regulated transcription, and carries poly(A) mRNA from nucleus to cytoplasm
Yrb1	Yes	5.50	1	Ran GTPase binding protein; involved in nuclear protein import and RNA export
Pbp1	Yes	18.01	2	Pab1p binding protein
Pbp4	Yes	10.80	2	Pbp1p binding protein
Sub1	Yes	7.50	2	Transcription coactivator; facilitates elongation through factors that modify RNAPII; role in hyperosmotic stress response through polymerase recruitment at RNAP II and RNAP III genes
Spt5	Yes	1.50	1	Transcription elongation factor; component of the universally conserved Spt4/5 complex (DSIF complex); the complex has multiple roles in concert with RNA polymerases I and II, including regulation of transcription elongation, RNA processing, quality control and transcription-coupled DNA repair
Rpb6	Yes	14.2	1	RNA polymerase subunit ABC23, common to RNA polymerases I, II and III; part of a central core
RPAC1	Yes	7.20	2	Subunit of the DNA-directed RNA polymerases I and III
RPA49	Yes	4.30	9	Subunit of the DNA-directed RNA polymerase I
Nsp1	Yes	4.10	4	FG-nucleoporin component of central core of the nuclear pore complex; also part of the NPC nuclear basket; contributes directly to nucleocytoplasmic transport and maintenance of the NPC permeability barrier
Nup2	Yes	2.60	2	Nucleoporin involved in nucleocytoplasmic transport; binds to either nucleoplasmic or cytoplasmic faces of the nuclear pore complex depending on Ran-GTP levels
Yra1	No	17.70/19.47	3/4	Nuclear polyadenylated RNA-binding protein; required for export of poly(A)+ mRNA from the nucleus; proposed to couple mRNA export with 3' end processing via its interactions with Mex67p and Pcf11p.
Pab1	No	1.73/6.93	1/3	Poly(A) binding protein; part of the 3'-end RNA-processing complex, mediates interactions between the 5' CAP structure and the 3' mRNA poly(A) tail, involved in control of poly(A) tail length, interacts with translation factor eIF-4G.
Gpp1	No	5.99/5.99	1/1	Constitutively expressed DL-glycerol-3-phosphate phosphatase; also known as glycerol-1-phosphatase; involved in glycerol biosynthesis, induced in response to both anaerobic and osmotic stress; GPP1 has a paralog, GPP2.
Gpp2	No	5.99/5.99	1/1	DL-glycerol-3-phosphate phosphatase involved in glycerol biosynthesis; also known as glycerol-1-phosphatase; induced in response to hyperosmotic or oxidative stress, and during diauxic shift; GPP2 has a paralog, GPP1.
Yap1	No	2.61/5.99	1/6	Basic leucine zipper (bZIP) transcription factor; required for oxidative stress tolerance; activated by H_2O_2 through the multistep formation of disulfide bonds and transit from the cytoplasm to the nucleus.
Hot1	No	63/46	71/53	Transcription factor for glycerol biosynthetic genes; required for the transient induction of glycerol biosynthetic genes GPD1 and GPP2 in response to high osmolarity; targets Hog1p to osmostress responsive promoters.

(1)   This indicates if the proteins interact with Hot1 only under the stress conditions tested (Yes) or also during exponential growth in the absence of osmolytes (No).
(2)   According to the information available in SGD (*Saccharomyces Genome Database*)

Taken together the data obtained from this analysis and others previosly described in the introduction section, Hot1 could be involved in interactions with the transcription machinery, not only at the initiation step, and could also be linked to phosphatases and kinases involved in several signal transduction pathways.

Hot1 phosphorylation is not affected by Gpp1/2, Ssk2, Rim11 and Kcs1

Hot1 phosphorylation in several residues has been previously described (Alepuz *et al.*, 2003; Burns *et al.*, 2014). Panel A in Figure 2 shows how the Hot1 phosphorylation pattern presents some differences depending on the osmo-stress considered. In order to demonstrate that independently of the osmolyte it is mainly caused by phosphorylation, total protein extracts were treated with lambda phosphatase, resulting in all the cases in an important decrease of the phosphorylation patern, being similar to that observed under non stress conditions (time 0).

According to this, the main Hot1 postranslational modification seems to be phosphorylation although the role of Hog1 phosphorylation in the control of Hot1-dependent gene expression is unclear (Alepuz *et al.*, 2003, Gomar-Alba *et al.*, 2013). In our TAP analyses we have not detected any Hot1-interacting kinase but in previous studies, Rim11 (Bandyopadhyay *et al.*, 2010) and Ssk2 (Rep *et al.*, 1999b) have been reported to interact with this transcription factor. Besides, CK2 inhibits Hot1 by phosphorylation under stress.

(Burns and Wente, 2014). We, hence, analyzed wether or not some kinases related with signal transduction processes have a role in this matter. For this purpose, under sorbitol 1M osmostress conditions (were phosphorylation levels seems to be higher) mutants in Ssk2 (MAPKKK of the HOG pathway), Rim11 (kinase required for signal transduction during entry in meiosis) and Kcs1 (inositol hexakisphosphate kinase) were considered. In any of the strains considered the phosphorylation of Hot1 under non-stress and hyperosmotic stress conditions was significantly affected (Fig. 2B). However the two catalític subunits of CK2, Cka1 and Cka2, influence the phosphorylated status of Hot1 under non stress conditions (Fig. 2C), becoming the transcription factor phosphorylated. This result confirms the data recently described by Burns and Wente (2014) that suggest a role of the CK2 kinase in the Hot1 stokastic regulation under non-stress conditions. Since changes in the Hot1 phosphorylation are not observed undes stress conditions in this mutant, both proteins do not seem to interact in the Hot1 functional conditions of osmostress.

The detection of interactions both under stress and non-stress conditions between Hot1 and Gpp1/2 prompted us to determine if these phosphatases could affect the phosphorylation status of this transcription factor. As shown in Figure 2 panel B, no changes in Hot1 phosphorylated forms could be detected under exponential growth and after an osmotic shock in a deletion mutant in these enzymes, since the same electrophoretic migration pattern is found than in the wild type strain.
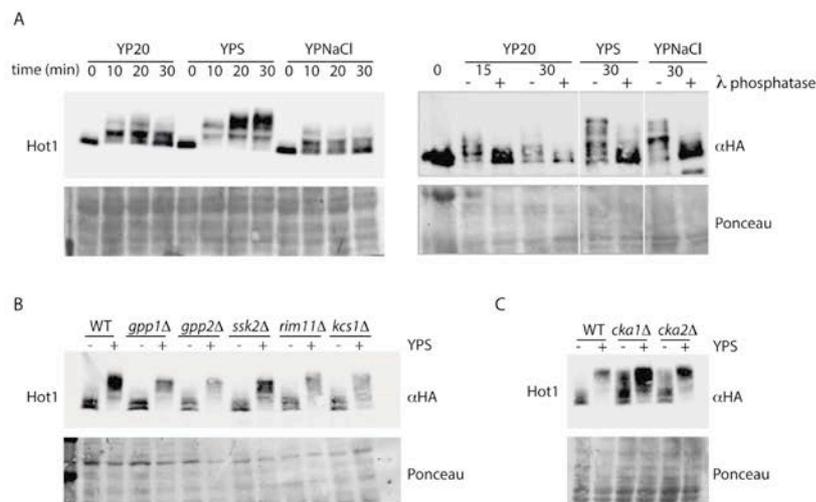

Figure 2. Proteins Cka1 and Cka2 but not Gpp1/2, Ssk2, Rim11 and Kcs1 are involved in the control of Hot1p phosphorylation. The Figure shows the Western blot analysis of the Hot1 protein in whole cell extracs prepared as described in the *Materials and Methods* section. Panel A shows the detection of Hot1-HA expressed from the wild type strain BY4741 during the exposure of yeast cells to osmotic stress caused by several osmolytes: 20% w/v (1.1M) glucose (YP20), 1M sorbitol (YPS) or 0.6M NaCl (YPNaCl) at different times with (+) or without (-) treatment with λ phosphatase. In panel B and C the result obtained for wild type and the mutant strains considered in osmostress due to 1M sorbitol for 15 min is visualized. Ponceau staining of the filters was carried out as a loading control and is shown in the lower figures of each panel. This figure shows the result of a representative experiment.

Interplay between Hot1 and Sub1 in the regulation of the expression of genes controlled by the transcription factor

One of the proteins found in this analysis as a Hot1-interacting protein, Sub1p, has been reported to interact with Hog1p, and a role for the recruitment of RNA polymerase II to osmostress genes in response to osmotic stress has been described for it (Rosonina *et al.*, 2009). To determine the recruitment of Hot1p and Sub1p to the promoter of target genes, ChIP experiments were carried out with *STL1* and *HGI1* genes; the first one is completely dependent for its regulation on Hot1, while for the latter other transcription factors are involved (Alepuz *et al.*, 2003; Gomar-Alba *et al.*, 2012).

As shown in Figure 3A, the recruitment of Sub1p to *STL1*

promoter under osmotic stress due to salt or high glucose concentration is completely abolished in the absence of Hot1p, while it was reduced in the case of *HGI1* promoter, according to the different regulatory mechanisms of both genes. Recruitment of the Rpb1p subunit of RNA polymerase II follows the same pattern (Figure 3B). This suggests that the involvement of Sub1p in the recruitment of the polymerase requires a previous binding of Hot1p. On the other hand, binding of Hot1p to the promoter of both genes is reduced in a *sub1Δ* mutant (Fig. 3C) and also their mRNA levels, but gene induction is still very high (Fig. 3D). According to this Hot1p binding is not strictly dependent on Sub1p.
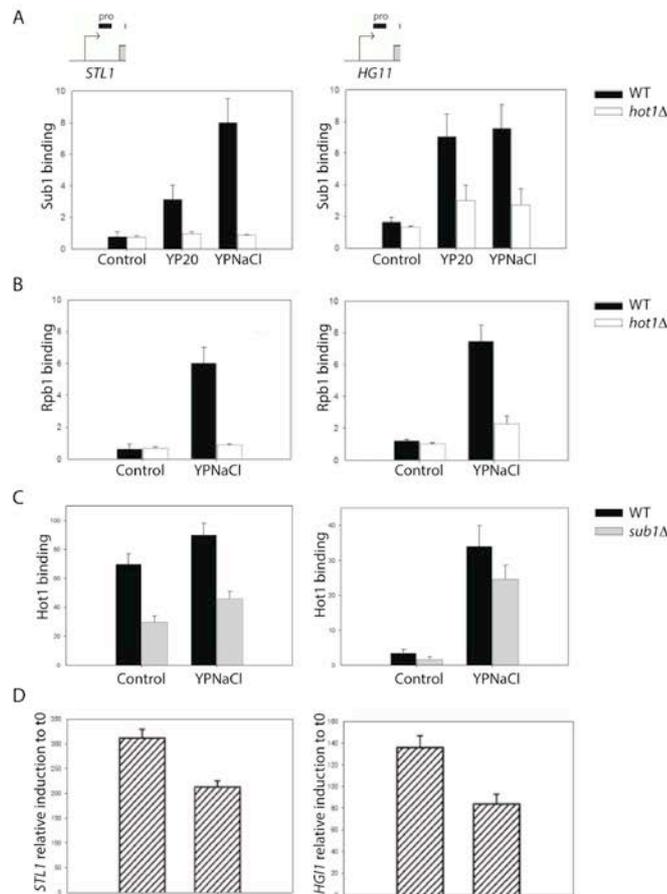

Figure 3. Hot1 is required for Sub1 and Rpb1 binding to *STL1* promoter while Hot1 binding and gene expression is only partially affected in a *sub1Δ* mutant. This Figure shows the association of these proteins over the *STL1* promoter between -372 and -313, determined by chromatin immunoprecipitation (ChIP) followed by Real-Time RT-PCR (panels A, B and C). The experiments were carried out with the FY86 *hot1Δ* or *sub1Δ* strains expressing HA tagged versions of Sub1 and Hot1 respectively. The cells from the exponentially growing cultures were incubated, or not (Control), for 10 min in 20% glucose (YP20) or 0.6 M NaCl (YPNaCl) as described in the *Materials and Methods section*. For immunoprecipitation, α-HA antibody was used for Hot1 and Sub1 and, in the case of Rpb1, specific antibodies against this subunit were used. Panel D shows the mRNA levels corresponding to *STL1* gene under stress due to glucose in wild type and *sub1Δ* strains determined by Real-Time RT-PCR as described in *Materials and Methods* section. This Figure shows the expression levels under high salt or glucose stress normalized to those obtained for the *ACT1* gene, referring to the value found at time 0 in each strain. All these experiments were carried out in triplicate; the average and standard deviation data are included.

Hot1 still associates with chromatin up to the 3'UTR region, although to a lower extent than at the promoter

Chromatin immunoprecipitation analyses were carried out in order to determine the distribution of Hot1 throughout the different regions of the *STL1* gene. The extension of this gene allows the selection of regions corresponding to promoter, upper (5'), middle (m), and farther (3') coding region, and the 3'UTR (Figure 4A). In these experiments osmotic stress due to 0.4 M NaCl was considered because of the higher induction of *STL1* gene under saline stress than under high glucose stress (Gomar-Alba *et al.*, 2013). As shown in Figure 4B, highest Hot1 binding is detected at the promoter with a progressive

decay through the coding region. This pattern is similar to that found for Sub1 (Panel C in this Figure), although the percentage of protein bound through the different regions when compared to the promoter is lower for Hot1 than for Sub1 (Figure 4 Panel D). As a control other two proteins were considered in these experiments: the biggest subunit of the RNA polymerase II and a protein involved in mRNA export. In the case of Rpb1 protein, binding is higher through the coding region than in the promoter without significant differences between the regions considered. In the case of Yra1 an enrichment from the middle part of the coding region is found without differences up to the 3' UTR, as expected for a protein involved in export.
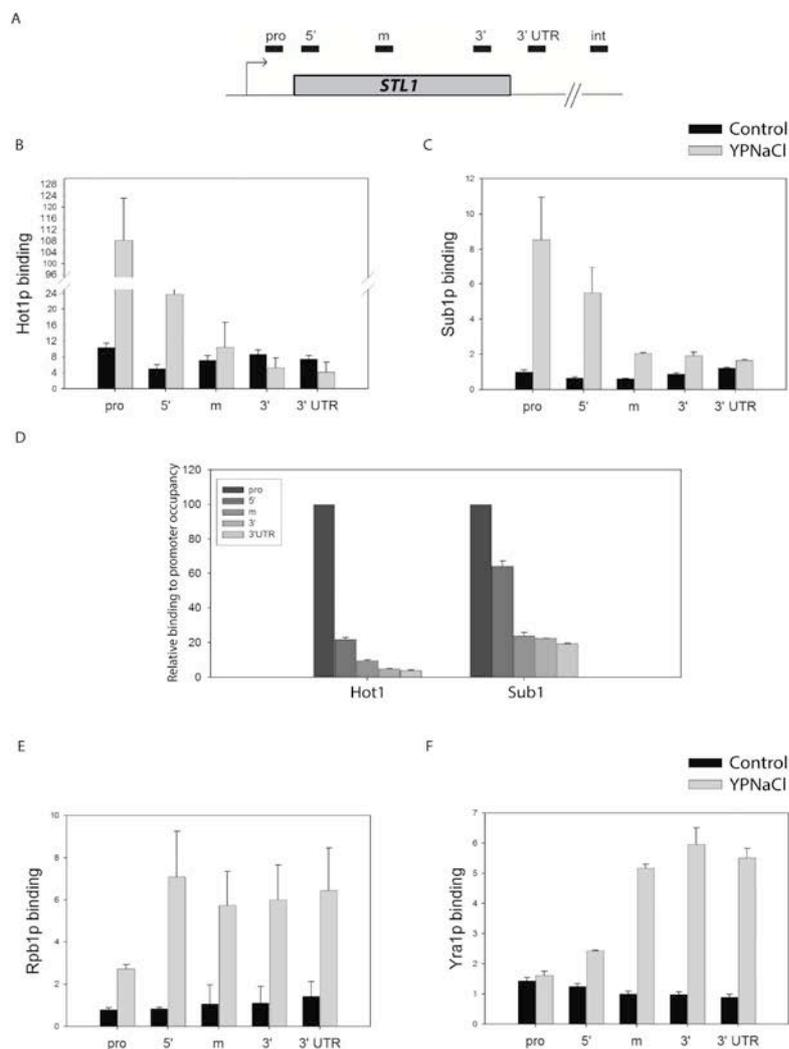
Figure 4. Association of Hot1, Sub1, Rpb1 and Yra1 to the different regions of *STL1* gene under osmostress. This Figure shows the occupancy of these proteins over the regions of *STL1* gene indicated in the Figure, determined by chromatin immunoprecipitation (ChIP) followed by Real-Time RT-PCR. Data in panel D is shown as percentage of Hot1 and Sub1 association to each one of the regions calculated considering as 100% of the occupancy of the promoter region in each case. The experiments were carried out with the FY86 strain expressing HA tagged versions of Hot1, Sub1 and Yra1. The cells from the exponentially growing cultures were incubated, or not (Control), for 10 min in 0.6 M NaCl (YPNaCl) as described in the *Materials and Methods section*. For immunoprecipitation, α-HA antibody was used for Hot1, Sub1 and Yra1 and, in the case of Rpb1, specific antibodies against this subunit were used.

Hot1 is genetically linked to the Spt4/5 elongation complex

The transcriptional coactivator Sub1 has been implicated in several aspects of mRNA metabolism in yeast including activation of transcription, preinitiation, regulation of RNA polymerase II phosphorylation, termination and 3'-end formation and, more recently, transcription elongation (Henry *et al.*, 1996; Knaus *et al.*, 1996; Calvo and Manley, 2001; He *et al.*, 2003; Sikorski *et al.*, 2011; García *et al.*, 2012).

One of the evidences supporting the role of Sub1 in transcription elongation is its genetic interaction with the factor Spt5, that stimulates transcription elongation and RNA polymerase processivity (Burova *et al.*, 1995; Chen *et al.*, 2009). Spt5 associates with Spt4 and the Spt4/5 complex is required for efficient elongation by RNA polymerase II (Hartzog *et al.*, 1998; Wada *et al.*, 1998). Actually Sub1 also affects transcription elongation efficiency and elongation rate (García *et al.*, 2012). In our experiments it has been possible to detect interactions Hot1-Sub1, Hot1-Spt5 and Hot1-Gim5 (subunit of the heterohexameric cochaperone prefoldin complex, which localizes in the cytosol but also to chromatin of actively transcribed genes in the nucleus and facilitates transcriptional elongation, Millan-Zambrano *et al.*, 2013).

In order to determine wether or not Hot1 could have some participation in elongation, we carried out dilution plate assays with several strains in the presence of the elongation inhibitor 6-AU, which is thought to promote transcription pausing and arrest by limiting the intracellular pools of GTP and UTP (Exinger and Lacroute, 1992). As a control several strains were considered: *dst1Δ*, which carries a deletion of the general transcription elongation factor TFIIS (Kipling and Kearsey, 1993), a deletion mutant *spt4Δ*, a termosentive mutant in *SPT5* (Spt5-194, García *et al.*, 2012), and *sub1Δ* mutant. The results found for the controls (Figure 5) are in accordance with those expected for these strains: *dst1Δ* mutant was dramatically sensitive to the lowest concentration of 6-AU considered in this

experiment (25 μg/mL) and the same occurred for the termosentive mutant in *SPT5* at the non-permissive temperature, while for *spt4Δ* growth defects are detected from this concentration above, and *sub1Δ* mutant is not sensitive to this drug. In the case of *hot1Δ* strain, cells grew normally and sensitivity to 6-AU was not detected. However, in the double mutant *hot1Δsub1Δ* it was possible to identify some growth defects even at the lowest concentration of 6-AU and the combination of a mutation in *HOT1* and in *SPT4* resulted in extreme 6-AU sensitivity, similar to that found for *dst1Δ* mutant. In the double mutant *hot1Δ spt5ᵗˢ* the residual growth detected in minimal medium containing 25 μg/mL of 6-AU at the non-permissive temperature is completely abolished. According to these results Hot1, as occurs for Sub1, is genetically linked to Spt4 and Spt5 and might be functionally linked to the elongation complex formed by these factors.

Discussion

Hot1 transcription factor was identified in a two-hybrid screen in which *HOG1* coding sequences were used as a bait (Rep *et al.*, 1999). Under osmotic stress, it has been seen to be involved in the transcriptional induction of several genes, including *STL1*, *GPD1*, *GPP2* and *HGI1* (Rep *et al.*, 1999, 2000; Gomar-Alba *et al.*, 2012). According to its role in the response to this adverse condition, cells with *HOT1* deletion are sensitive to severe osmotic stress conditions although in a much lower degree than those in which *HOG1* is not expressed (Rep *et al.*, 1999).

Hot1 activity is linked to its interaction with other proteins and for this reason in this work we have focused on the detection of undescribed targets of this transcription factor. Hot1 is a direct target of the MAPK Hog1, and alanine substitution of three serine/proline residues that can serve as phosphoacceptor sites reduces Hot1 hyperphosphorylation (Alepuz *et al.*, 2001). An interplay between Hot1 and Hog1
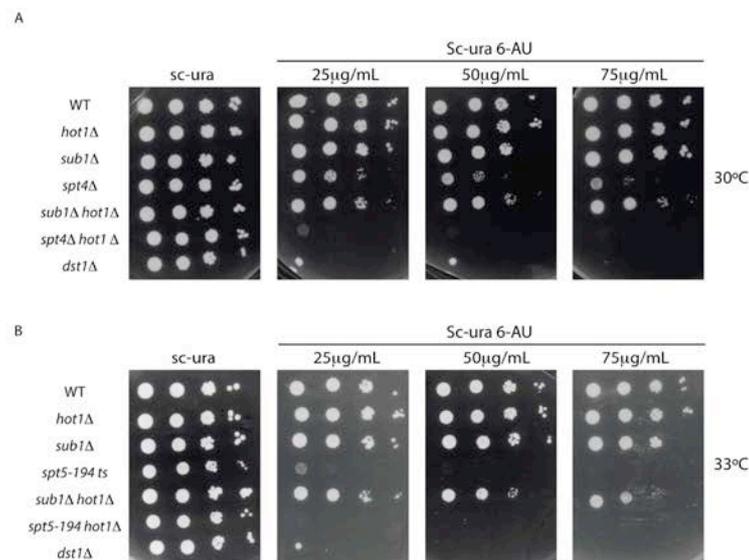
Figure 5. Genetic interaction between *HOT1*, *SPT4* and *SPT5*. Serial dilutions of yeast strains with the indicated genotypes were spotted on SC-ura and SC-ura media with 25, 50 or 75 μg/mL of 6-AU and grown at 30°C or 33°C for 3 days. The result of a representative experiment is shown.

exists in the transcription of genes regulated by this transcription factor under osmostress. By one hand, Hog1-mediated recruitment of RNA polymerase II is needed. Despite the situation found for transcription factors such Sko1 and Smp1 (Proft *et al.*, 2001; Proft and Struhl, 2002, de Nadal *et al.*, 2003), this recruitment does not depend on the phosphorylation of Hot1 by the MAPK (Alepuz *et al.*, 2001), but requires a KRRRR Hot1 sequence that serves as a docking site for Hog1 (Gomar-Alba *et al.*, 2013). On the other hand Hog1 is not recruited to promoters, ORFs or downstream regions when Hot1 (and Sko1) are absent (Alepuz *et al.*, 2003; Cook and O'Shea (2012)). These last authors have proposed that Hog1 is loaded to the set of genes with the nearby Sko1 and Hot1 binding sites along with RNA polymerase II during transcription initiation.

As can be deduced from the results shown in Table 1, the number of proteins interacting with Hot1 under osmotic stress conditions caused by high glucose concentrations is very reduced, probably because of its function as a transcription factor. However, it can not be ruled out that this protein could be involved in short-lived interactions non detected by means of this methodology. It is worth mentioning that the TAP analysis has been carried out just 10 min after the application of the osmotic stress and transient interactions in wich the transcription factor is involved could have not been detected. This is the case, for instance, of the Hog1-Hot1 interaction, clearly demonstrated (Rep *et al.*, 1999b; Gomar-Alba *et al.*, 2012) but not observed with this approach.

Hot1 contacts with other kinases according to global analyses of yeast interactome. Several authors have reported interactions with the four subunits of the CK2 kinase (Ho *et al.*, 2002; Gavin *et al.*, 2006; Krogan *et al.*, 2006; Collins *et al.*, 2007; Breikrentz *et al.*, 2010,). Actually, this enzyme directly phosphorylates and inhibits Hot1 during salt stress (Burns and Wente, 2014). Kinases Rim11 and Kcs1 (Bandyopadhyay *et al.*, 2010) and Ssk2 (Rep *et al.*, 1999) have also been reported to interact with Hot1. In the *Tandem Affinity Purification* analysis carried out in this work we have not detected any other Hot1-interacting kinase, although the glycerol-1-phosphatase Gpp1 has been identified both under stress and non-stress conditions. Experiments carried out with mutants in this phosphatase and in Rim11 and Kcs1 kinases have not revealed any role of these enzymes on the control of the Hot1 phosphorylation status under stress.

According to this, Hog1 and CK2 seem to be the only proteins that regulate Hot1 activity although in the former case not by the phosphorylation itself. We do not observe in the CKA mutants the reduction of Hot1 phosphorylation under osmostress conditions reported recently by Burns and Went (2014). However, it is worth mentioning that they observe this reduction in the doble mutant *Δhog1Δcka2*, but not in the single *Δcka2*. This is an interesting point because the MAPK Hog1 could mask the effect of some kinases, if weak, in single mutants. As the demonstration of the transcient interaction associated to phosphorylation is difficult we have focused our work just in those kinases that have a relevant effect. In this sense, it is quite clear from the results presented here that CK2 is involved in the Hot1 dephosphorylation under non-stress conditions and suggests the involvement of phosphatase(s) regulated by CK2 under physiological conditions.

Among the proteins with which Hot1 could interact under osmotic stress conditions, subunits of RNA polymerases and components of the machinery involved in mRNA 3'-end processing and export have been identified in our analyses. In the former case the contacts can be simply related with the process of transcription itself, which involves recruitment of the RNA polymerase when this transcription factor is bound to its target sequence (Alepuz *et al.*, 2003; Cook and O'Shea, 2012; Gomar-Alba *et al.*, 2013). On the other hand, in the case of export factors, Yra1 has been shown to interact with Hot1 also under non-stress conditions in the TAP analysis presented in this work, and this interaction has not been confirmed by coimmunoprecipitation experiments (Figure 1). In the case of proteins which bind to the poly(A) tail and are involved in the regulation of its length, such Pab1 and Pbp1, they interact under both stress and non-stressed conditions but the coimmunoprecipitation experiments do not sustain undoubtively the contacts with Hot1. Regarding Rat8 and Yrb1, related to mRNA export, interactions with this transcription factor have not been confirmed by other procedures; besides, Hot1 mutant has not displayed any deffect in this step of gene expression in preliminary experiments carried out in our laboratory (data not shown). The interaction between these proteins could be simply related to their association to chromatin inside the coding region.

The most interesting Hot1 interaction discovered in this work is with the transcription elongation protein Sub1. This interaction has been supported by coimmunoprecipitation experiments (Fig. 1), by genetic interactions (Fig. 5) and by a similar association with chromatin through the coding region and up to the 3'-UTR (Fig. 4B and 4C), although in a much lower extent than at promoters and at the beginning of the coding region (Figure 4D). Sub1 protein is a transcription activator that facilitates elongation and mRNA 3'-end processing through factors that modify RNA polymerase II (Henry *et al.*, 1996; Knaus *et al.*, 1996; Calvo and Manley, 2001; He *et al.*, 2003; Sikorski *et al.*, 2011; García *et al.*, 2012). According to this interaction, in this work we provide some evidences suggesting a putative role of Hot1 not only in transcription initiation but also in elongation: the interaction with the Spt5 elongation factor (García *et al.*, 2012), the growth deffects displayed by the double mutant *hot1Δsub1Δ* in plates containing 25-75 μg/mL of 6-azauracil (Figure 5), the extreme sensitivity to this compound of the combination of a mutation in *HOT1* and in *SPT4*, and the absence of growth, even at the low AU concentration, of the double mutant *hot1Δ spt5^ts* (Fig. 5). Several approaches have been described in the literature to get further insights about the involvement of a particular protein in transcription elongation. Among them there are the measurements of the RNA polymerase II processivity and the elongation rate (Mason and Struhl, 2005). Unfortunately these methodologies cannot be applied to the case of the Hot1 transcription factor because it is absolutely required for polymerase binding and hence it is not possible to see the relative Rpb1 occupancy in *hot1Δ* cells after the introduction of osmotic stress conditions or a few minutes after the last wave of transcription.

It is worth mentioning that the interaction of Hot1 with the MAPK Hog1 also sustains a putative role in elongation for this transcription factor. Several evidences about the involvement of Hog1 in the regulation of transcriptional elongation have accumulated during the last years (reviewed in de Nadal and Posas, 2011). This SAPK associates with RNA polymerase subunit Rpb1 phosphorylated at serine 2 or serine 5 in the CTD and with other proteins (Spt4, TFIIs, Paf1 and Thp1) that travel with the elongation form of this polymerase (Proft *et al.*, 2006). Besides, these authors also found that Hog1 is recruited to coding and 3' regions of osmoresponsive genes but not to any
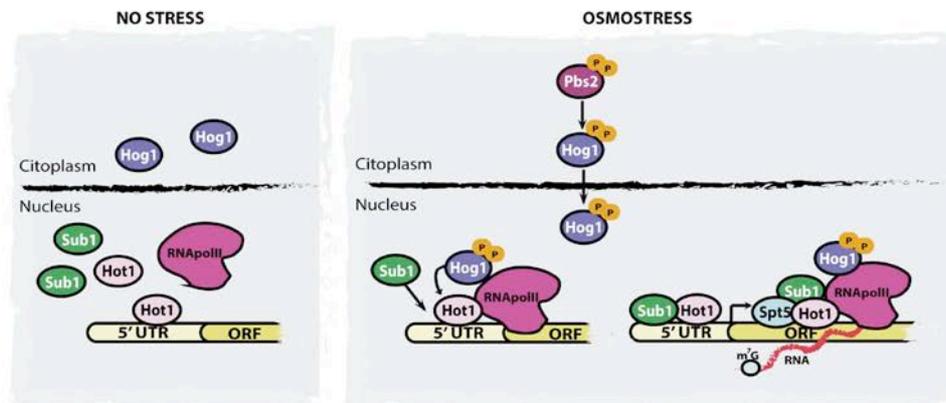
Figure 6. Model of Sub1-Hot1 role during transcription initiation and early elongation in Hot1-regulated genes. Phosphorylation status has only be indicated in case of Pbs2 and Hog1 proteins.

region of constitutively expressed genes, being this binding dependent on the 3'UTR regions. ChIP-Chip analyses have confirmed the recruitment of Hog1 with both the 5' upstream and the 3' downstream sequences of osmoinducible genes (Pascual-Ahuir *et al.*, 2006).

Rosonina et al. (2009) have reported a role of Sub1 in the regulation of gene expression under osmostress. However when these authors analyzed the expression of several genes controlled by this transcription factor in these conditions (*ALD3, STL1, GPD1, CTT1, HSP12* and *HSP26*) in a *sub1Δ* mutant, the reduction in mRNA levels when compared to the wild-type strain is undetectable in most of them and only in two cases (*CTT1* and *HSP26*) it arrived to 30 to 40%. Regarding recruitment of RNA polymerase to the promoters of these genes, it is diminished in about 20% for most of them. On the other hand, ChIP experiments carried out in this work demonstrate that Sub1 binding to the promoter of these genes is severely affected in a *hot1Δ* mutant while Hot1 binding and gene expression is less affected in a *sub1Δ* mutant (Fig. 3). According to this the contribution of Sub1 to the expression of Hot1-dependent genes has lower relevance for osmostress response and, according to the model described in Figure 6, requires the previous association of this transcription factor.

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Material suplementario

Table 3.4.S1. Yeast strains used in this work

Strain	Description	Origin
FY86	*MATα leu2Δ1 ura3-52 his3Δ200*	Lab Stock
FY86 HOT::TAP	*MATα leu2Δ1 ura3-52 his3Δ200 HOT1-TAP::kanMX6*	M. Gomar (Gomar-Alba y col., 2013)
FY86 HOT::TAP SUB1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 HOT1-TAP::kanMX6 SUB1::HA::HIS*	This work
FY86 SUB1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 SUB1::HA::HIS*	This work
FY86 HOT::TAP PAB1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 HOT1-TAP::kanMX6 PAB1::HA::HIS*	This work
FY86 PAB1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 PAB1::HA::HIS*	This work
FY86 HOT::TAP PBP1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 HOT1-TAP::kanMX6 PBP1::HA::HIS*	This work
FY86 PBP1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 PBP1::HA::HIS*	This work
FY86 HOT::TAP RAT8::HA	*MATα leu2Δ1 ura3-52 his3Δ200 HOT1-TAP::kanMX6 RAT8::HA::HIS*	This work
FY86 RAT8::HA	*MATα leu2Δ1 ura3-52 his3Δ200 RAT8::HA::HIS*	This work
FY86 HOT::TAP YRB1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 HOT1-TAP::kanMX6 YRB1::HA::HIS*	This work
FY86 YRB1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 YRB1::HA::HIS*	This work
FY86 HOT::TAP YRA1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 HOT1-TAP::kanMX6 YRA1::HA::HIS*	This work
FY86 YRA1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 YRA1::HA::HIS*	This work
W303 RPB6::MYC	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 RPB6::MYC::kanMX6*	Paco Navarro
W303 RPB6::MYC HOT::HA	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 RPB6::MYC::kanMX6 [pRS313-HOTwt::HA]*	Paco Navarro
BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Lab Stock
BY4741 *cka1Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cka1::kanMX4*	Euroscarf
BY4741 *cka2Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cka2::kanMX4*	Euroscarf
BY4741 *gpp1Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 gpp1::kanMX4*	Euroscarf
BY4741 *gpp2Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 gpp1::kanMX4*	Euroscarf
BY4741 *ssk2Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 ssk2::kanMX4*	Euroscarf
BY4741 *rim11Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 rim11::kanMX4*	Euroscarf
BY4741 *kcs1Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 kcs1::kanMX4*	Euroscarf
BY4741 HOT1::HA	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 [pRS313-HOTwt::HA]*	This work
BY4741 *cka1Δ* HOT1::HA	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cka1::kanMX4 [pRS313-HOTwt::HA]*	This work
BY4741 *cka2Δ* HOT1::HA	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cka2::kanMX4 [pRS313-HOTwt::HA]*	This work
BY4741 *gpp1Δ* HOT1::HA	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 gpp1::kanMX4 [pRS313-HOTwt::HA]*	This work
BY4741 *gpp2Δ* HOT1::HA	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 gpp1::kanMX4 [pRS313-HOTwt::HA]*	This work

BY4741 *ssk2Δ* HOT1::HA	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 ssk2::kanMX4 [pRS313-HOTwt::HA]*	This work
BY4741 *rim11Δ* HOT1::HA	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 rim11::kanMX4 [pRS313-HOTwt::HA]*	This work
BY4741 *kcs1Δ* HOT1::HA	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 kcs1::kanMX4 [pRS313-HOTwt::HA]*	This work
FY86 HOT1::HA	*MATα leu2Δ1 ura3-52 his3Δ200 [pRS313-HOTwt::HA]*	This work
FY86 HOT1::HA *sub1Δ*	*MATα leu2Δ1 ura3-52 his3Δ200 sub1::kanMX4 [pRS313-HOTwt::HA]*	This work
FY86 SUB1::HA *hot1Δ*	*MATα leu2Δ1 ura3-52 his3Δ200 SUB1::HA::HIS hot1::ura3*	This work
BY4741 *sub1Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 sub1::kanMX4*	Euroscarf
BY4741 *hot1Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 hot1::kanMX4*	Euroscarf
BY4741 *spt4Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 spt4::kanMX4*	Olga Calvo
BY4741 SPT5-194 (ts)	MATa his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys-128d *spt5-194*	G. Hartzog
BY4741 *dst1Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 dst1::kanMX4*	Euroscarf
BY4741 *sub1Δ hot1Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 sub1::kanMX4 hot1::ura3*	This work
BY4741 *spt4Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 spt4::kanMX4 hot1::ura3*	This work
BY4741 SPT5-194 (ts)	MATa his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys-128d *spt5-194* **hot1::ura3**	This work

Table 3.4.S2. Oligonucleotides used in this work

Oligonucleotide	Sequence (5' to 3')	Use
HOT-TAP L	AAAAGAACCATTGGCTGGTTGCAAGAGAGCCTTGCTGGAATAACCATGGAAAAGAGAAG	TAP-tagging of *HOT1*
HOT-TAP R	AAAACAGCACTAACGATTTTTGTAGATTTACAGAATGTATTTATGGACTCACTATAGGG	"
HOT-200	GTATGGTAACAAGTGGAG	Confirmation of TAP-tagging of *HOT1*
HOT+200	CTAAGGGGCGATTTGTCA	"
OBS 292	TTGTATTTTCAGGGTGAGCTC	Confirmation of TAP-taggings
DELHOTURA A	TACAAAAAAGATTATATTTAGGGTACATATGGCTGGAGCATAATTCGGTAATCTCCGAGC	*HOT1* gene disruption with *URA3*
DELHOTURA B	TCCTTCCTATGATTGTAAACGATTATTTACTATCGTACGTGCCCCGGGTAATAACTGATA	"
URA3 UP	CTTAACTGTGCCCTCCAT	Confirmation of *HOT1* disruption
URA3 CHECK	GCGAAGAGCGACAAAGA	"
YHRPRO-2A	GTCCCTTTCTGACAATAAGACC	Chromatin inmunoprecipitation. PCR of *HGI1* promoter
YHRPRO-2B	TGTTTCTGGCGATCCCTTCG	"
STL1PRO-A	TTGGTTAATCCTCGCCAGGT	Chromatin inmunoprecipitation. PCR of *STL1* promoter
STL1PRO-B	TATGAGTGTGACTACTCCTG	"

STL1-1 5'	GGCTTCTCCCTGTTTGGATA	PCR of *STL1* 5' region
STL1-2 5'	CTACAGTTGCGTGTCTGTCA	"
STL1-1 CEN	TCGCCACGTTGGCTGATTTC	PCR of *STL1* centromeric region
STL1-2 CEN	TGTTTGGTCCTGTTAACAGC	"
STL1-1 3'	CGGAAGAAGTTTGGAGGAAA	PCR of *STL1* 3' region
STL1-2 3'	GGCATGATCTTCGACTTCTT	"
STL1-1 3' UTR	GGATCTTCTACTACATCAGC	PCR of *STL1* 3' UTR region
STL1-2 3' UTR	CACATACCAGAACCGTTATC	"
INT-A	GGCTGTCAGAATATGGGGCCGTAGTA	Chromatin inmunoprecipitation. PCR of an intergenic region
INT-B	CACCCCGAAGCTGCTTTCACAATAC	"
SUB-HA F2	AAATGAACAAGGCTGAAGACGACATAAGTGAAGAAG AACGGATCCCCGGGTTAATTAA	HA-tagging of *SUB1*
SUB-HA R1	ATATGAATTGTTAAAATGAATTTGACAAACAGAAAAG TAGAATTCGAGCTCGTTTAAAC	„
YRA1-F2	ATCTGGACAAGGAAATGGCGGACTATTTCGAAAAGAA ACGGATCCCCGGGTTAATTAA	HA-tagging of *YRA1*
YRA1-R1	TTTAAATTTTCTGGAAACTAATACATAATGAAACTCGG GAATTCGAGCTCGTTTAAAC	„
PAB1-HA F2	GTCTTTCAAAAAGGAGCAAGAACAACAAACTGAGCAA GCTCGGATCCCCGGGTTAATTAA	HA-tagging of *PAB1*
PAB1-HA R1	TAAGTTTGTTGAGTAGGGAAGTAGGTGATTACATAGA GCAGAATTCGAGCTCGTTTAAAC	"
PBP1-HA F2	ACCATGGCCACCATAATAGTAGTACCAGTGGCCATAA ACGGATCCCCGGGTTAATTAA	HA-tagging of *PBP1*
PBP1-HA R1	TAAAAGTTCCATTATTTATGGGTAAGTAAGTACCACTG GAATTCGAGCTCGTTAAC	„
RAT8-HA F2	AGTCAAACTATGGTGGTAACAGTACATGGGGAGGTCA TCGGATCCCCGGGTTAATTAA	HA-tagging of *RAT81*
RAT8-HA R1	ATCGAGATTCAGTATTTCATCGAAACATCGAAATCTTC GAATTCGAGCTCGTTTAAAC	„
YRB1-HA F2	AAGAATTTGAAAAAGCTCAAGAAATCAACAAAAAGGC TCGGATCCCCGGGTTAATTAA	HA-tagging of *YRB1*
YRB1-HA R1	TTTTATATAATTAAAGAGCAACTTTTCCTCACAGTAGA GAATTCGAGCTCGTTTAAAC	„

3.5. Capítulo 5

Response of yeast cells to high glucose involves molecular and physiological differences when compared to other osmostress conditions

RESEARCH ARTICLE

Response of yeast cells to high glucose involves molecular and physiological differences when compared to other osmostress conditions

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One sentence summary: This manuscript describes the main molecular and physiological differences in the response of yeast cells to osmostress due to high glucose when compared to sorbitol or salt.
Editor: Ian Dawes

ABSTRACT

Yeast cells can be affected by several causes of osmotic stress, such as high salt, sorbitol or glucose concentrations. The last condition is particularly interesting during natural processes where this microorganism participates. Response to osmostress requires the HOG pathway and several transcription factors, including Hot1, which plays a key role in high glucose concentrations. In this work, we describe how the yeast response to osmotic stress shows differences in accordance with the stress agent responsible for it. Compared with other conditions, under high glucose stress, delocalization of MAPK Hog1 is slower, induction of HOT1 expression is higher and Msn2/4 transcription factors are involved to a lesser extent. Besides, the transcriptomic analyses carried out with samples incubated for 30 min in the presence of high glucose or sorbitol reveal the presence of two functional categories with a differential expression between these conditions: glycogen biosynthesis and mobilization and membrane-anchored proteins. We present data to demonstrate that the cells treated with 20% (w/v) (1.11 M) glucose contain higher chitin levels and are more sensitive to calcofluor white and ethanol.

Keywords: Chitin; gene expression; Hot1; osmotic stress; *Saccharomyces cerevisiae*

INTRODUCTION

Osmotic stress is a well-studied adverse condition that can affect *Saccharomyces cerevisiae* (reviewed in Hohmann and Mager 2003; Martínez-Montañés, Pascual-Ahuir and Proft 2010; Saito and Posas 2012). During growth for the production of alcoholic beverages and other industrially relevant processes, cells must cope with osmotic stress given the high sugar concentrations (Attfield 1997; Bauer and Pretorius 2000).

The MAPK HOG (high osmolarity glycerol) pathway (Capaldi *et al.* 2008; Saito and Posas 2012; Brewster and Gustin 2014) allows S. *cerevisiae* cells to perceive and quickly respond to altered osmolarity. When a hyperosmotic stress condition is detected by an osmosensing system (the Sln1 or Sho1 branch of the pathway), MAPKK Pbs2 phosphorylates Hog1 in the cytosol and promotes its rapid nuclear accumulation (Ferrigno *et al.* 1998; Reiser, Ruis and Ammerer 1999; Tanaka *et al.* 2014). Hog1 regulates several unrelated transcription factors (Hot1, Msn1, Smp1, Gcn4, Skn7, Sko1, Msn2/4 and Rtg1/3), and each one is responsible for controlling the expression of a subset of osmoresponsive genes (Rep *et al.* 1999a,b, 2000; Posas *et al.* 2000; de Nadal and Posas 2008; Ni *et al.* 2009). There is also substantial evidence to demonstrate the involvement of the HOG pathway in the

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45 response to high sugar concentrations (Remize *et al.* 2003; Jiménez-Martí *et al.* 2011).

Most of the transcriptomic studies that have been carried out to understand the osmostress response have focused on salt and/or sorbitol as osmolytes (Gasch *et al.* 2000; Posas *et al.* 2000; 50 Rep *et al.* 2000; Causton *et al.* 2001; Hirasawa *et al.* 2006). They have reported changes in the expression of the genes involved in glucose, trehalose and/or glycogen metabolism, redox regulation and stress response.

Although the molecular response of yeast to high sugar con- 55 centrations shares common features when compared to other osmostress conditions, differences have been found, related to the functionality of the Protein kinase A pathway and the catabolite repression mechanism (Conrad *et al.* 2014 and references therein). Several transcriptomic analyses have focused on 60 the response to high sugar stress. Kaeberlein *et al.* (2002) described an increase in the expression of glycerol and trehalose biosynthetic genes in cells from strain PSY316 exposed to 20% (w/v) glucose. In a more recent study conducted by our group (Jiménez-Martí *et al.* 2011), an expression higher than 2-fold was 65 also found under similar conditions for genes that participate in response to chemical stimuli. Most of the genes showing the opposite behavior were related to oxidative phosphorylation, and both the Krebs and urea cycles, which suggests an additional repressing effect of increasing glucose concentration. 70 Some reports have described unexpected changes in gene expression, not found in the presence of other osmolytes, during processes of alcoholic fermentation in high sugar must, that affect for instance, genes regulated by carbon catabolite repression (Rossignol *et al.* 2003; Zuzuarregui *et al.* 2006; Mendes-Ferreira 75 *et al.* 2007; Marks *et al.* 2008) or some stress response genes (Pérez-Torrado *et al.* 2002; Jiménez-Martí *et al.* 2011). Marks *et al.* (2008) have described a partial attenuation of classic glucose repression during fermentation which would be the result of the response to both ethanol and oxygen in the environment. Ca- 80 paldi *et al.* (2008) suggested that differences in the response to osmotic stress due to high sugar can be related to the inhibition of Msn2/4 activity under these conditions, which shifts the Hog1-dependent expression program toward the genes regulated by Sko1 and Hot1. Accordingly, entry of Hog1 into the nu- 85 cleus upon this adverse condition would allow these transcription factors to be recruited in regulatory regions of stress responsive genes, to thus target the Hog1-RNA PolII complex to them (Cook and O'Shea 2012; Nadal-Ribelles *et al.* 2012).

Several studies have looked at genes and functions consid- 90 ered important to yeast during wine fermentation, when cells are exposed to high sugar concentrations. Marks *et al.* (2008) identified 223 genes, designated as fermentation stress response genes that were dramatically induced at various points during fermentation and are mainly associated with transport, or- 95 ganelle organization, protein modification, RNA metabolism, response to stress and transcription. Zakrzewska *et al.* (2011), using the yeast knockout collection to systematically investigate the genes involved in stress survival, found an inverse correlation between mutant growth rate and stress survival. Using 100 chemostat cultures, these authors have demonstrated that the reduction of growth rate leads to a redistribution of resources toward stress tolerance functions and have revealed an important role of the Rpd3 histone desacetylase in the adquisition of tolerance to conditions particularly considered by these authors, 105 such acetic acid and heat stress. More recently, Walker *et al.* (2014) have identified a set of 93 yeast genes (designated as Fermentation Essential Genes) whose deletion resulted in increases of at least 20% in the duration of fermentation. Genes involved in maintenance of vacuolar acidification, microautophagy and 110 sugar-related signaling are included in this set.

In post-transcriptional processes, differences between high glucose concentrations and other hyperosmotic stress conditions have also been described. The polysome profile results obtained in our laboratory indicate that the temporary shut- 115 down of translation under high salinity conditions described by Melamed, Pnueli and Arava (2008) also occurs with osmotic stress due to 20% (w/v) glucose, but over a shorter time (Gomar-Alba, Jiménez-Martí and del Olmo 2012). Most of the *S. cerevisiae* proteins involved in glycolysis and pentose phosphate path- 120 ways, and also peroxiredoxin, which participates in protection against oxidative stress insult, are upregulated under 20% (w/v) glucose (Pham and Wright 2008; Guidi *et al.* 2010).

Despite all this information about the response to several forms of osmotic stress, a direct comparative analysis between 125 the effects of glucose and sorbitol has not yet been reported. In this work, we provide the results of this study by considering several aspects which in this sense remain uncharacterized; e.g. entry in the nucleus of Hog1, Hot1 regulation and the changes in gene expression and yeast physiology that take place as a result.

MATERIALS AND METHODS 130

Yeast strains and growth conditions

All the *S. cerevisiae* strains used for these experiments are described in Table S1 (Supporting Information). Growth of yeast strains was carried out in YPD medium [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose] at 30°C with orbital 135 shaking (200 rpm). The yeast cells carrying plasmids were grown in SC medium (0.17% (w/v) nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) *Drop out* mix without the selected amino acid or nucleobase, 2% (w/v) glucose]. For strains with the *ade2* mutation (derived of W303– 140 1a), adenine was added at a final concentration of 20 μg mL^{-1}. The solid medium also contained 2% (w/v) agar.

For most experiments, yeast cultures were kept in the exponential growth phase for 16 h. Then cells were exposed to the considered osmotic stress conditions, these being 1.11 M (20% 145 (w/v)) glucose, 1 M sorbitol or 0.6 M NaCl. For this purpose, they were transferred to YPD or SC-derived media containing glucose as the carbon source and the above-mentioned osmolyte concentrations (YP20/SC20, YPS/SCS and YPNaCl/SCNaCl, respectively), and incubation continued for different times depend- 150 ing on each experiment. In the stress response studies, short times (under 90 min) were used. Growing cells were incubated for 48–96 h in the growth and viability, calcofluor white (CFW) microscopy and chitin determination experiments. Finally, incubation continued up to 12 days in the glycogen content anal- 155 ysis.

To determine yeast cell resistance to ethanol or CWF, dilution plating assays were carried out on YPD plates with 10% (v/v) ethanol (v/v), 20 μg CWF mL^{-1} or without any of these additives. For this purpose, 5 μL of the 10-fold serial dilutions of cultures 160 were spotted onto these plates and incubation was carried out at 30°C for 3 days.

Strain construction

The description of all the plasmids used in this work is included in Table S1 (Supporting Information). For the fluores- 165 cence microscopy experiments, the green fluorescent protein (GFP) epitope was added at the C-terminus of Hot1, Hog1

or Sko1 using the procedure described by Longtine *et al.* (1998) and the oligonucleotides indicated in Table S1(Supporting Information).

Gene expression analyses

For RNA isolation and quantification and cDNA preparation, protocols explained elsewhere were followed (Jiménez-Martí *et al.* 2011). cDNA samples were utilized for the gene expression analysis by semiquantitative or real-time RT-PCR, as previously described (Jiménez-Martí *et al.* 2011). The ACT1 gene was employed as a reference gene given its constitutive expression under osmotic stress due to high glucose concentrations (Jiménez-Martí *et al.* 2011).

For the microarray analyses, the strategy described in Jiménez-Martí *et al.* (2011) was adopted. In this case, the Cy3–Cy5 combinations for hybridization were YPD.2-YPS.2, YPS.3-YPD.3, YPD.2-YP20.2 and YP20.3-YPD.3. Reproducibility of replicates was tested by the ARRAYSTAT software (Imaging Research Inc., Ontario, Canada). The entire set of supporting microarray data has been deposited in the GEO (gene expression omnibus) database (http://www.ncbi.nlm.nih.gov/projects/geo/) with accession number GSE59659. To validate the microarray results, the expression of some genes was analyzed by semiquantitative RT-PCR. Table S1 (Supporting Information) includes the sequence of the oligonucleotides used in these amplification reactions.

Methods of protein manipulation and analysis

Protein extracts for the routine analyses and phosphorylation studies were prepared as described in Gomar-Alba, Alepuz and Olmo (2013). The primary antibody employed in this study was anti-GFP (Roche). Blots were developed with horseradish peroxidase-labeled secondary antibodies using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Little Chalfont, UK) and the ImageQuant LAS 4000 mini Biomolecular Imager instrument (GE Healthcare, Little Chalfont, UK).

Determination of chitin content

Flow cytometry experiments were carried out following the procedure described by Costa-de-Oliveira *et al.* (2013) with the samples obtained from the cultures of strain W303–1a after growth under the previously described conditions. Approximately, 2×10^6 yeast cells were collected, washed with PBS 1% (w/v) and incubated with 2.5 μg CFW mL^{-1} (Fluka, St. Louis, MO, USA) in a final volume of 500 μL for 15 min at 25°C. Then they were analyzed in a BD LSR Fortessa flow cytometer with the FACS-Diva software v7 (BD Biosciences, Franklin Lakes, USA). Fluorescence intensities were measured using excitation/emission at 355/433 nm.

To confirm the flow cytometry results, an epifluorescence microscopy analysis was performed following the procedure described by Costa-de-Oliveira *et al.* (2013). Yeast cells were grown and prepared as described for the flow cytometry assays, but were stained with 25 μg CFW mL^{-1} for 15 min. After placing the cell suspension on a glass slide, it was observed under an epifluorescence microscope (400×) *Axioskop 2* (Zeiss Inc., Jena, Germany), coupled with the AxioVision acquisition image system (Zeiss, Barcelona, Spain). Pictures were taken with a SPOT camera (Diagnostic Instruments Inc., Sterling Heights, USA).

Fluorescence microscopy

The yeast strains expressing proteins labeled with the GFP were grown to OD$_{600}$ 0.4 and transferred to YP20, YPS and YPNaCl. The cells from these cultures were observed every five minutes under the above-described fluorescence microscope.

Glycogen content determination

For these experiments, cells (10 mg of dry weight) were collected at several growth stages from cultures in YPD, YPS and YP20 media. The glycogen content in these samples was determined following the protocol described by Pérez-Torrado, Gimeno-Alcañiz and Matallana (2002) with no modifications. For this purpose, a commercial *Aspergillus niger* amyloglucosidase (Boehringer Mannheim GmbH) was used for enzymatic breakage of glycogen and the glucose oxidase–peroxidase reaction (Boehringer Mannheim GmbH) for glucose analysis.

RESULTS

Nuclear localization of MAPK Hog1 is more short-lived in the presence of sorbitol than under similar osmolarity conditions caused by glucose or salt

As reviewed in the section 'Introduction', considerable evidence sustains the involvement of the HOG pathway, and, of Hog1 phosphorylation in particular, in the response to high sugar concentrations (Remize *et al.* 2003; Jiménez-Martí *et al.* 2011), similarly to what occurs under other osmotic stress conditions. As activation of this MAPK by Pbs2p results in its nuclear accumulation (Ferrigno *et al.* 1998; Reiser, Ruis and Ammerer 1999), the first of our analyses focused on determining putative changes in the kinetics of Hog1 localization when 20% (w/v) glucose was the agent responsible for osmotic stress. For this purpose, fluorescence microscopy experiments with yeast cells expressing Hog1-GFP were done after osmotic shock caused by 1.11 M glucose, 1 M sorbitol or 0.6 M NaCl (YP20, YPS and YPNaCl media, respectively). These concentrations were considered because they result in similar water activity values (Chen 1989; Comesaña, Correia and Sereno 2001). According to the data obtained (Fig. 1), Hog1 was detected in nuclei in 5–15 min after osmotic shock; the protein was delocalized at longer times. A close inspection of the results found in the presence of the osmolytes considered herein indicated that the stress caused by sorbitol resulted in quicker Hog1 delocalization, which can be clearly detected even at 15 min. At this time point, the location of MAPK was strictly nuclear in around 10% of the cells in the presence of 1 M sorbitol, in approximately 50% under 0.6 M NaCl and in more than 90% when 1.11 M glucose was used as an osmolyte.

Regulation of the Hot1 transcription factor is affected by the nature of the osmolyte

Although Hog1 regulates several transcription factors, Sko1, Hot1 and Msn2/4 are required for 88% of Hog1-dependent gene activation, and Hot1, together with Sko1, is the main Hog1-regulated transcription factor involved in response to osmotic stress caused by high sugar concentrations (Capaldi *et al.* 2008). In order to ascertain whether the nature of the osmolyte can affect HOT1 and SKO1 expression or not, real-time RT-PCR analyses were carried out in strain W303–1a under the three osmotic stress conditions tested in this work. The expression of Hot1 was slightly higher (around 5-fold) when using 20% (w/v) glucose
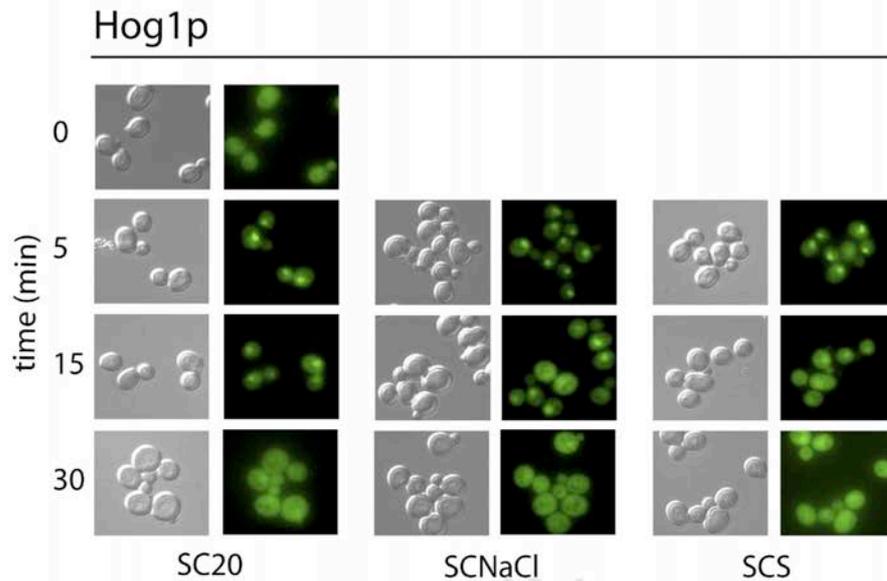
Figure 1. The kinetics of Hog1 entry into the nucleus in the presence of different osmolytes. Cells from the cultures in the exponential growth phase of strain FY86 in SC medium were transferred to fresh SC medium containing 1.11 M (20% (w/v)) glucose (SC20), 0.6 M NaCl (SCNaCl) and 1 M sorbitol (SCS), and were observed by fluorescence microscopy (GFP signal) at the indicated times. Differential interference contrast (DIC) images are also included. Time 0 corresponds to the time of culture transfer. The figure shows representative images of the obtained results.

as an osmolyte than when similar osmotic stress was caused by sorbitol or salt (around three to four times, Fig. 2A). In all three cases, however, the induction kinetics was similar, with the maximal expression values being found 30 min after applying the stress. For *SKO1* (Fig. 2B), the expression levels increased somewhat (around 2-fold) under all the tested conditions and no significant differences according to the nature of the osmolyte were observed.

According to the data found in the literature (Harbison *et al.* 2004; Reimand *et al.* 2010; Huebert *et al.* 2012), several of the proteins involved in stress response (Msn2, Skn7, Cin5, Tup1 and Spf1) play a role in the transcription of *HOT1*. Under the adverse conditions considered herein, the expression of this gene was analyzed in the mutants in all these factors by real-time RT-PCR. For this purpose, and after considering that its maximal expression was found after 30 min of osmostress (see above), this time was considered. Spf1 and Cin5 were not involved in the regulation of *HOT1* expression under osmotic stress but an interplay was noted between Msn2/4, Skn7 and Tup1 in this control (Fig. 2C). Some statistically significant differences appeared for the involvement of all these transcription factors according to the nature of the osmolyte: under osmostress due to high glucose and salt concentrations Msn2/4 partially controlled *HOT1* expression (transcription reduced to half in strain *msn2Δmsn4Δ* compared to its wild type), while when osmotic stress was caused by high glucose and sorbitol Tup1 (expression reduced to 65–80%, depending on the osmolyte) and Skn7 (reduced expression to around 80%) participated in *HOT1* regulation. It is worth mentioning that *HOT1* expression under salt stress increased in the absence of Tup1.

Additional controls were carried out with other genes regulated by these transcription factors (Fig. 2D), to confirm that the considered mutants displayed the expected patterns. The expression of gene *HSP26*, which is completely dependent on Msn2 under salt stress (Amorós and Estruch 2001), lowered to 20% under this condition, to 40% under sorbitol and to 70% when glucose was used as an osmolyte. Accordingly, the expression of *HOT1* and *HSP26* was similarly controlled by Msn2 under high glucose stress, but the contribution of this transcription factor was greater under salt stress for the latter gene. The expression of *CTT1*, a Tup1-controlled gene (Márquez *et al.* 1998; Proft and Struhl 2002), followed the expected pattern according to its regulation in a *tup1Δ* mutant (Fig. 2E), and its mRNA levels lowered substantially more than those corresponding to *HOT1* under all the tested conditions. Similar results were obtained for another Tup1-regulated gene, *GPD1* (data not shown).

Nuclear localization of Msn2 is more short-lived in the presence of high glucose than under similar osmolarity conditions caused by sorbitol or salt, while no changes are detected for Hot1 and Sko1

According to the results shown in Fig. 3A, Hot1 was always located in the nucleus, irrespective of the osmostress agent considered. Analyses were also carried out for the other two Hog1-regulated transcription factors, Sko1 and Msn2, which are also involved mainly in recruiting RNA polymerase II to osmostress-induced genes. Sko1 was located in the nucleus under all the tested conditions (Fig. 3B). For Msn2 (Fig. 3C), and as previously described (Görner *et al.* 1998), the protein entered the nucleus transiently, but became delocalized after a few minutes if osmotic shock was caused by 20% (w/v) glucose. In fact, 15 minutes after introducing this osmolyte, the percentage of cells with the protein in this compartment was very low (less than 10%), while 60–70% of the cells still contained this transcription factor in the nucleus if salt was present. An intermediate value (30%) was found when stress was due to sorbitol.
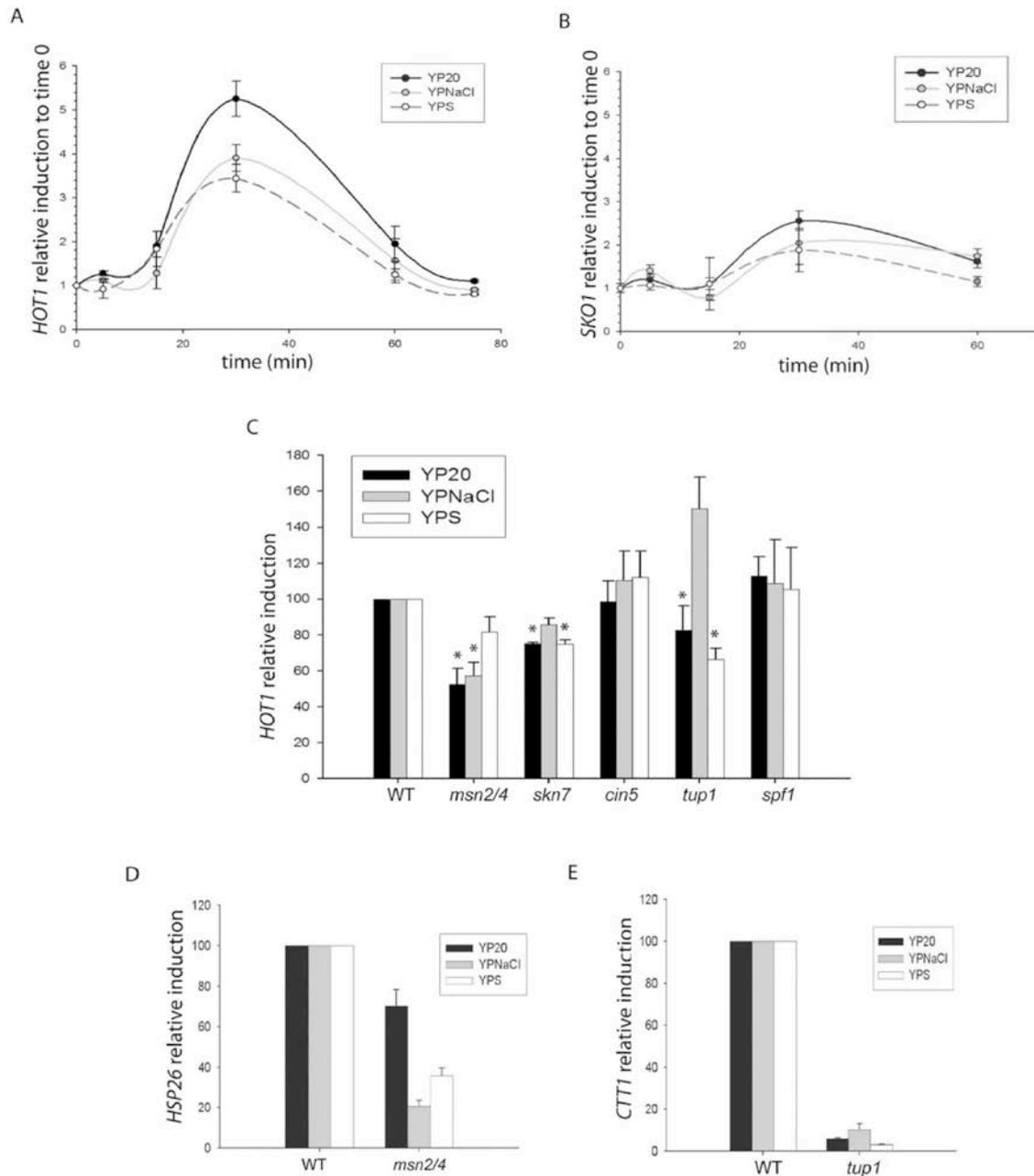
Figure 2. Expression of HOT1 and SKO1 under osmostress caused by glucose, sorbitol or salt. Cells from exponential cultures grown at 30°C in YPD medium of several yeast strains were transferred to YP20 (YPD-derived medium containing 1.11 M (20% (w/v)) glucose), YPS (YPD-derived medium with 1 M sorbitol) or YPNaCl (YPD-derived medium with 0.6 M NaCl) for 30 min before total RNA was isolated and cDNA was prepared. In all cases, gene expression was determined by real-time RT-PCR and data were normalized with the ACT1 gene. Quantifications were carried out from the data obtained in three independent experiments; the average value and the standard deviation appear. Panels A and B show changes at the HOT1 and SKO1 mRNA levels, respectively, in strain W303–1a under the three stress conditions indicated above. Values shown are relative to those found at the time of cell transfer to YP20, YPNaCl or YPS (time 0). In panel C, HOT1expression was determined using BY4741 or W303–1a mutants of MSN2/4, SKN7, CIN5, TUP1 and SPF1. In this case, the data shown are relative to the value found in the samples obtained from the cultures grown in YPD simultaneously and to that found in the corresponding wild-type strain (W303–1a for msn2/4Δ and BY4741 for the other mutants). The figure also includes the expression of both the HSP26 gene in the msn2/4Δ mutant (panel D) and the CTT1 gene in a tup1Δ mutant (panel E) when yeast cells were exposed to different osmostress conditions. Values in panels D and E were determined as in panel C. Asterisk indicates those conditions in Fig. 2C in which statistically significant differences were found when compared to the third condition (P-value ≤ 0.05 calculated with the Student's t-test facility of the Microsoft Excel program considering two tails and unequal variance).
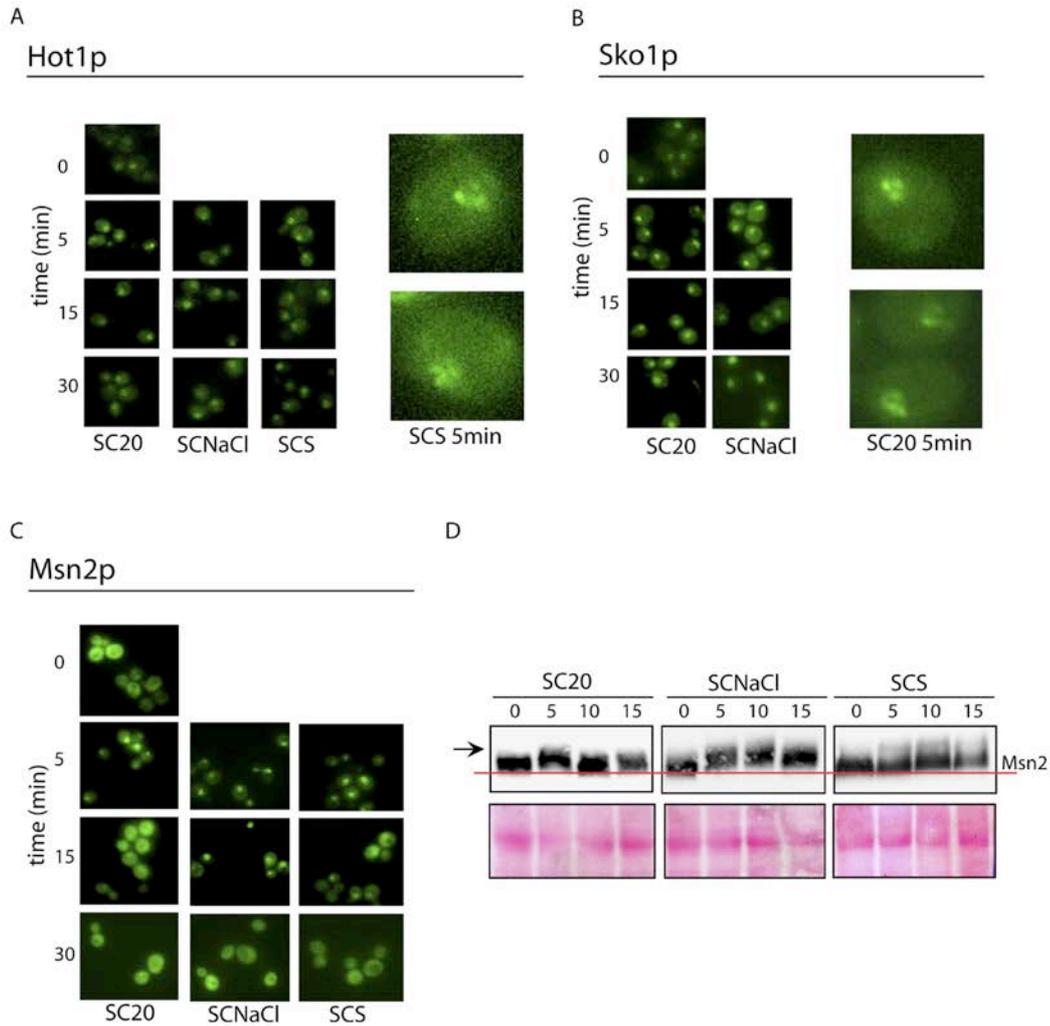
Figure 3. Location of Hot1 (panel A), Sko1 (panel B) and Msn2 (panel C) under several osmostress conditions. Cell cultures were prepared, treated and analyzed as described in Fig. 1, and GFP fluorescent signals are shown. This figure also includes a western blot representative image (panel D) showing the detection of the hyperphosphorylated forms of Msn2 in the samples obtained at different incubation times in SC20, SCNaCl and SCS, and applied to a 7% polyacrylamide-SDS gel. These samples correspond to the extracts prepared as described in the Materials and Methods section from strain W303–1a *msn2Δmsn4Δ* that expresses the GFP-tagged Msn2 protein. Detection was carried out with anti-GFP (Roche). The arrow shows the position of the hyperphosphorylated forms of Msn2, which show a shift when compared to the forms that had lost this high degree of phosphorylation (horizontal bar).

Garreau et al. (2000) described a direct correlation between hyperphosphorylation of Msn2/4p in both diauxic transition and heat shock, and also induced transcriptional activity of these factors and nuclear localization. Under osmotic stress conditions, Msn2 phosphorylation followed a pattern which correlated with the location data: under high glucose stress, phosphorylation reduced sooner than when stress was due to sorbitol or salt (Fig. 3D). Actually, after 10 min of high sugar osmostress, the hyperphosphorylation of Msn2 significantly reduced while under sorbitol stress this occurred at 15 min, and longer times were required in the presence of 0.6 M NaCl. Hence,

this result suggests that Msn2 is less involved in the regulation of osmostress genes under high glucose concentrations and agrees with the data reported previously by Capaldi et al. (2008).

A punctate pattern in the nucleus was detected for Hot1 and Sko1 (see the enlarged images in panels A and B of Fig. 3), which suggests that these transcription factors are distributed in speckles or interchromatin granule clusters. In actively transcribing cells, Zeng et al. (1997) described that transcription and splicing factors are dispersed throughout the nucleus with abundant sites of preferred localization.

Table 1. Genes considered for microarray data validation by semiquantitative RT-PCR.

Functional category	Gene	YP20 (1)	YPS (2)	Description
Proteins anchored to membrane	YGR189C (CRH1)	4.055	0.575	Chitin transglycoxylase
	YNL192W (CHS1)	3.102	0.600	Chitin synthase I
Energy reserve	YKR058W (GLG1)	0.575	3.365	Glycogenin glucosyltransferase (initiator of glycogen synthesis)
	YFR015C (GSY1)	0.325	3.310	Glycogen synthase

(1) Value corresponding to the ratio between the expression in YP20 (YPD-derived medium containing 1.11 M (20% (w/v)) glucose and YPD in accordance with the obtained microarray analysis data.
(2) Value corresponding to the ratio between the expression in YPS (YPD-derived medium containing 1 M sorbitol) and YPD in accordance with the obtained microarray analysis data.

Differences in global gene expression between 1.11 M glucose and 1 M sorbitol

Once the transcription factors involved in the response to osmotic stress have been activated, they are capable of recruiting the transcription machinery to the gene promoters that contain appropriate cis-acting elements to allow their expression. Hirasawa *et al.* (2006) described slight differences between transcriptional responses to osmotic stress caused by NaCl and sorbitol by DNA microarray technology. As a direct comparison between responses to high glucose and sorbitol has not yet been described, we followed the same approach for this purpose in this work. The exponentially growing yeast cells of strain W303–1a were transferred to the same medium (YPD) or to this medium containing 1 M sorbitol (YPS) or 1.11 M (20% (w/v)) glucose (YP20), and incubation continued for 30 min. Afterward RNA was isolated and processed as described in the section 'Materials and Methods'.

From the whole set of microarray data obtained, we considered that genes differentially upregulated in high glucose should sequentially meet three conditions: (i) show expression levels higher than 3-fold in YP20 than in YPD (this occurred for 647 genes), (ii) show higher values in YP20 than in YPS (this was the case for 81 genes out of those 647) and (iii) show expression levels lower than 1.5-fold in YPS than in YPD. The last condition could only be applied to 18 genes of the previous group. Accordingly, the expression levels of these 18 genes (known as Group 1 from this point onward; Table S2, Supporting Information) increased after osmotic shock caused by high glucose concentrations, but not under similar water activity conditions due to sorbitol. The application of the FUNC ASSOCIATE tool (http://llama.mshri.on.ca/funcassociate/) allowed the determination of statistically significant functional categories, in which the genes of this group were included. Only one was detected in our analysis, which corresponded to 'membrane anchored proteins'. It contains 61 genes and includes YGR189C (CRH1),

YJL171C, YLR194C and YNL192W (CHS1). The proteins encoded by the first and the last of these genes are required for chitin biosynthesis. A *P*-value of 2.19×10^{-5} supported the statistical significance of this functional category. It is worth mentioning that Group 1 included two more genes related with yeast cell wall organization: YGR032W (GSC2) and YJR150C (DAN1).

In addition, 1138 genes showed 3-fold higher expression values in YPS than in YPD. Of these genes, 309 displayed higher levels in YPS than in YP20, and the expression level in the latter was lower than 1.5-fold for 57 of them when compared to YPD. This group of 57 differentially upregulated in sorbitol genes (Table S3, Supporting Information) referred to Group 2 in this work. In this case, the statistically overrepresented category (*P*-value of 5.79×10^{-7}) corresponded to the 'glycogen metabolic process'. Of the 29 genes included in this category, YEL011W (GLC3), YFR015C (GSY1), YFR017C, YKR058W (GLG1) and YPR160W (GPH1) appeared in our microarray data as being upregulated, but only under osmotic shock caused by sorbitol. This group also included several genes that encode mitochondrial ribosomal proteins.

To validate the data obtained in this microarray analysis, the expression of some of the above-described differentially expressed genes was followed by semiquantitative RT-PCR. The genes selected for this purpose are included in Table 1 and they gave the widest variations in expression according to the conditions tested. The obtained results are shown in Fig. 4, and they are consistent with the data described above and with the information shown in this table.

HOG1-dependent transcription factors Msn2/4, Sko1 and Skn7 are responsible mainly for the control of genes specifically upregulated at high glucose concentrations

A close inspection of the genes belonging to Group 1 (upregulated in high glucose, but not in sorbitol), for which the YEAS-
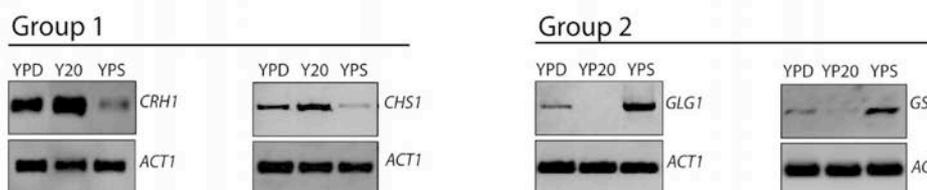

Figure 4. Validation of the microarray data by semiquantitative RT-PCR. Samples were prepared as described in Fig. 2 from the exponentially growing cultures of the strain W303–1a transferred for 30 min to YPS or YP20. Panel A: Group 1. Genes differentially upregulated in 1.11 M glucose. Panel B: Group 2. Genes differentially upregulated in 1 M sorbitol. The oligonucleotides used for the PCR reactions are described in Table S1 (Supporting Information). Experiments were carried out in triplicate. This figure shows the result of a representative experiment. As a reference, the results obtained for the ACT1 gene are included.
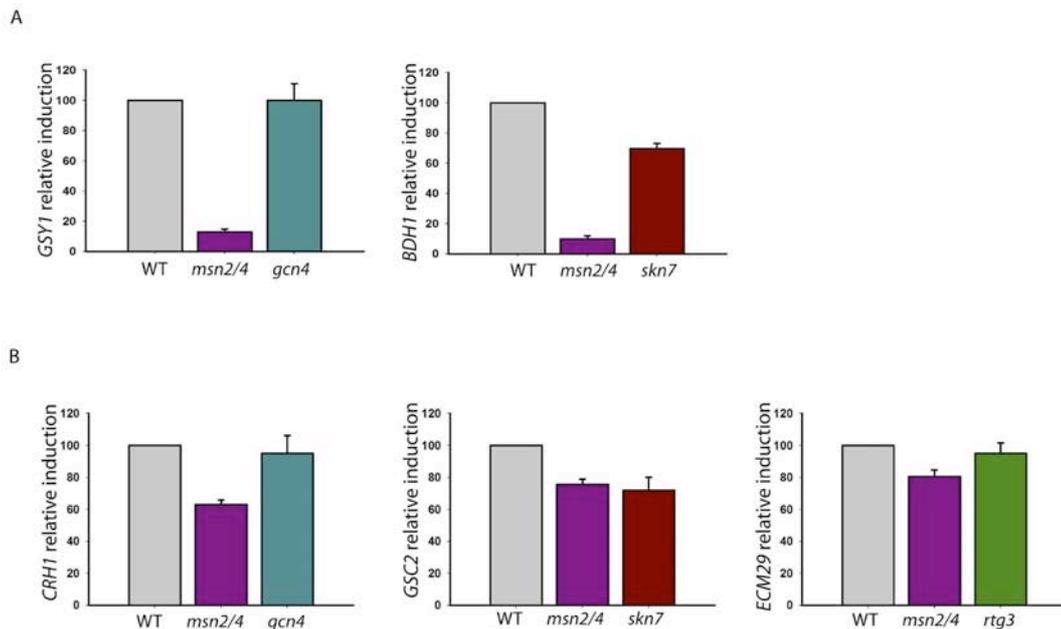
A

B

Figure 5. Contribution of Msn2/4, Gcn4, Rtg3 and Skn7 to the upregulation of several genes belonging to Groups 1 and 2 under the osmotic stress caused by 1 M sorbitol or 1.11 M glucose. This figure shows the data obtained for Group 2 genes *GSY1* and *BDH1* (panel A) and Group 1 genes *CRH1*, *GSC2* and *ECM29* (panel B). The cells from exponential cultures grown at 30°C in YPD medium of several yeast strains were transferred to YP20 (Group 1 genes) or YPS (Group 2 genes) for 30 min before total RNA was isolated and cDNA was prepared. In all cases, gene expression was determined by real-time RT-PCR and data were normalized with the ACT1 gene. The values shown are relative to those found in the samples grown simultaneously in YPD and to the ones obtained in the corresponding wild-type strain (W303–1a for *msn2/4Δ* and BY4741 for the other mutants), which have been expressed as 100%. Quantifications were carried out from the data obtained in three independent experiments; the average value and standard deviation appear.

TRACT tool (http://www.yeastract.com/rankbytf.php) was used, indicated that almost all of them require Msn2/4 (100% are Msn2
440 dependent and 72% Msn4 dependent) for their expression, 33% are regulated by Sko1, and the Hot1 transcription factor participates in regulation of only one (CHA1). Other HOG1-regulated transcription factors also seemed important in controlling of the expression of this group of genes: Gcn4 (56%), Skn7 (28%) and
445 Rtg3 (22%). Table S4 (Supporting Information) shows the contribution of all these transcription factors to the Group 1 genes according to YEASTRACT and references therein.

For group 2 (upregulated in sorbitol, but not in 20% (w/v) glucose), most are regulated by Msn2/4 (around 81% for Msn2 and
450 65% for Msn4) while only two genes (GSY1 and GLG1) depend on Hot1 for their induction, and an intermediate value is found for the Sko1 targets (25%). Once again, Gcn4 (67%) and Skn7 (25%) play a role in controlling the expression of many of these genes. Rtg3 has no targets in this group.

455 The information described above and obtained from YEAS-TRACT is based in most cases on global gene expression analyses. To determine the contribution of transcription factors Msn2/4, Gcn4, Rtg3 and Skn7 to the transcription regulation of the genes belonging to Groups 1 and 2 under the experi-
460 mental conditions considered herein, real-time RT-PCR analyses were carried out with the samples obtained from the mutants in these factors and their corresponding wild-type strains. For Group 1, CRH1, GSC2 and ECM29 were considered because they are representative of the involvement of Gcn4, Skn7 and Rtg3,
465 respectively, in controlling their expression (see Table S4, Sup-

porting Information); besides, they are all targets of Msn2/4. For Group 2, GSY1 (regulated by Msn2/4 and Gcn4) and BDH1 (controlled by Msn2/4 and Skn7) were analyzed.

As shown in Fig. 5, the expression of the Group 2 genes considered (panel A) was absolutely dependent on Msn2/4 for them
470 to be upregulated under the osmotic stress caused by 1 M sorbitol, while Skn7 contributed partially and Gcn4 was not involved at all. However, in Group 1 (panel B), the involvement of the Msn2/4 transcription factors to control gene expression under high glucose stress was only partial (around 60–80% of
475 the mRNA levels were retained in the mutant, depending on the gene considered), Skn7 and Gcn4 played a similar role as under sorbitol stress (partial and null respectively), and Rtg3 did not appear to contribute to ECM29 regulation.

According to these results, the role of transcription factors
480 Msn2/4 appear to be less important under high glucose stress, although still partially contribute to the expression of their target genes. Besides, Skn7 becomes also relevant, at least in those genes specifically upregulated under this condition.

The physiological effects on yeast cells of exposure to
485 ### 1.11 M glucose or 1 M sorbitol as osmolytes

The microarray analyses results suggest that the osmotic shock produced by 1.1 M glucose can result in differences in the glycogen and chitin levels when compared to that caused by sorbitol. When osmotic stress was due to 1.11 M glucose (YP20), very high
490 glycogen levels were detected and were maintained for at least

A

B
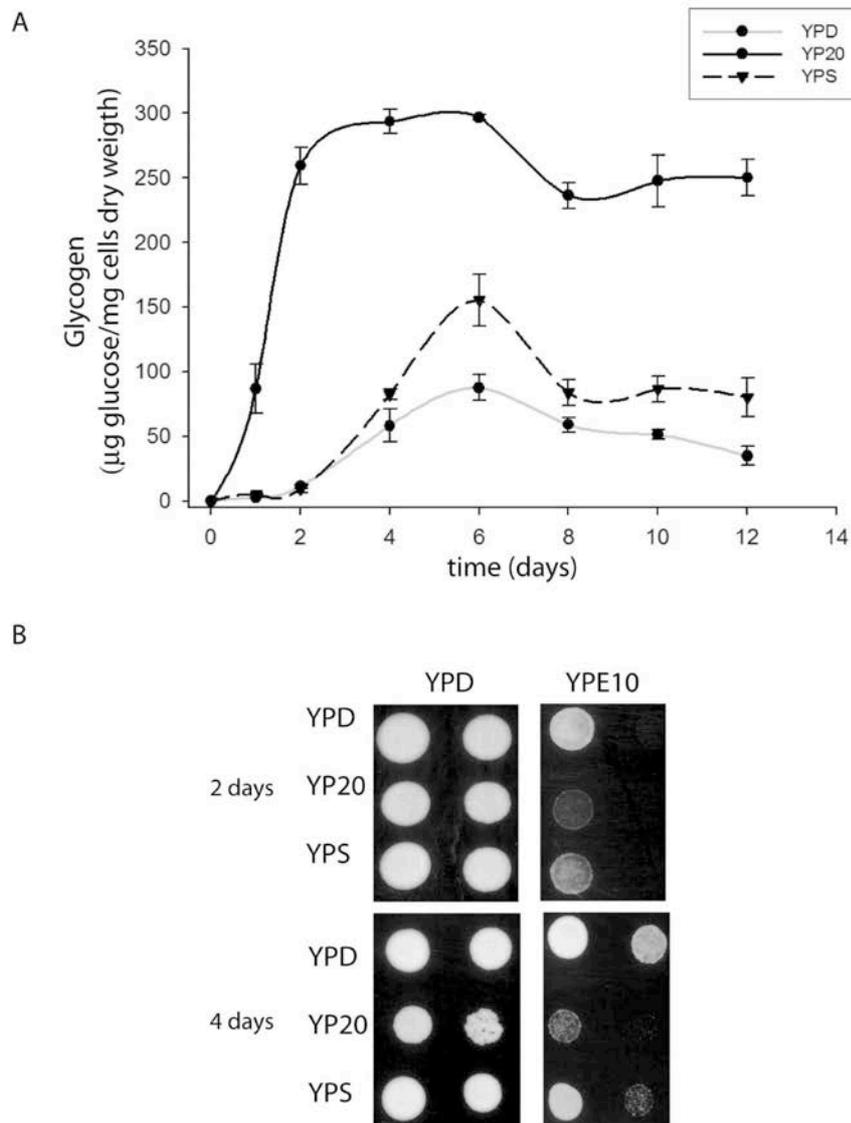
Figure 6. The effect of long-term exposure to the high sorbitol or glucose concentrations on glycogen levels and stress resistance in strain W303–1a. Panel A: comparison of the kinetics of glycogen levels in YPD or in derived media with high concentrations of glucose (YP20) or sorbitol (YPS). Experiments were carried out as described in the 'Materials and Methods' section and were repeated at least three times. This figure shows the amount of glucose released from glycogen and, for each piece of data, the mean and standard deviation are included. Panel B: resistance to ethanol of the cells incubated for 2 and 4 days in YPD, YP20 and YPS, as determined by plate dilution assay in YPD plates containing 0% or 10% (v/v) ethanol (YPD and YPE10 respectively). This figure shows the result of a representative experiment in which 10-fold serial dilutions from the cultures were applied on those plates and incubation continued for 3 days at 30°C.

12 days after shock, with maximal levels held around 4–6 days (Fig. 6A). For YPD, these levels increased from the time of entry into the stationary phase. In the presence of sorbitol as osmolyte and 2% (w/v) glucose as a carbon source (YPS), the changes in glycogen amounts were similar to those found in YPD but levels were higher after 2 days. All these results indicate that yeast cells show a higher capacity to synthesize and mobilize glycogen in the presence of 1 M sorbitol, when GSY1 and GPH1 genes are more expressed. Due to the relationship between glycogen availability and stress resistance (Pérez-Torrado, Gimeno-Alcañiz and Matallana 2002), the sensitivity of the cells transferred to YPD, YP20 or YPS to stress due to 10% ethanol was determined. The

cells affected by 1 M sorbitol for 2 and 4 days were more resistant to ethanol than those incubated for the same time in a high glucose concentration (Fig. 6B), being the survival improved at 4 days, when increased glycogen levels were found. The highest resistance was displayed by the cells grown in YPD.

CWF is a fluorescent dye that preferentially binds to polysaccharides containing β-1,4-linked D-glucopyranosyl units (Wood 1980) which, in yeast, binds primarily to chitin (Elorza et al. 1993). Flow cytometry analyses using CFW were carried out to determine putative differences in the number of molecules per cell of this polysaccharide (Fig. 7A). In these experiments, the population on the left corresponded to the cells with no reagent incorporated; this population can be found only for the control in which CFW was not included. The peak on the right was due to the chitin found on the cell surface, to which CFW was able to bind. It was the only population found in the other images included in this figure. The position of the peak is related to the number of chitin molecules. Thus, higher chitin content is indicated by a longer distance of the peak on the right. As seen in this figure, when glucose was used as an osmolyte (YP20), for times of 24 h and beyond, this peak clearly appeared toward higher values, compared to when sorbitol was added (YPS). In the case of YPD although the peak was in a position very similar to that found for YP20, the shape is indicative of a very heterogeneous population of cells regarding chitin content, and the number of cells with high fluorescence intensity is lower than in YP20.

Cellular chitin content was also examined microscopically by staining cells with CFW. In agreement with previous reports (Imai et al. 2005, and references therein), the fluorescent dye bound strongly to bud necks and scars in cells grown for 48 h in YP medium containing 2 or 20% (w/v) glucose (Fig. 7B), although in the former case important differences between cells were found. However, when this incubation was carried out in the presence of sorbitol, staining at bud necks and scars became weak, and fluorescence appeared more diffuse. The presence of lower chitin levels can be confirmed by analyzing resistance to CFW. As CFW is toxic to yeast cells (Roncero and Durán 1985), mutants with low chitin levels exhibit a CFW resistance phenotype. The data shown in Fig. 8 coincide with the results described above as the cells incubated for 48 h in 1 M sorbitol (YPS) displayed more resistant to this dye than those grown in 1.11 glucose (YP20) for the same time.

DISCUSSION

This work has focused on changes that occur in yeast cells when they are affected by osmotic stress due to high glucose concentrations by comparing with what happens when the same degree of osmotic stress (water activity of 0.981; Chen 1989) is caused by sorbitol. The relevance of this stress condition for many biotechnological processes makes its consideration very interesting. To our knowledge, no similar work has been carried out previously. In some cases, we also made comparisons with the osmotic stress due to 0.6 M NaCl. We describe differences between osmostress due to high glucose concentrations and that caused by other osmolytes in all the stages involved in the molecular response to adverse conditions: from activation of the HOG pathway to synthesis of functional proteins with metabolic or cell wall organization roles.

The comparative analyses reported here between transcriptomic responses to high glucose and sorbitol have revealed two categories of genes that become statistically overrepresented under different conditions: cell wall organization (for the genes that were specifically upregulated under high glucose stress) and glycogen biosynthesis and mobilization (for the genes that were upregulated in 1 M sorbitol but not in 1.11 M glucose). Two of these genes (CHS1 and GSY2) belong to the 'Fermentation Stress response Genes' (FSG) described by Marks et al. (2008). Experiments have been carried out to determine if a correlation exists between the transcriptomic data and yeast physiology. Glycogen content has been determined in YPD, YP20 and YPS. The results shown in Fig. 6A indicate that the levels were much higher in YP20 than in YPS and YPD. Glycogen content depends on enzymes Gsy2 (glycogen synthase) and Gph1 (glycogen phosphorylase), whose activity is regulated by glucose repression (François, Villanueva and Hers 1988). The results found in YPD correlated with the data available in the literature because glycogen content was low in cells in the exponential growth phase, but increased as cells approached the stationary phase (Pérez-Torrado, Gimeno-Alcañiz and Matallana 2002). This pattern was similar for YPS and much higher levels were found in YP20, because of glycogen mobilization defects. It is worth mentioning that the cells grown in 1 M sorbitol were more resistant to the application of another stress condition, e.g. 10% ethanol stress (Fig. 6B), than those incubated in the presence of 1.1 M glucose, due probably to their better ability to mobilize glycogen (Pérez-Torrado, Gimeno-Alcañiz and Matallana 2002). Growth defects were detected in YPS (data not shown) due to the high osmotic stress applied. This could explain the better ability of cells grown in YPD to survive in the presence of ethanol despite the similar glycogen content found in both media at the times considered.

Another interesting difference detected between glucose or sorbitol used as osmolytes is related to changes in the cell wall, which appeared to be more affected when osmotic stress was due to sorbitol. According to the expression of the chitin biosynthesis genes being lower in sorbitol than in glucose, the amount of this polymer lowered in sorbitol, as shown by the FACS and microscopic analyses (Fig. 7). Although chitin constitutes only 2–3% of the cell wall, it plays a vital role in S. cerevisiae. Chitin and other cell wall components (β-1,3- and β-1,6-glucan and mannoproteins) are dynamically controlled and highly regulated by stress and the cell cycle. When they decrease in one component, they are compensated by increases in others (Shaw et al. 1991; Popolo et al. 1997; Ram et al. 1998). It is noteworthy that sensitivity to CFW closely relates to chitin content: mutants with increased chitin levels show greater sensitivity to this reagent, while reduced chitin content makes cells more resistant to it (Ram et al. 1994, 1998). In agreement with this, more resistance to this compound was detected in the cells incubated for 48 h in the presence of 1 M sorbitol than when exposed to a high glucose stress for the same time (Fig. 8).

The complete microarray data analysis provides some other interesting gene expression control features under hyperosmotic stress. According to the information about many of the genes that appear in Table S3 (Supporting Information; corresponding to Group 2, upregulated in YPS, but not in YP20) available in SGD (Saccharomyces Genome Database, http://www.yeastgenome.org/) and the ability of the Mig1p repressor to bind to their promoters (YEASTRACT, http://www.yeastract.com/rankbytf.php), their expression is controlled by carbon catabolite repression. In fact, some of these genes encode the proteins involved in respiration and its control or in glucose sensing (MTH1, HAP2, RGI2 and COX5B). This finding reinforces the usefulness of the data obtained in the microarray analyses. Besides, it suggests an increased carbon catabolite repression under the growth conditions considered in this work,
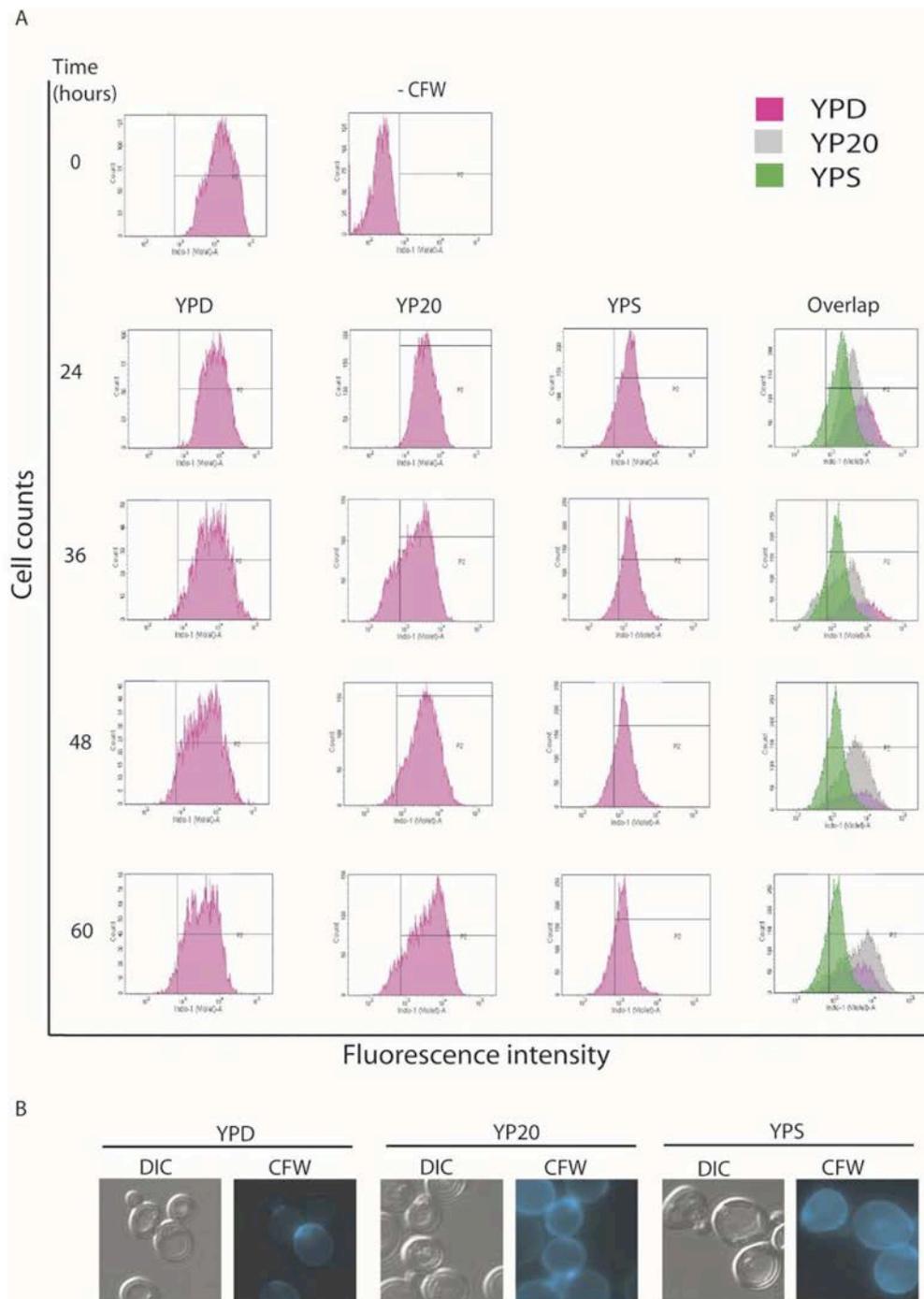
Figure 7. Determination of the chitin content of yeast cells after long-term incubation in media containing 0.11 M glucose (YPD), 1.1 M glucose (YP20) or 1 M sorbitol (YPS). Panel A: FACS (Fluorescence-activated cell sorting) analyses following the procedure described in the 'Materials and Methods' section. Experiments were carried out at least in triplicate and this figure shows the result of a representative analysis followed for 60 h after adding the osmostress agent. A total of 2.5 μg CFW (Calcofluor white) mL^{-1} were used as a final concentration. An overlap between the images corresponding to each condition at each time is included on the right. A negative control with no CFW addition is included at the top. For each sample, 10 000 cells were counted by the citometer. Panel B: fluorescence microscopy visualization of the chitin content in the cells incubated in the presence of the osmostress agents considered for 48 h and then treated for 15 min with 25 μg CFW mL^{-1} as a final concentration. The images corresponding to fluorescent signal (CFW) and differential interference contrast (DIC) are shown.
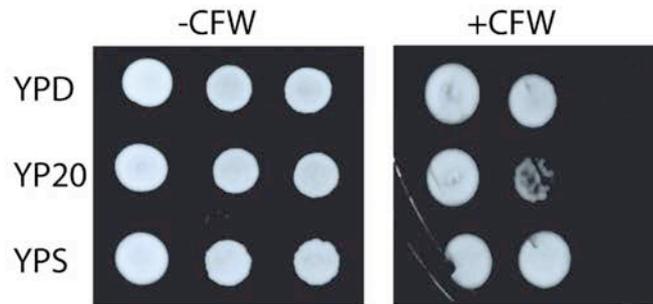
Figure 8. Resistance of the cells incubated for 48 h in YPD or in derived media containing 1.11 M glucose (YP20) or 1 M sorbitol (YPS) to CFW (calcofluor white). A total of 5 μL of the 10-fold serial dilutions of the cultures were spotted on YPD plates with (+) or without (−) 20 μg CFW mL^{-1}. Plates were incubated at 30°C for 3 days. Experiments were carried out in triplicate. This figure shows the result of a representative experiment.

with glucose concentrations higher than 2% (w/v), and helps identify new genes controlled by this mechanism.

The analyses performed in this work provide new features about the transcription factors involved in the osmostress response. Differences were observed in the mRNA and protein levels of one of the transcription factors which is more clearly involved in the response to high glucose concentrations (Capaldi et al. 2008): Hot1 (Fig. 2). A previous work described that *HOT1* expression increases 2-fold when yeast cells are affected by osmotic stress due to 0.7 M NaCl (Rep et al. 1999a). Our results confirm this result and indicate that the maximal expression of this transcription factor is higher in the presence of a high glucose concentration than when similar stress is caused by sorbitol or salt. Besides, *HOT1* expression regulation differs according to the osmotic stress condition (Fig. 2): Msn2 is involved to a similar extent under high glucose and salt stress, while Skn7 participates in the presence of high glucose concentrations and sorbitol. The mutants in repressor-activator Tup1, required for osmostress responses, display lower *HOT1* mRNA levels under high glucose and sorbitol stress conditions and higher ones when osmotic stress was caused by salt. This suggests that this protein plays a dual role in the regulation of this transcription factor.

Capaldi et al. (2008) suggested that at high glucose concentrations, inhibition of Msn2/4 leads to an overall decrease in the activation of the general stress response, and shifts the Hog1-dependent expression program toward the genes regulated by Sko1 and Hot1. The kinetics of the nuclear localization of Msn2 carried out in this work (Fig. 3) and the data obtained about the expression of *HOT1* and the Group 1 genes (Fig. 2 and Fig. 5) agree with this possibility. Besides, according to reports by other authors and the results of the experiments done in this work, *MSN2* expression is lower in the presence of high glucose concentrations than under salt or sorbitol stress (Posas et al. 2000; Kaeberlein et al. 2002, and other data not shown). However, *HOT1* expression is still controlled by Msn2/4 under high sugar stress (Fig. 2) and, according to the analysis performed with the YEASTRACT tool, all the genes specifically upregulated under this condition are controlled by Msn2/4. The results reported in this work about the transcriptional control of the genes specifically upregulated under high glucose or sorbitol stress (belonging to Groups 1 and 2, Fig. 5 and other data included in the text) indicate that transcription factor Skn7 can also play a relevant role, and one not previously described, when osmostress is caused by high sugar concentrations.

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Material suplementario

Table 3.5.S1. Yeast strains, plasmids and oligonucleotides used in this work

Strain	Description	Origin
FY86	*MATα leu2Δ1 ura3-52 his3Δ200*	Lab Stock
FY86 HOG1::GFP	*MATα leu2Δ1 ura3-52 his3Δ200 HOG1::GFP::KANMX4*	This work
FY86 YCPlac111-MSN2::GFP	*MATα leu2Δ1 ura3-52 his3Δ200 [YCPlac111-Msn2::GFP]*	This work
FY86 HOT1::GFP	*MATα leu2Δ1 ura3-52 his3Δ200 HOT1::GFP::KANMX4*	This work
FY86 SKO1::GFP	*MATα leu2Δ1 ura3-52 his3Δ200 SKO1::GFP::KANMX4*	This work
FY86 SUB1::GFP	*MATα leu2Δ1 ura3-52 his3Δ200 SUB1::GFP::KANMX4*	This work
FY86 pRS313-HOT::3HA	*MATα leu2Δ1 ura3-52 his3Δ200 [pRS313-HOT::HA]*	This work
FY86 YCplac111-HOG::3HA	*MATα leu2Δ1 ura3-52 his3Δ200 [YCplac111-HOG::HA]*	This work
W303	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1*	Lab Stock
W303 hot1Δ	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 hot1::kanMX4*	P. Alepuz (Alepuz et al., 2003)
W303 pRS315	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 [pRS315]*	This work
W303 pRS315-HOT	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 [pRS315-HOT]*	This work
W303 YEP352-HOT	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 [YEP352-HOT]*	This work
W303 *msn2Δ msn4Δ*	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 msn2Δ3::HIS3, msn4-1::TRP1*	F. Estruch (Estruch and Calson 1993)
W303 *msn2Δ msn4Δ* YCPlac111-MSN2::GFP	*MATa leu2-3, can1-100, ura3-1, ade2-1, his3-11,15, trp1-1 msn2Δ3::HIS3, msn4-1::TRP1 [YCPlac111-Msn2::GFP]*	This work
BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Lab Stock
BY4741 *skn7Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 skn7::kanMX4*	Euroscarf
BY4741 *cin5Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cin51::kanMX4*	Euroscarf
BY4741 *tup1Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 tup1::kanMX4*	Euroscarf
BY4741 *spf1Δ*	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 spf1::kanMX4*	Euroscarf

Plasmid	Description	Origin
YEp352	multicopy shuttle vector with URA3 marker	Lab stock
PRS313	centromeric yeast vector with HIS3 marker	Lab stock
pRS315	centromeric yeast vector with LEU2 marker	Lab stock
YCplac111	centromeric yeast vector with LEU2 marker	Lab stock
pRS313-HOT::HA	The entire Hot1 ORF with its endogenous promoter fused to a 3xHA tag before the stop codon cloned in pRS313	M. Gomar (Gomar-Alba et al., 2013)
YCplac111-HOG::HA	The entire Hog1 ORF with its endogenous promoter fused to a 3xHA tag before the stop codon cloned in YCplac111	M. Gomar (Gomar-Alba et al., 2013)
YEp352-HOT	The entire Hot1 ORF with its endogenous promoter in YEP352	This work
pRS315-HOT	The entire Hot1 ORF with its endogenous promoter in PRS315	This work
YCplac111-MSN2::GFP	The entire Msn2 ORF with its endogenous promoter fused to a 3xGFP tag before the stop codon cloned in YCplac111	F. Estruch (Estruch and Calson 1993)

Oligonucleotide	Sequence (5' to 3')	Use
STL1PRO-A	TTGGTTAATCCTCGCCAGGT	Chromatin inmunoprecipitation. PCR of STL1 promoter
STL1PRO-B	TATGAGTGTGACTACTCCTG	"
INT-A	GGCTGTCAGAATATGGGGCCGTAGTA	PCR of an intergenic
INT-B	CACCCCGAAGCTGCTTTCACAATAC	"
HOT-A	GGCGAGTCGGCTACTAAG	PCR of HOT1 gene
HOT-B	TGAGCCTCCACTTGTTAC	"
HSP26-A	GTCATCACTTTGCCAGACTAC	PCR of HSP26 gene
HSP26-B	ACCTCAATCTTCTTGACGTGG	
ACT-1	GGATCTTCTACTACATCAGC	PCR of ACT1 gene
ACT-2	CACATACCAGAACCGTTATC	"
GPD1-A	TTGAATGCTGGTAGAAAG	PCR of GPD1 gene
GPD1-B	TGACCGAATCTGATGATC	"
CTT1-A	AGACCAGACGGCCCTATCTT	PCR of CTT1 gene
CTT1-B	GACTACACGCTCCGGAACTC	"
STL1-1	CGGAAGAAGTTTGGAGGAAA	PCR of STL1 gene
STL1-2	GGCATGATCTTCGACTTCTT	"
HOT675	AAGCACAAACAATGCCCA	PCR semiquantitative of HOT1 gene
HOT1360rev	GCTGGGCAAACTTAAAGC	"
YGR189C-F	TTCTTCCTCCGAAGCCAGCT	PCR semiquantitative of YGR189C gene
YGR189C-R	GCAAGGCTAGTAAAGCTACGA	"
CHS1-1	GTGATTCAAACCACTCCAGA	PCR semiquantitative of YNL192W (CHS1) gene
CHS1-2	AGTTGTATTGGAACGGTACC	"
GSY1-1	TGGCTCGTGACTACAAAATC	PCR semiquantitative of YFR015C (GSY1) gene
GSY1-2	TCGTAGTATGCAGACGTATCG	"
GLG1-1	AAGCTGGCTATTGCCACATT	PCR semiquantitative of YKR058W (GLG1) gene
GLG1-2	ACTCAAAAATATCATCAGGGA	"
HXT6-1	ACGCTGCTATTGCAGAGCAA	PCR semiquantitative of YDR343C (HXT6) gene
HXT6-2	TTGGTGCTGAACATTCTCTT	"
YER067W-1	GACAAAGAAGGATAAGAAGGA	PCR semiquantitative of YER067W gene
YER067W-2	AATTTGCGCCTACAGGATGT	"
ITR1-1	CACATACCATATCTCACGTCA	PCR semiquantitative of YDR497C (ITR1) gene
ITR1-2	TAATCTCTTGAGTTGGTTCAT	"
TPO2-1	GAGTGATCAAGAATCTGTTGT	PCR semiquantitative of YGR138C (TPO2) gene
TPO2-2	TCTCAACACTGTCGATGGAA	"
Hot-BamHI	CGCGGATCCCCATGTTAAGTT	HOT1 entire ORF and promoter clonation in pRS315 and YEp352
Hot-Pst	TGCACTGCAGGGCCTACTCTA	"

Table 3.5.S2. Genes differentially overexpressed under 20% glucose

Standard Name	Systematic Name	YP20[1]	YPS[2]	Description
ATG8	*YBL078C*	2,98	1,49	Component of autophagosomes and Cvt vesicles
UGA2	*YBR006W*	3,22	1,41	Succinate semialdehyde dehydrogenase
CHA1	*YCL064C*	5,03	1,41	Catabolic L-serine (L-threonine) deaminase
YDR053W	*YDR053W*	3,07	1,28	Unkown function
YER064C	*YER064C*	2,74	1,39	Uncharacterized ORF; non-essential nuclear protein
GSC2	*YGR032W*	3,37	1,48	Catalytic subunit of 1,3-beta-glucan synthase
MUP1	*YGR055W*	3,70	0,92	High affinity methionine permease
CRH1	*YGR189C*	4,05	0,58	Chitin transglycosylate
ECM29	*YHL030W*	2,95	1,01	Major component of the proteasome
FSH1	*YHR049W*	2,91	0,91	Putative serine hydrolase
YJL171C	*YJL171C*	3,04	0,92	GPI-anchored cell wall protein of unknown function
DAN1	*YJR150C*	2,84	0,63	Cell wall mannoprotein
YKL161C	*YKL161C*	4,37	1,41	Protein kinase implicated in the Slt2p mitogen-activated kinase signal
YLR194C	*YLR194C*	4,59	0,52	Structural constituent of the cell wall
MSS11	*YMR164C*	3,30	1,48	Transcription factor involved in regulation of invasive growth and starch degradation
CHS1	*YNL192W*	3,10	0,60	Chitin synthase I
PCL1	*YNL289W*	3,35	1,00	Pho85 Cyclin
HPA2	*YPR193C*	3,17	1,09	Tetrameric histone acetyltransferase

(1) It shows the value corresponding to the ratio between the induction level in YP20 and YPD according to the data obtained from the microarray analysis

(2) It shows the value corresponding to the ratio between the induction level in YPS and YPD according to the data obtained from the microarray analysis

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Table 3.5.S3. Genes differentially overexpressed under 1M sorbitol

Standard Name	Systematic Name	YP20[1]	YPS[2]	Description
FRT2	*YAL028W*	1,09	3,32	Tail-anchored ER membrane protein of unknown function
BDH1	*YAL060W*	1,02	4,65	NAD-dependent (R,R)-butanediol dehydrogenase
MRPL16	*YBL038W*	1,35	3,26	Mitochondrial ribosomal protein
MAL31	*YBR298C*	0,64	3,16	Maltose permease
MRK1	*YDL079C*	0,84	17,09	Glycogen synthase kinase 3
YDL159W-A	*YDL159W-A*	1,44	4,68	Putative protein of unknown function
YDL218W	*YDL218W*	1,41	4,47	Putative protein of unknown function
GIS1	*YDR096W*	1,16	3,02	Histone demethylase and transcription factor
RSM24	*YDR175C*	1,05	3,74	Mitochondrial ribosomal protein
MTH1	*YDR277C*	0,23	9,81	Negative-regulator of the glucose-sensing signal
SHE9	*YDR393W*	1,25	3,03	Mitochondrial inner membrane protein
MRP20	*YDR405W*	1,06	3,11	Mitochondrial ribosomal protein
MRPL28	*YDR462W*	0,95	4,22	Mitochondrial ribosomal protein
UGO1	*YDR470C*	1,28	3,72	Outer membrane component of the mitochondrial fusion machinery
DIG2	*YDR480W*	1,46	3,00	MAP-kinase-responsive inhibitor of the Ste12p transcription factor
GLC3	*YEL011W*	0,49	4,63	Glycogen branching enzyme
YER067W	*YER067W*	0,18	10,50	Protein of unknown function
FMP10	*YER182W*	1,06	3,09	Putative protein of unknown function
GSY1	*YFR015C*	0,33	3,31	Glycogen synthase
YFR017C	*YFR017C*	0,72	8,13	Cytoplasmic protein that inhibits Gdbp1 glycogen debranching activity
HXK1	*YFR053C*	0,87	21,89	Hexokinase isoenzyme 1
SDS23	*YGL056C*	1,40	3,12	Protein implicated in APC/cyclosome regulation
SPO74	*YGL170C*	1,30	3,75	Component of the meiotic outer plaque of the spindle pole body
HAP2	*YGL237C*	1,46	3,22	Transcriptional activator and global regulator of respiratory gene expression
YGR130C	*YGR130C*	1,43	3,11	Component of the eiosome with unknown function
TDH3	*YGR192C*	1,39	3,13	Glyceraldehyde-3-phosphate dehydrogenase
DOG2	*YHR043C*	0,95	3,45	2-deoxyglucose-6-phosphate phosphatase
GIC1	*YHR061C*	0,52	3,78	Protein of unknown function involved in initation of pudding and cellular polarization
RGI2	*YIL057C*	1,29	77,52	Protein of unknown function involved in energy metabolism under respiratory conditions

COX5B	*YIL111W*	1,43	3,61	Subunit Vb of cytochrome c oxidase
SUC2	*YIL162W*	0,45	3,10	Invertase, sucrose hydrolyzing enzyme
YIR016W	*YIR016W*	0,84	3,82	Putative protein of unknown function
MET28	*YIR017C*	0,87	4,68	Basic leucine zipper transcription activator what participates in the regulation of sulfur metabolism
YJL144W	*YJL144W*	1,49	3,95	Cytoplasmic hydrophilin essential in dessication-rehidration process
YJR115W	*YJR115W*	0,45	7,55	Putative protein of unknown function
GLG1	*YKR058W*	0,58	3,37	Glycogenin glucosyltransferase (iniatiator of glycogen synthesis)
YKR075C	*YKR075C*	0,51	7,93	Protein of unknown function
UPS2	*YLR168C*	1,48	5,76	Mitochondrial protein involved in phospholipid metabolism
YLR177W	*YLR177W*	1,12	8,45	Putative protein of unknown function
NDL1	*YLR254C*	1,36	4,00	Homolog of nuclear distribution factor NudE
SUR7	*YML052W*	0,80	3,06	Plasma membrane protein that localize to furrow-like invaginations
YML119W	*YML119W*	0,70	3,17	Putative protein of unknown function
ARA2	*YMR041C*	1,50	3,24	NAD-dependent arabinose dehydrogenase
ICY1	*YMR195W*	0,48	3,16	Protein of unknown function
YMR206W	*YMR206W*	0,96	12,06	Putative protein of unknown function
YNL144C	*YNL144C*	0,37	4,49	Putative protein of unknown function
YNR034W-A	*YNR034W-A*	1,04	18,03	Putative protein of unknown function
YNR073C	*YNR073C*	0,97	3,21	Putative manitol dehydrogenase
HST3	*YOR025W*	1,34	5,27	NAD(+)-dependent protein deacetylase
FYV12	*YOR183W*	1,19	3,03	Protein of unknown function
RFM1	*YOR279C*	1,37	5,34	DNA-binding protein required for vegetative repression of middle sporulation genes
MPD1	*YOR288C*	1,33	3,14	Protein disulfide isomerase (PDI)
DBP1	*YPL119C*	1,34	3,73	Putative ATP-dependent RNA helicase of the DEAD-box protein family
YIG1	*YPL201C*	1,06	4,28	Protein that interacts with glycerol 3-phosphatase
PUF2	*YPR042C*	1,39	3,71	Member of the PUF (Pumilio homology domains) protein family
NAT3	*YPR131C*	1,48	3,08	Catalytic subunit of the NatB N-terminal acetyltransferase
GPH1	*YPR160W*	0,49	5,33	Non-essential glycogen phosphorylase

(1) It shows the value corresponding to the ratio between the induction level in YP20 and YPD according to the data obtained from the microarray analysis

(2) It shows the value corresponding to the ratio between the induction level in YPS and YPD according to the data obtained from the microarray analysis

Table 3.5.S4. Genes differentially repressed under 20% glucose

Standard Name	Systematic Name	YP20[1]	YPS[2]	Description
RPL4A	*YBR031W*	3,68	0,92	Ribosomal 60S subunit protein L4A
YBR090C	*YBR090C*	3,55	0,91	Putative protein of unknown function
NOP6	*YDL213C*	4,42	1,27	rRNA-binding protein required for 40S ribosomal subunit biogenesis
MTH1	*YDR277C*	4,34	0,10	Negative regulator of the glucose-sensing signal transduction pathway
SUR2	*YDR297W*	5,78	1,20	Sphinganine C4-hydrolase
HXT7	*YDR342C*	13,44	0,35	High-affinity glucose transporter
HXT6	*YDR343C*	14,09	0,41	High-affinity glucose transporter
ATO3	*YDR384C*	3,78	0,96	Putative ammonium transporter
YEL076C	*YEL076C*	4,44	0,68	Putative protein of unknown function
YEL076C-A	*YEL076C-A*	4,05	0,68	Putative protein of unknown function
YER067W	*YER067W*	5,70	0,10	Protein of unknown function, involved in energy metabolism under respiratory conditions
STE2	*YFL026W*	3,11	1,27	Receptor for alpha-factor pheromone
RPL22B	*YFL034C-A*	3,24	1,29	Ribosomal 60S subunit protein L22B
YFL052W	*YFL052W*	4,09	0,97	Putative zinc cluster protein that contains a DNA binding domain
YFL064C	*YFL064C*	3,07	0,67	Putative protein of unknown function
YHB1	*YGR234W*	3,05	0,85	Nitric oxide oxidoreductase
HXT4	*YHR092C*	12,20	0,39	High-affinity glucose transporter
DSE2	*YHR143W*	3,58	1,45	Daughter cell-specific secreted protein with similarity to glucanases
LIA1	*YJR070C*	6,72	1,48	Deoxyhypusine hydroxylase
NNF1	*YJR112W*	3,04	1,09	Essential component of the MIND kinetochore complex
MDN1	*YLR106C*	4,93	1,45	Huge dynein-related AAA-type ATPase (midasin)
PDC5	*YLR134W*	3,01	1,38	Minor isoform of pyruvate decarboxylase
YLR464W	*YLR464W*	4,19	0,72	Putative protein of unknown function
ISF1	*YMR081C*	6,21	0,73	Serine-rich, hydrophilic protein
TMA23	*YMR269W*	3,31	1,48	Nuclear protein implicated in ribosome biogenesis
YNL058C	*YNL058C*	3,32	1,43	Putative protein of unknown function
IMP4	*YNL075W*	3,11	1,18	Component of the SSU processome
MFA2	*YNL145W*	6,30	1,45	Mating pheromone a-factor
EGT2	*YNL327W*	5,44	1,38	Glycosylphosphatidylinositol (GPI)-anchored cell wall endoglucanase
YOR008C-A	*YOR008C-A*	4,67	0,89	Putative protein of unknown function
DSE3	*YOR264W*	3,19	1,05	Daughter cell-specific protein
RPL7B	*YPL198W*	10,28	1,43	Ribosomal 60S subunit protein L7B
YPR202W	*YPR202W*	3,93	0,53	Putative protein of unknown function

(1) It shows the value corresponding to the ratio between the induction level in YP20 and YPD according to the data obtained from the microarray analysis

(2) It shows the value corresponding to the ratio between the induction level in YPS and YPD according to the data obtained from the microarray analysis

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Table 3.5.S5. Genes differentially repressed under 1M sorbitol

Standard Name	Systematic Name	YP20[1]	YPS[2]	Description
LTE1	*YAL024C*	1,39	3,64	Protein similar to GDP/GTP exchange factors but without detectable GEF activity
ITR1	*YDR497C*	1,06	3,06	Myo-inositol transporter
PAN2	*YGL094C*	1,25	3,88	Essential subunit of the Pan2p-Pan3p Poly(A)-ribonuclease complex
TPO2	*YGR138C*	1,39	9,92	Polyamine transporter
TOP3	*YLR234W*	1,18	4,99	DNA topoisomerase III
SPO77	*YLR341W*	1,45	3,43	Meiosis-specific protein of unknown function
FAP1	*YNL023C*	1,12	3,48	Protein that binds to Fpr1p and competes with rapamycin
YNR064C	*YNR064C*	0,93	3,58	Epoxide hydrolase
YOR385W	*YOR385W*	1,01	4,15	Putative protein of unknown function

(1) It shows the value corresponding to the ratio between the induction level in YP20 and YPD according to the data obtained from the microarray analysis

(2) It shows the value corresponding to the ratio between the induction level in YPS and YPD according to the data obtained from the microarray analysis

4. Resultados y Discusión

Durante el desarrollo de esta Tesis Doctoral se ha intentado avanzar en el conocimiento de cómo responden las levaduras al estrés provocado por elevadas concentraciones de glucosa, de gran interés biotecnológico. Este estudio nos ha llevado a identificar y profundizar en la función de un gen de papel desconocido hasta ahora, *YHR087W*, conocido también como *RTC3*, y al que nosotros hemos denominado *HGI1*. Además, una gran parte del trabajo presentado se ha destinado a entender aspectos muy poco conocidos sobre el factor transcripcional Hot1 y su conexión con la ruta HOG, dada la relación particular que presenta con el control de la respuesta al estrés hiperosmótico provocado por alta glucosa. Tras la inclusión en los capítulos anteriores de las publicaciones obtenidas y los manuscritos en fase de preparación en relación con todos estos temas, se procede al análisis y la discusión de los resultados más relevantes. Se incluyen, además, algunas figuras adicionales, no incluidas en los apartados anteriores, que se consideran interesantes en el contexto de esta discusión.

La respuesta de las células de levadura a elevadas concentraciones glucosa implica diferencias moleculares y fisiológicas respecto a otras condiciones de osmoestrés

En los distintos trabajos realizados durante el desarrollo de esta Tesis, una de las condiciones de osmoestrés más estudiada ha sido la causada por altas concentraciones de glucosa. Tal como se comenta en la Introducción, la presencia y abundancia de este nutriente en el medio, fuente de carbono fermentable preferida por *S. cerevisae*, supone la activación de dos importantes rutas de señalización, la vía de la PKA (Marchler y col., 1993) y la vía de represión por glucosa (revisado recientemente en Conrad y col., 2014). La levadura entra entonces en un paradójico conflicto pues, aunque tenga abundancia de nutrientes, está bajo la presión ejercida por el estrés osmótico, con la consiguiente activación de la ruta HOG, y de todas las respuestas adaptativas que ello conlleva (véase apartado correspondiente de la Introducción de este trabajo). Además de los aspectos moleculares diferenciales presentes en el osmoestrés causado por glucosa con respecto al ejercido por otros osmolitos como sal o sorbitol, en el Capítulo 5 también se presentan interesantes diferencias fisiológicas que nos ayudarán a entender la complejidad de las

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respuestas adaptativas ejercidas por las células de levadura para sobrevivir a estas condiciones desfavorables concretas.

Diferencias moleculares en la respuesta a osmoestrés provocado por alta glucosa

Gran parte de los resultados de esta Tesis se focalizan en el estudio del factor transcripcional de respuesta a estrés osmótico Hot1. Junto con Sko1 y Msn2/4, dicha proteína regula la expresión del 88% de los genes de respuesta a osmoestrés dependientes de Hog1. Por eso, es de especial interés analizar si, tal como sugieren Capaldi y col. (2008), en presencia de alta glucosa hay un cambio en el programa transcripcional orquestado por la MAPK Hog1 hacia genes regulados por Hot1 y Sko1, que tendrían mayor relevancia en la respuesta a estrés en esta condición.

Uno de los resultados presentados en el Capítulo 5 (Gomar-Alba y col., 2015) muestra que Hot1 se induce más en estrés causado por glucosa que bajo el equivalente estrés (en términos de actividad de agua) causado por sal o sorbitol (Figura 3.5.2)[2], hecho que se repite, aunque no de forma tan significativa, en el caso de Sko1, que presenta niveles más bajos de inducción en general. Esta inducción del mRNA de *HOT1* causada por glucosa se puede observar en las distintas construcciones que se muestran en la Figura 4.1[3].
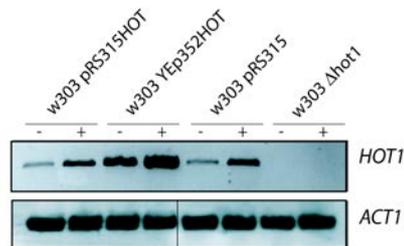
Figura 4.1. Cambios en los niveles de mRNA de *HOT1* en condiciones de osmoestrés causado por altas concentraciones de glucosa (YP20, glucosa 20% (p/v)). Células procedentes de un cultivo exponencial de cepas derivadas de W303-1a (-) fueron transferidas a medio YP20 durante 30 minutos (+). Se recogieron, se procedió a la extracción de RNA total y se sintetizó cDNA. La expression génica se determinó mediante RT-PCR semicuantitativa, y se normalizaron los datos frente al gen *ACT1*.

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[2] Las Figuras que hacen referencia al apartado *3. Publicaciones* de esta Tesis se numeran de la siguiente forma: primero con el número 3, a continuación el Capítulo (artículo) al que pertenecen y finalmente la Figura dentro de artículo en cuestión al que hacen referencia. Así, la Fig. 3.5.2 se refiere a la Figura 2 del Capítulo 5, dentro del apartado de Publicaciones.

[3] Las Figuras correspondientes al apartado *4. Resultados y Discusión* se numeran siempre empezando con el número 4 del apartado, y después consecutivamente con el correspondiente número de Figura.

Para analizar si las diferencias de expresión de *HOT1* son debidas a una regulación diferencial, se precedió a estudiar el efecto de distintos mutantes en proteínas candidatas a regular *HOT1*, como Msn2, Skn7, Cin5, Tup1 and Spf1 (Harbison y col., 2004; Reimand y col., 2010; Huebert y col., 2012). Los resultados obtenidos dan soporte a esta hipótesis, ya que mientras que Msn2/4 regula mayoritariamente la expresión del gen en presencia de alta glucosa y sal, en esta última condición el mutante *Δtup1* muestra un incremento considerable de la expresión del gen, lo que sugiere un mecanismo de represión diferencial entre estas dos condiciones. En sorbitol, Tup1 y Skn7 serían los principales activadores de la expresión del gen (Figura 3.5.2 Panel C). Además de esta conclusión, en este trabajo, por primera vez, se aportan datos directos sobre los factores implicados en la expresión de *HOT1*.

El otro factor transcripcional que puede tener un papel diferencial en el estrés causado por glucosa es Msn2/4 (Martínez-Pastor y col., 1996), implicado en la respuesta general a estrés. Tener la vía PKA activa implica ejercer una represión sobre este factor transcripcional, mientras que, como consecuencia de la activación de la vía HOG, es una de las dianas activadas por la MAPK. Independientemente del osmolito causante del estrés, Msn2 se activa y es translocado rápidamente al núcleo (Figura 3.5.3), aunque esta localización nuclear es más transitoria en presencia de glucosa, aspecto observado también por otros investigadores (Capaldi y col., 2008). Esto sugiere una desactivación más rápida del factor transcripcional, y se correlaciona con su cinética de fosforilación, que también es más corta en estas condiciones (Figura 3.5.3). Estos resultados demuestran una menor implicación de Msn2/4 en la respuesta a estrés causada por altas concentraciones de glucosa, y son coherentes con los obtenidos en el estudio transcriptómico global llevado a cabo por Capaldi y col. (2008).

La mayor inducción de *HOT1* en presencia de glucosa parece sugerir, por tanto, una mayor implicación del factor transcripcional en este tipo de estrés, contrariamente a lo que sucede con Msn2/4. Cuando se realiza un análisis comparativo mediante micromatrices, entre los genes diferencialmente sobreexpresados en glucosa y sorbitol, se obtienen dos grupos claramente diferenciados, aquellos sobreexpresados en glucosa (y no en sorbitol) (Grupo 1, Tabla 3.5.S2) y aquellos sobreexpresados en sorbitol (y no en glucosa) (Grupo 2, Tabla 3.5.S3). Cuando el Grupo 1 de genes es sometido a análisis con la herramienta

YEASTRACT se deduce que Msn2 participa en el control de la expresión de todos ellos, y Hot1 sólo es responsable de regular uno (*CHA1*). En el Grupo 2, Msn2 controla en cierta medida el 81%, mientras que Hot1 únicamente interviene en la regulación de dos (*GSY1* y *GLG1*). Estos resultados no parecen reflejar el mayor papel de Hot1 en osmoestrés causado por glucosa, ni aparentemente tampoco la menor implicación de Msn2/4 en este tipo de osmoestrés. Sin embargo, un análisis más detallado de algunos casos concretos (Figura 3.5.5) muestra que, aunque Msn2/4 esté implicado en la regulación de los genes de ambos grupos, lo hace de manera mucho más relevante y significativa en los genes del Grupo 2 (*GSY1* y *BDH1*), donde en el mutante *Δmsn2/4* la expresión de los mismos se reduce hasta un 10%, mientras que en el Grupo 1 (*CRH1, GSC2* y *ECM29*) sólo disminuye hasta un 60-80%. Estos resultados, por tanto, indican que Msn2/4 parece tener, efectivamente, un papel menos importante en la respuesta a estrés por alta glucosa, aunque sigue contribuyendo parcialmente a la expresión de sus genes diana. Además, tal como muestra la Figura 3.5.5, Skn7 (factor transcripcional implicado en osmorregulación y respuesta a estrés oxidativo y choque térmico), presenta una importante contribución en esta condición, al menos sobre los genes que regula, aspecto desconocido hasta el momento.

Sería necesario realizar un análisis similar a éste con los genes diana de Hot1 (cuyo número, como se ha comentado, es bastante inferior al de los dependientes del factor transcripcional Msn2/4) para obtener más conclusiones sobre la implicación relativa de los distintos factores transcripcionales en los genes parcialmente regulados por Hot1 (que se describen en el siguiente apartado) en los distintos tipos de estrés osmótico. También sería interesante profundizar en la relevancia del factor Sko1 en las diferentes condiciones de estrés.

Es importante señalar que durante el desarrollo de este trabajo también se han obtenido otras evidencias (no incluidas en el Capítulo 5) sobre diferencias moleculares en la respuesta a alta glucosa en comparación con situaciones similares de hiperósmosis causada por otros osmolitos. Se han podido detectar diferencias en el estado de fosforilación de Hot1 (Figura 3.4.2), así como en la recuperación de la actividad traduccional tras la aplicación del estrés. (Figura 3.1.8).

Diferencias fisiológicas en la respuesta a osmoestrés provocado por alta glucosa

Los resultados de las micromatrices realizadas en este trabajo nos muestran una serie de genes que se expresan diferencialmente en glucosa 1.1M y en sorbitol 1M (Tablas 3.5.S2, S3, S4 y S5). Estos genes se pueden agrupar en dos categorías estadísticamente significativas, relacionadas con del metabolismo de los carbohidratos de reserva, particularmente glucógeno, y con la composición de la pared celular (y, de forma especial, con su contenido en quitina).

Tal como se muestra en la Figura 3.5.6, la levadura presenta niveles mucho más elevados de glucógeno en osmoestrés por glucosa que en sorbitol o YPD, durante al menos 12 días. Cuando las células se exponen a sorbitol 1M como osmolito, presentan diferencialmente sobreexpresados genes implicados en la biosíntesis y movilización de glucógeno, por lo que los resultados obtenidos (Figura 3.5.6) demuestran que, a nivel fisiológico, al alcanzarse niveles más elevados de expresión de estos genes (*GSY1* y *GPH1*) en sorbitol se producen mayores niveles de glucógeno que en YPD, pero a su vez, menores que en osmoestrés provocado por alta glucosa, donde se detecta acumulación pero no movilización de dicho polisacárido de reserva. Estos resultados son coherentes son los trabajos que relacionan una mayor disponibilidad de glucógeno con una tolerancia a estrés mejorada (Pérez-Torrado y col., 2002b), ya que, tal como se demuestra en la Figura 3.5.6 (Panel B) células afectadas por osmoestrés causado por sorbitol 1M (durante 2 y 4 días) son más resistentes a etanol 10% (v/v) que las incubadas durante el mismo tiempo en glucosa 1.1M. El hecho de que las mantenidas en YPD durante los períodos de tiempo del análisis sean aún más resistentes puede explicarse principalmente por la no exposición a condiciones tan adversas de osmoestrés durante ese largo periodo de tiempo.

En cuanto a los genes implicados en la organización de la pared celular, la sobreexpresión diferencial encontrada en glucosa 1.1M repercute directamente en un mayor contenido de quitina en la pared celular de las células de levadura expuestas a esta condición (Figura 3.5.7). La quitina constituye solamente entre un 2 y un 3% de la pared celular de *S. cerevisae* y, no obstante, juega un papel vital en la levadura. Bajo condiciones de osmoestrés causadas por sorbitol 1M, se expresan menos (en comparación con alta glucosa) los genes implicados en la biosíntesis de quitina, lo que implica un menor contenido del polímero y una mayor resistencia a *calcofluor white* (Figura 3.5.8).

Todos estos resultados demuestran que existe una correlación entre los datos transcriptómicos obtenidos en este trabajo y diversos aspectos fisiológicos de *S. cerevisiae* y aportan, en su conjunto, una amplia visión que abarca, desde los factores transcripcionales implicados y los genes diferencialmente expresados hasta las consecuencias a nivel fisiológico de las particularidades del osmoestrés causado por altas concentraciones de glucosa. Aunque todavía quedan muchos aspectos por dilucidar en relación al proceso de señalización en dichas condiciones, nuestros estudios aportan datos novedosos que ayudan a entender mejor cómo se adaptan las levaduras las mismas. La Figura 4.2 modeliza de forma simplificada las principales conclusiones obtenidas en el Capítulo 5 de esta Tesis.
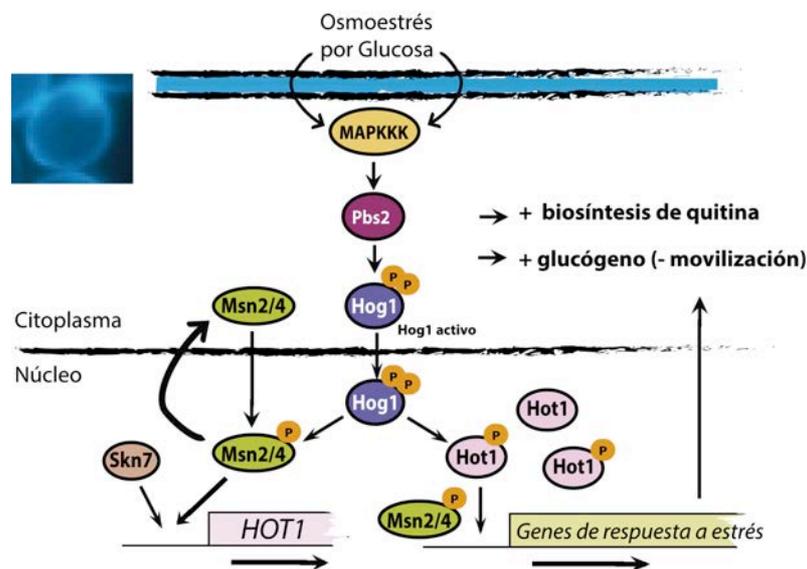
Figura 4.2. Modelización de la respuesta de las células de *S. cerevisiae* al estrés osmótico causado por altas concentraciones de glucosa. En respuesta a este osmoestrés se activa la vía HOG, la MAPK Hog1 se transloca al núcleo, y se mantiene en este compartimento más tiempo que en osmoestrés causado por sorbitol 1M. Tanto Msn2/4 como Hot1 son dianas sujetas a activación por Hog1. Msn2/4 regula, junto con Skn7, la expression del gen *HOT1*, que se induce significativamente más en esta condición de osmoestrés que en otras como sal (NaCl 0.6M) o sorbitol (1M). Msn2/4 también participa activando otros genes de respuesta a estrés, aunque su papel como activador transcripcional es menos relevante que en las otras condiciones y su cinética de activación y permanencia en el núcleo es más transitoria. Entre los genes de respuesta a estrés diferencialmente expresados en alta glucosa, se encuentran genes de biosíntesis de la pared celular, especialmente de quitina, sobreexpresados en esta condición y no en sorbitol 1M. En cambio, se encuentran no inducidos (y sí en sorbitol 1M) genes de mobilización de glucógeno, por lo que en osmoestrés causado por glucosa las células de levadura acumulan mayores niveles de este polisacárido en su interior. En la Figura solamente se indica el estado de fosforilación de las proteínas Hog1, Hot1 y Msn2/4.

El gen de *S. cerevisiae* regulado por Hot1 *YHR087W/HGI1* esta implicado en la traducción en condiciones de osmostrés causadas por alta glucosa

Uno de los genes que presenta mayores niveles de inducción en osmoestrés causado por glucosa es *YHR087W*, gen en un principio de función desconocida al que, sin embargo, se le atribuyen relaciones en procesos muy diversos y en su mayoría desvinculados de la respuesta a estrés (Shavchenko y col., 2005; Adinall y col., 2008; Constanzo y col., 2010). En el primer Capítulo de esta Tesis se sugiere por primera vez una función de la proteína, relacionada con la respuesta a estrés osmótico, y se presenta un estudio detallado de su regulación transcripcional en estrés osmótico y térmico. Todo ello en su conjunto hará que en futuros análisis presentados en este trabajo sea empleado como gen modelo de respuesta a osmoestrés.

Papel de Yhr087w en la traducción

Los datos transcriptómicos globales proporcionan en muchos casos el punto de partida necesario para iniciar el estudio de genes de función desconocida. El abordaje del análisis funcional del gen *YHR087W* presentaba una notable complejidad por la gran variedad de procesos subcelulares distintos con los que se había relacionado en estudios previos de otros autores. Aunque había sido foco de consideración (casi siempre indirecta) de muchos trabajos anteriores, en un rastreo llevado a cabo por Adinall y col. (2008) para identificar mutantes por deleción que suprimieran el fenotipo del mutante termosensible *cdc13-1* (en el que, a temperatura no permisiva, el DNA telomérico es degradado) se sugirió para la proteína Yhr087w una función relacionada con la estabilidad telomérica. Estos datos fueron los responsables del nombre sistemático original del gen, *RTC3* (*Restriction of Telomere Capping*).

Varios estudios transcriptómicos previos habían descrito la notable inducción que experimenta este gen bajo distintas condiciones de estrés: térmico, oxidativo, fase estacionaria (Gasch y col., 2000), pHs ácidos y básicos (Causton y col., 2001) e hiperosmolaridad causada por sal, sorbitol (Causton y col., 2001) y glucosa (Kaerbelein y

col., 2002; Erasmus y col., 2003). Pero fue Capaldi y col. en 2008 quienes, en otro estudio de análisis de expresión génica mediante micromatrices, postularon que es la ruta HOG la que regula el incremento de expresión del gen bajo distintas condiciones de osmoestrés.

Estudios realizados en cepas vínicas en nuestro grupo de investigación (Jiménez-Martí y col., 2009) demostraron que la sobreexpresión del gen mejora su comportamiento fermentativo y la tolerancia a estrés, y que niveles altos de mRNA de *YHR087W* se correlacionan con una mayor resistencia a estrés osmótico (Jiménez-Martí y col., 2011). Estos resultados, junto con la elevada inducción del gen en condiciones de estrés, sugieren una función de la proteína relacionada con la respuesta a hiperosmolaridad aunque no permiten descartar en absoluto su implicación en otras condiciones de estrés.

El abordaje del estudio fenotípico del mutante por deleción en cepas de laboratorio refuerza esta hipótesis, ya que su disrupción causa problemas de crecimiento, menor viabilidad y disminución en el consumo de glucosa en presencia de concentraciones de dicho azúcar del 20% y 30% (p/v) (Jiménez-Martí y col., 2011). Sin embargo, estos fenotipos sólo se observan en cultivos en fase estacionaria y, tal como se muestra en la Figura 3.1.S1, los defectos de crecimiento del mutante en distintas condiciones de osmoestrés son difícilmente apreciables en ensayos de crecimiento en placa. Por tanto, se trata de un gen que se induce muy fuertemente en condiciones de estrés (Figura 4.3) pero cuya deleción no presenta un fenotipo claro en dichas condiciones.
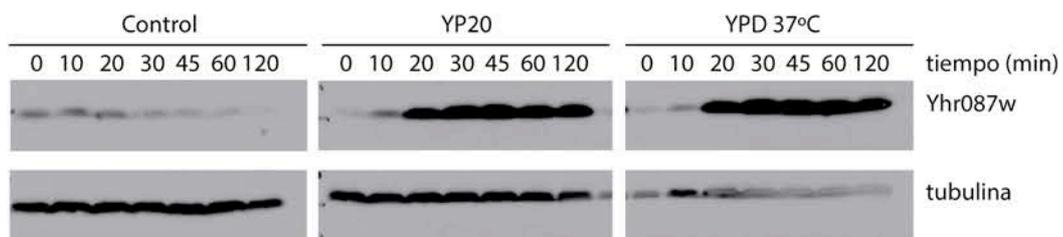
Figura 4.3. Cambios en los niveles de expresión de Yhr087w detectados mediante *western-blot* en condiciones de estrés osmótico causado por altas concentraciones de glucosa. Células procedentes de 16 horas en YPD fueron transferidas a YPD e incubadas a 30ºC (Control) o 37ºC (YPD 37ºC). Otra alícuota del cultivo fue transferida a YPD conteniendo 20% (p/v) de glucosa e incubada a 30ºC (YP20). Se recogieron células en los tiempos indicados en la Figura y se procedió a realizar extractos proteicos totales que fueron analizados mediante *western-blot* y detectados con el anticuerpo α-PAP. Se utilizó el anticuerpo anti tubulina como control de carga. Figura procedente de Mercè Gomar, Tesis de Máster (2011).

Es importante señalar, sin embargo, que esto no es en absoluto contradictorio ni sugiere una escasa relevancia funcional de Yhr087w, ya que el mutante *Δhot1*, por citar un ejemplo directamente relacionado con este trabajo, no presenta defectos de crecimiento en presencia de osmoestrés (tan sólo una ligera reducción de la viabilidad celular después de 24 horas de incubación en presencia de NaCl 1.7M) y son necesarios ensayos indirectos mediante la ruta HOG (como el descrito con *ssk2Δnt,* Rep y col., 1999b) para observar un fenotipo en el mutante del factor transcripcional.

Por todo ello, resultan de especial relevancia los resultados del estudio proteómico realizados en nuestro laboratorio con el mutante *Δyhr087w* en el que aparecen disminuidos (entre otros) los niveles de las proteínas de respuesta a estrés Hsp104 y Hsp78 (Jiménez-Martí y col., 2011; Figura 3.1.S3). Ello atribuye un papel a Yhr087w en alguna de las etapas del proceso de expresión génica en respuesta a osmoestrés, y posiblemente también en respuesta a choque térmico (Figura 3.1.S3). Las conclusiones logradas en el Capítulo 1 de esta Tesis, que atribuyen a Yhr087w un papel en procesos post-transcripcionales, concretamente en la traducción de mRNAs de genes de respuesta a estrés, derivan inicialmente de varios estudios previos que atribuyen un papel negativo a la proteína en otras etapas del proceso de expresión génica y se ven reforzados por los resultados obtenidos en las interacciones físicas, sensibilidad a drogas que inhiben la traducción y análisis de niveles de mensajeros asociados a las fracciones polisómicas.

En este sentido, se han realizado experimentos de inmunoprecipitación de la cromatina (ChIP) que demuestran que Yhr087w no es capaz de unirse ni al promotor ni a la región codificante de *HSP104* (Figura 3.1.S2), y que en el mutante *Δyhr087w* no se ve afectado el reclutamiento de la polimerasa (Rpb1) a este gen (Gomar-Alba, Tesis fin de Máster, 2011). Ensayos con tiolutina demuestran que en este mutante no se ve disminuida la estabilidad del mRNA del gen *HSP104*, (Gomar-Alba y del Olmo, resultados no publicados). Tampoco la exportación de mensajeros en general se ve alterada en *Δyhr087w* (Jiménez-Martí, Tesis Doctoral, 2011), aunque esto no descarta un papel de la proteína en el caso de mRNAs concretos. Todos estos resultados sugieren que Yhr087w no tiene una función transcripcional ni en exportación, a pesar de las interacciones génicas descritas entre *YHR087W* y proteínas implicadas en la transcripción y su control (Nut1, Set3, Npl3,

Cna1 o Bcy1, Constanzo y col., 2010) o en procesamiento y exportación de mRNAs (Nat3, Nsr1, Yra2, Air1, Savchenco y col., 2005; McClellan y col., 2007, Wilmes y col., 2008).

Pero posiblemente el estudio más interesante que descarta una función transcripcional de la proteína son las micromatrices realizadas en las mismas condiciones que el estudio proteómico con el mutante *Δyhr087w* (tras 1h de incubación en 20% (p/v) de glucosa) (Jiménez-Martí y del Olmo, resultados no publicados), en el que se atribuye una mínima relevancia a Yhr087w en el control transcripcional en *S. cerevisiae*, ya que sólo 8 genes aparecen menos expresados en la cepa mutante que en la silvestre, y entre ellos no se encuentran ni *HSP104* ni *HSP78*.

El hecho de que en el mutante *Δyhr087w* no haya niveles inferiores de mRNAs de *HSP104* ni *HSP78* (Figura 4.4) pero sí de las proteínas codificadas por estos genes (Figura 3.1.S3), atribuye fuertemente a Yhr087w un papel post-transcripcional.
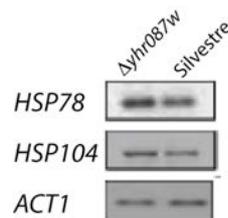
Figura 4.4. Análisis mediante RT-PCR semicuantitativa de los niveles de mRNA de los genes de respuesta a estrés *HSP78* y *HSP104* en una cepa silvestre (BY4742) y un mutante *Δyhr087w*. Células procedentes de 16 horas en YPD fueron transferidas a YP20 (Glucosa 20% (p/v)) durante 1 hora. Se recogieron, se procedió a la extracción de RNA total y se sintetizó cDNA. La expresión génica se determinó mediante RT-PCR semicuantitativa, y se normalizaron los datos frente al gen *ACT1*. Figura adaptada de Jiménez-Martí, Tesis Doctoral (2010).

En el Capítulo 1 de esta Tesis se demuestra que, efectivamente, Yhr087w participa en la traducción de mRNAs concretos de genes de respuesta a estrés. El estudio de los perfiles de polisomas (Figura 3.1.8) permite deducir que en la cepa mutante por deleción la tasa de traducción general se ve ligeramente afectada en condiciones de estrés, es decir, existe una disminución de los niveles de mensajeros traducidos activamente (asociados a ribosomas en la fracción polisomal, P) con respecto a tiempo 0. Que el mutante presente una tasa de traducción inferior, junto con el hecho de que Yhr087w esté asociado a la fracción ribosomal, refuerza claramente el papel de la proteína en la traducción, y podría

explicar los niveles inferiores de Hsp104 y Hsp78 en el mutante detectados en el estudio proteómico. La comprobación de esta hipótesis proviene de los datos obtenidos del análisis de los perfiles de polisomas a los 45 minutos de aplicar el estrés causado por 20% de glucosa. Según estos resultados la relación P/FM es inferior en el mutante, lo que indica que hay más mRNAs asociados a las fracciones libre y monosomal (FM), es decir, no activamente traducidos. El aislamiento y purificación de estos mRNAs, y su cuantificación mediante RT-PCR a tiempo real (Figura 3.1.9) demuestran que, efectivamente, en el mutante *Δyhr087w* hay niveles inferiores de mRNAs en proceso de traducción de transcritos correspondientes a *HSP104* y *HSP78*. Además, y este es el punto realmente interesante, ocurre lo mismo en el caso de otros genes de respuesta a estrés como *GPD1* pero no en el de genes de expresión constitutiva y no relacionados con estrés, como *IPP1* y *PDA1*. Este conjunto de resultados implica que la disminución de la tasa de traducción general observada en la cepa mutante no afecta a todos los genes por igual, sino solamente a mRNAs particulares de respuesta a estrés, atribuyendo por primera vez a Yhr087w una clara funcionalidad en dicho proceso.

Del análisis de estos resultados, se deriva una cuestión interesante. ¿Porqué viéndose tan afectada la traducción de transcritos de un gen de respuesta a estrés tan sobreexpresado como *GPD1*, no se han detectado niveles menores de la proteína codificada en el estudio proteómico comparativo realizado entre las cepas silvestre y mutante (cuyos resultados completos se encuentran publicados en Jiménez-Martí y col., 2011). En este sentido, es muy importante resaltar, que, en la respuesta a estrés, cada gen posee una cinética particular de inducción. Como bien se ha descrito en este trabajo, mientras que *STL1* se induce rápidamente (a los 5 minutos empieza la expresión del gen, inexistente sin estrés, apareciendo el pico de máxima expresión a los 15 minutos para luego decaer de nuevo a los 30, Figura 3.2.6), otros genes como *YHR087W* empiezan a inducirse a los 20 minutos manteniéndose su expresión durante horas. Consecuentemente, existen diferencias también en sus cinéticas de traducción. Es por ello, que para analizar correctamente la expresión de genes concretos, o la traducción de sus mRNAs, es esencial tener en cuenta este aspecto. Se deberían realizar cinéticas particulares, tanto de traducción de los mRNAs asociados a fracciones polisomales, como de expresión de la proteína de interés por *western-blot*, considerándose también mediante esta última técnica su cinética de degradación (por ejemplo mediante ensayos de inhibición con cicloheximida).

La implicación de Yhr087w en el proceso de traducción queda, además, demostrada en este trabajo, por las interacciones físicas y la sensibilidad del mutante a drogas que inhiben la traducción. Los resultados del experimento de TAP (*Tandem Affinity Purification*), confirmados individualmente por coinmunoprecipitación, muestran que Yhr087w interacciona físicamente con una serie de proteínas relacionadas con el inicio de la traducción, como Tif32 (eIF3a), Tif4631 (eIF4G) y Cdc33 (eIF4E, que además une la caperuza del mRNA), detectándose también interacciones génicas con la última de ellas en presencia de la droga inhibidora de la traducción higromicina. Muchos mutantes en proteínas implicadas en esta etapa de la expresión génica muestran defectos de crecimiento en presencia de este tipo de drogas, y el hecho de que el mutante *Δyhr087w* presente una clara sensibilidad a cicloheximida (Figura 3.1.7) refuerza una vez más su implicación en este proceso post-transcripcional. Es importante destacar que estos defectos de crecimiento son mucho más evidentes que los que presenta bajo distintas condiciones de osmoestrés, lo cual permite atribuir al mutante *Δyhr087w* un fenotipo claro para futuros análisis.

Regulación del gen *YHR087W* por el factor transcricional Hot1 en respuesta a osmoestrés

La otra aportación significativa de este trabajo radica en describir la regulación de un gen tan inducido en respuesta a estrés (y bajo condiciones tan variadas) como *YHR087W* (Figuras 4.5 y 3.1.2).
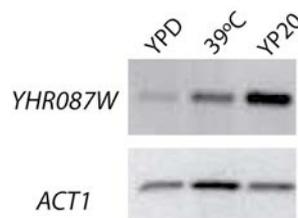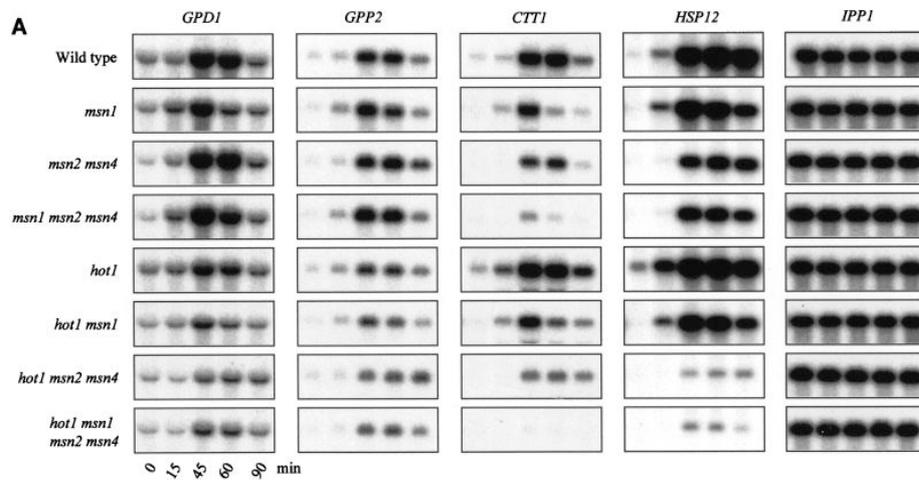

Figura 4.5. Cambios en los niveles de mRNA de *YHR087W* en condiciones de estrés causado por choque térmico (39ºC) y altas concentraciones de glucosa (YP20, glucosa 20% (p/v)). Células procedentes de un cultivo exponencial de la cepa W303-1a fueron transferidas a 39ºC o medio YP20 durante 30 minutos. Se recogieron, se procedió a la extracción de RNA total y se sintetizó cDNA. La expression génica se determinó mediante RT-PCR semicuantitativa, y se normalizaron los datos frente al gen *ACT1*.

En el estudio transcriptómico global llevado a cabo en 2008 por Capaldi y col. se sugería que era la ruta HOG la encargada de dirigir la expresión del gen en condiciones de osmoestrés. Los resultados obtenidos en el mutante *Δhog1* confirman claramente esta hipótesis (Figura 3.1.2) en condiciones de estrés causado por 20% de glucosa, viéndose en estudios posteriores (Gomar-Alba y col., 2013, Figura 3.2.6) que ocurre lo mismo en osmoestrés por sal (NaCl 0.4M). En todo caso, es significativo que el factor transcripcional de respuesta a estrés Hot1 sea el principal regulador del gen en estas condiciones de estrés, reduciéndose su expresión a un 20% respecto de la cepa silvestre. Este hecho constituye una prueba adicional de la relevancia de dicho factor transcripcional en la respuesta a altas concentraciones de azúcares discutida en el apartado anterior de la discusión. Sko1 sería el otro factor implicado en la regulación del gen, así como Msn2/4. De hecho *YHR087W* tiene el elemento STRE reconocido por dichas proteínas en posiciones -301 y -199 entre otras (Bai y col., 2015). Los tres reguladores transcripcionales están controlados a su vez por la ruta HOG en condiciones de osmoestrés, y por eso es en el mutante *Δhog1* donde se observa la disminución más significativa de la expresión del gen.

GPD1, *GPP2*, *HSP12* y *CTT1* son genes claramente vinculados a la respuesta a estrés osmótico que han sido tradicionalmente muy estudiados y también están regulados por Hot1 (Tabla 3.3.1). Es importante destacar el hecho de que ninguno de ellos está completamente controlado por el factor transcripcional, y es sólo en las cepas con la combinación de varios mutantes en factores de transcripción (Msn2/4 y Msn1; Rep y col., 2009b, Figura 4.6) cuando se logra que disminuyan considerablemente los niveles de expresión de este gen. Y, sorprendentemente, sólo con *CTT1* se logra, en el cuádruple mutante *Δhot1 Δmsn2 Δmsn4 Δmsn1* que la expresión del gen sea nula. Estos cuatro genes son, al igual que *YHR087W,* genes muy inducidos en respuesta a estrés y con una cinética de inducción bastante larga, ya que su expresión dura hasta los 90 minutos tras la aplicación del estrés y, en el caso de *HSP12*, como en el de *YHR087W,* se prolonga incluso más. Esto es un claro ejemplo de cómo la levadura *S. cerevisiae* hace uso tanto de la redundancia funcional como de la interconexión entre distintos mecanismos de regulación, para asegurar que se mantenga la expresión de genes tan necesarios en condiciones adversas de respuesta a estrés. En ese sentido hubiese sido interesante estudiar la expresión con varias

combinaciones en mutantes de factores de transcripción del gen *YHR087W*, y ver si se conseguía reducir completamente su expresión.

Figura 4.6. Cambios en los niveles de mRNA de varios genes de respuesta a estrés regulados por Hot1 en condiciones de estrés osmótico (NaCl 0.7M) detectados por *Northern-blot*. Niveles de transcrito observados en la combinación de distintos mutantes de factores de transcripción de respuesta a estrés. El gen *IPP1* se usa como referencia para normalizar. Figura procedente de Rep y col. (1999b).

Es importante destacar que, desde que se publicó este trabajo, numerosos estudios realizados sobre Hot1 usan como gen modelo el *YHR087W* (Gomar-Alba y col., 2013, Burns y Wente, 2014; Bai y col., 2015), aunque no está relacionado con el metabolismo y transporte de glicerol, como muchos de los genes que regula el factor transcripcional. Otro gen modelo comúnmente utilizado en los trabajos relacionados con Hot1 es precisamente *STL1,* que codifica una proteína de membrana plasmática implicada en la importación de glicerol al interior celular (Figuras 1.8 y 1.11). A lo largo de los distintos Capítulos de esta Tesis, se han usado ambos como ejemplos de genes regulados por Hot1. La elección no es en absoluto arbitraria y es que estos dos genes tienen peculiaridades que hacen que se complementen a la perfección. Por una parte, *STL1* presenta la gran ventaja de estar completamente regulado por el factor transcripcional, es decir, en el mutante *Δhot1* no hay mensajero de *STL1* (Alepuz y col., 2003; Figura 3.2.6). Esta es una importante particularidad que ninguno de los genes comentados anteriormente posee, y hace que sea muy útil en el estudio del factor transcripcional, pues no deja margen a confusión si se trabaja con un Hot1 no funcional, como lo harían los distintos porcentajes de disminución de la expresión de otros genes. Presenta en cambio un notable inconveniente, y es que su

cinética de expresión es realmente rápida (Alepuz y col., 2003; Gomar-Alba y col., 2013; Figura 3.2.6) siendo muy distintos los niveles de mRNA encontrados entre los 5, 10, 15 y 20 min, lo que complica trabajar con precisión con muchos cultivos a la vez, e implica mantener siempre las mismas concentraciones de osmolito para no alterar la cinética entre distintos experimentos. Este inconveniente no lo encontramos en el caso del gen *YHR087W*, que mantiene niveles altos y constantes de mRNA desde los 15 minutos hasta las dos horas. En cambio, como ya se ha discutido, en el mutante *Δhot1* los niveles de expresión del gen caen a un 20-30%, pero no a 0. Por todo ello, ambos genes ofrecen el complemento ideal para asegurar el rigor de los resultados obtenidos en los distintos experimentos de análisis de la expresión génica que se realizan a lo largo de toda esta Tesis.

Que *STL1* sea completamente regulado por el factor transcripcional Hot1, nos remite al muy recientemente publicado trabajo de Bai y col. (2015), donde van más allá y afirman que es el único gen controlado por dicho factor transcripcional. Para llegar a esta afirmación, los investigadores se basan principalmente en que es el único gen en cuyo promotor se encuentra la secuencia HoRE (*Hog1 Responsive Element*). Este elemento de secuencia, situado en las posiciones -654 a -626, sería esencial para la activación del gen de forma Hog1-Hot1 dependiente y contiene dos repeticiones de la secuencia 5'-CATTGGC-3' que sería la reconocida por el factor transcripcional. Dado que Hot1 se puede unir al promotor de otros genes de respuesta a estrés anteriormente citados (Figura 3.3.2) y regular, al menos parcialmente, su expresión (Tabla 3.3.1; Rep y col., 1999b; Alepuz y col., 2001; Capaldi y col., 2008; Cook y O'Shea, 2012; esta Tesis Doctoral), debería existir en todos ellos un elemento de reconocimiento para Hot1 que Bai y col. (2015) no consiguen localizar, lo cual deja abierta la especulación a otras posibilidades. Más adelante se retomará esta discusión al revisar cómo interacciona el factor transcripcional con el DNA.

### Implicación funcional de Yhr087w en otros procesos distintos a la respuesta a estrés osmótico

En el apartado anterior sólo se ha hecho referencia a la regulación de *YHR087W* en respuesta a estrés osmótico. Sin embargo, este gen está también inducido por otros tipos de condiciones adversas, como el choque térmico, donde el factor transcripcional Msn2/4

tendría el papel principal, probablemente casi exclusivo, en la regulación del gen (es en el único mutante en el que en dichas condiciones la expresión del gen se reduce hasta un 25%). No deja de ser curioso el hecho que, bajo estas situaciones, en los mutantes *Δhot1* y *Δsko1* (principales reguladores en osmoestrés) se incrementen aproximadamente el doble los niveles de mRNA de *YHR087w*. Aunque este Capítulo y la Tesis en general está centrada a la respuesta a estrés osmótico, sería muy interesante ver si Yhr087w regula de algún modo la traducción de transcritos en respuesta a choque térmico, ya que, al menos en el caso de Hsp104 (chaperona que también está implicada en el plegamiento en este tipo de estrés) los niveles de proteína se ven disminuidos en el mutante *Δyhr087w* (Figura 3.1.S3).

Más allá de la relación de Yhr087w con la traducción de mRNAs, está el hecho de que varias evidencias vinculan de algún modo a la proteína en funciones no relacionadas directamente con la respuesta a estrés. En primer lugar, el hecho de que *Δyhr087w* suprima el fenotipo del mutante termosensible *cdc13-1* (Adinall y col., 2008) es sorprendente y no carece en absoluto de interés. Por ese motivo, paralelamente a los experimentos descritos en este capítulo, se realizaron ChIPs para ver si Yhr087w podía asociar el DNA de regiones teloméricas o subteloméricas (Gomar-Alba y del Olmo, resultados no publicados). En ninguno de los ensayos realizados, en los distintos cromosomas ensayados (V, VI y VII), se observó inmunoprecipitación de la proteína, por lo que si de algún modo puede regular negativamente la estabilidad telomérica, debe ser de forma indirecta, y no mediante su asociación directa al DNA en estas regiones cromosómicas.

El otro punto de especial interés con respecto a Yhr087w radica en la gran similitud estructural que presenta con la región N-terminal de la proteína de *S. cerevisiae* Sdo1. Sdo1 une RNA, interacciona con factores nucleares que participan en el procesamiento de rRNAs, y está implicada en la maduración de la subunidad 60S ribosomal (Menne y col., 2007; Luz y col., 2009). Esta claramente relacionada con el metabolismo del RNA y con el inicio de traducción, por lo que se pensó en la posibilidad de que Yhr087w fuera su redundante funcional (Savchenko y col., 2005), pues Sdo1 es una proteína esencial. Durante el desarrollo de este proyecto se trabajó con distintos mutantes de *SDO1* pero en ningún caso se observaron interacciones génicas con *YHR087W* (Figura 4.7), ni en condiciones de estrés, ni con drogas inhibidoras de la traducción (al contrario que el mutante *Δyhr087w*, mutantes en *SDO1* son más sensibles a higromicina que a

cicloheximida, ver Figura 4.7). El hecho de que Sdo1 no esté regulado por estrés ni condiciones adversas hace pensar que, aunque ambos realicen funciones relacionadas con la traducción y presenten similitudes estructurales, cada uno tiene su rol particular en esta etapa, estando el de Yhr087w vinculado con la respuesta a estrés, como se ha ido demostrando a lo largo de este trabajo.
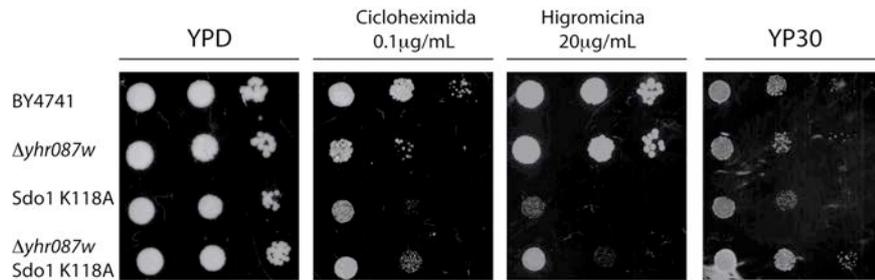
Figura 4.7. Sensibilidad a inhibidores de la elongación traduccional y a osmoestrés por glucosa al 30% (p/v) en el mutante *Δyhr087w* y Sdo[K118A]. Se realizaron diluciones seriadas (1:10) que fueron goteadas sobre placas de YPD, YPD con cicloheximida 0,1μg/mL o higromicina 20 μg/mL y YP30 (glucosa 30% (p/v)), y se incubaron a 30ºC durante 48-72 horas.

Con todo, desde que se publicó este trabajo en 2012 no han habido nuevas aportaciones sobre la funcionalidad de esta proteína, aunque, como ya se ha comentado anteriormente, *YHR087W* ha sido usado como modelo de gen regulado por Hot1 en la respuesta a osmoestrés en distintas ocasiones, y todos los datos obtenidos hacen pensar en un papel relevante de la proteína en respuesta a estrés, más allá de las pequeñas diferencias observadas por el momento en el mutante y la complejidad que entraña su estudio.

Interacciones del factor transcripcional Hot1 con el DNA y con otras proteínas en el contexto de su función en la respuesta a estrés hiperosmótico en *S. cerevisiae*

Uno de los objetivos de este trabajo, y que da título a la Tesis, es el análisis del factor transcripcional de respuesta a estrés osmótico Hot1. Gran parte de los estudios realizados en esta Tesis Doctoral (Capítulos 2, 3 y 4) han estado dirigidos a entender cómo para llevar a cabo el control adecuado de sus genes diana, dicho factor transcripcional interacciona con otras proteínas y con el DNA. Durante estos tres Capítulos se analizan con profundidad distintos aspectos relacionados con esta proteína, aportando claridad a algunas de las principales incógnitas sobre la regulación y activación de Hot1 y sobre los elementos necesarios para su funcionalidad, y su interacción con el DNA y con otras proteínas. El primero de los Capítulos (2) está publicado en Gomar-Alba y col. (2013), mientras que los otros dos están actualmente en proceso de revisión (en *BBA Gene Regulatory Mechanisms*) o preparación final.

Elementos de Hot1 necesarios para su funcionalidad: KR4 y ED5

Hot1 dirige la expresión de una serie de genes implicados en la biosíntesis y movilización del osmolito compatible glicerol, fundamentales para la adaptación de la levadura a las condiciones de alta osmolaridad del medio. Entre los factores transcripcionales activados por Hog1, Msn2/4 son los que tienen mayor efecto en los cambios en la expresión génica dependientes de osmoestrés, aunque en alta concentración de azúcares Hot1 y Sko1 presentan un papel importante, como se ha comentado anteriormente (Capaldi y col., 2008). Sin la MAPK Hog1, Hot1 no se activa, y no se expresa *STL1* (recordemos que está controlado únicamente por dicho factor). Además se observan disminuciones en la actividad transcripcional de los otros genes dependientes de Hot1 (Rep y col., 1999b; Alepuz y col., 2003; Gomar-Alba y col., 2012). Sin embargo, aunque Hog1 activa por fosforilación algunos factores transcripcionales como Smp1 y Sko1, la fosforilación de Hot1 dependiente de Hog1 no es esencial para la expresión de su gen diana *STL1*. Esta conclusión procede de experimentos llevados a cabo en un mutante en

el que las serinas susceptibles de fosforilación por la MAPK fueron sustituidas por alaninas, y, por tanto, Hot1 resulta incapaz de ser fosforilado; a pesar de ello esta cepa sigue manteniendo los mismos niveles de expresión de *STL1* que una cepa silvestre (Alepuz y col., 2003). En el Capítulo 2 se demuestra que la activación de Hot1 mediada por Hog1 es dependiente de la interacción entre ambas proteínas (y no de la fosforilación por la MAPK), aunque, evidentemente, si no interaccionan Hot1 permanece sin fosforilar, del mismo modo que ocurre en una cepa *Δhog1* (Figura 3.2.3). El dominio de interacción (*o docking site*) con Hog1, determinado en este trabajo mediante ensayos de doble híbrido y posteriormente confirmado por coinmunoprecipitación y ensayos de fosforilación, es una región básica, compuesta por los aminoácidos KRRRR (KR4) y situada en las posiciones 381-385. Esta secuencia es completamente esencial para la funcionalidad de Hot1: cuando es delecionada o sustituida por metioninas, Hot1 no interacciona con Hog1 (Figura 3.2.1) ni es capaz de activar la expresión de sus genes diana *STL1* y *HGI1* (Figura 3.2.6).

En este trabajo se demuestra, por tanto, que la activación de Hot1 por la MAPK Hog1 se logra por la interacción entre ambas proteínas, con la relevancia que esta interacción supone, no sólo para la reprogramación transcripcional que tiene lugar en la respuesta a estrés, sino también, y muy ligada a ella, en la habilidad de las células de levadura para superar esta condición adversa.

El otro elemento esencial para la funcionalidad del factor transcripcional que se estudia en el Capítulo 2 de este trabajo es una región acida, situada en posición 541-546 y compuesta por los residuos EDDDDD (ED5). En muchos casos, el módulo de transactivación de los factores transcripcionales está compuesto por residuos ácidos (Courey y Tijan, 1988). En la Figura 3.2.5 se muestran los resultados de un ensayo de Un-Híbrido[4] en el que distintos truncados de Hot1 se fusionaron al dominio de unión al DNA de LexA, por lo que sólo se activa la expresión del gen reportero *lacZ* si la región de Hot1 considerada funciona como dominio activador de la transcripción. El hecho de que el truncado *HOT.1*, que carece de la región carboxi-terminal de la proteína y del elemento ED5, sea capaz de activar la transcripción (tanto en la cepa silvestre como en la *Δhot1*) al mismo nivel que el truncado *HOT.0* (140-582) que sí posee ED5, sugiere que este elemento

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[4] Utilizamos esta terminología por similitud con doble-híbrido

no es indispensable para la capacidad activadora de la transcripción de Hot1. Curiosamente, en este mismo ensayo, en el mutante ΔED5, en el que estos aminoácidos han sido delecionados en HOT.0, sí cae la capacidad transactivadora del factor transcripcional (en cepas silvestre y *Δhot1*). Esto dificulta descartar completamente el papel de ED5 como activador de la transcripción.

Siguiendo con el papel de ED5, experimentos de inmunoprecipitación de la cromatina realizados en este trabajo demuestran que cuando esta región es delecionada Hot1 no puede unirse a los promotores de los genes que regula (Figura 3.2.7; Figura 3.3.1). Esto implica que este dominio también es esencial para la funcionalidad del factor transcripcional, pues cuando está ausente, la expresión de los genes regulados por Hot1 es idéntica a la encontrada en un mutante *Δhot1*. Resulta curioso que, en el caso del gen *STL1*, la mutación ΔED5 tiene incluso un mayor efecto en este sentido que la ΔKR4. Los resultados de estos experimentos de ChIP son de gran interés, ya que, dada la homología de secuencia entre la región C-terminal de Hot1 y el dominio de unión al DNA de los factores transcripcionales Gcr1 y Msn1 (Figura 3.3.1), se había considerado (Krantz y col., 2006) que el dominio de unión al DNA de Hot1 fuera la región comprendida entre los aminoácidos 610-719, que no incluye el elemento ED5. En el Capítulo 3 de esta Tesis, se demuestra *in vivo,* mediante ChIP y ensayos de Un-Híbrido, e *in vitro,* mediante EMSA, que efectivamente la región C-terminal de Hot1, sin incluir ED5, es suficiente para unir el DNA de sus promotores diana, aunque también lo puede unir una región más amplia (534-719) que sí incluye los aminoácidos ácidos. Una de las hipótesis que puede explicar que al delecionar ED5 Hot1 no pueda unir el DNA en los experimentos de ChIP es que la ausencia de estos residuos cambie el plegamiento de la región C-terminal, y, por tanto, del dominio de unión al DNA (DBD), en el contexto de la proteína completa, aunque sería necesario un estudio más profundo sobre la funcionalidad de este dominio para poder entender en su conjunto y complejidad, los distintos resultados obtenidos en el mutante ΔED5.

En ese sentido, otra evidencia obtenida en este trabajo, derivada de los distintos experimentos realizados con el mutante *hot1ΔED5*, es que éste, al igual que el *hot1ΔDBD*, siempre presenta un mayor nivel de proteína que la cepa silvestre (Figura 3.2.3, Figuras 4.8 y 4.9). El hecho de que mutantes en Hot1 que no pueden unir el DNA presenten mayor

nivel de la proteína hace pensar en la posibilidad de que no sean correctamente degradados. Por ese motivo, se realizó una cinética en ciclohexímida para ver la estabilidad de la proteína Hot1 (Figura 4.8, Panel A) y, al confirmar su cinética de degradación, se procedió a realizar el mismo experimento con los mutantes *hot1ΔDBD* y *hot1ΔED5*. El resultado obtenido (Figura 4.8B) muestra que, aunque es evidente la mayor cantidad de proteína Hot1 que presenta el mutante *hot1ΔDBD*, que no une el DNA, la cinética de degradación que sigue este factor transcripcional es idéntica a la de la cepa silvestre aunque, evidentemente, al partir de niveles proteicos mayores, tardan más tiempo en ser completamente degradados en presencia de ciclohexímida. Se obtuvieron los mismos resultados en el mutante *hot1ΔED5* (resultados no mostrados).
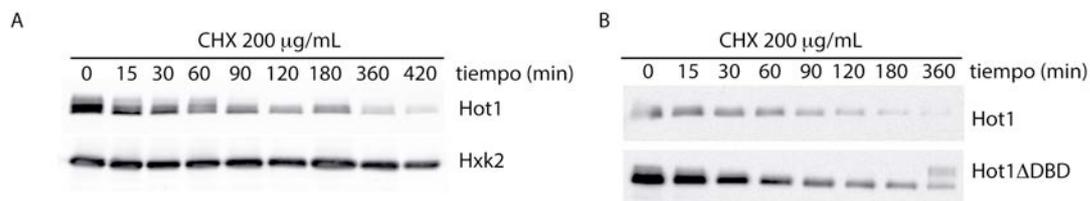
Figura 4.8. Cinética de degradación de Hot1 y Hot1ΔDBD en presenica de ciclohexímida (CHX) 200 μg/mL. Células procedentes de un cultivo exponencial (OD_{600} = 0.4) fueron tratadas con ciclohexímida y recogidas a los distintos tiempos indicados en la Figura. Se realizaron extractos proteicos totales y se inyectó en el gel siempre la misma cantidad de proteína total (25μg). Las muestras fueron analizadas mediante *western-blot* y la proteína Hot1 y sus derivadas detectadas con el anticuerpo α-HA peroxidasa 3F10 (1:10.000) de *Roche*. Se utilizó el anticuerpo hexoquinasa 2 (Hxk2) como control de carga.

Es importante considerar la posibilidad de que, del mismo modo que KR4 lo es para Hog1, el motivo ácido ED5 sea un sitio de unión con otras proteínas candidatas a interaccionar con Hot1. Burns y Wente (2014) propusieron recientemente que ED5 sería el *docking site* entre Hot1 y CK2, quinasa de la que se hablará después con mayor profundidad en el apartado de interacciones físicas en las que participa el factor transcripcional.

Finalmente, el último aspecto determinante a comentar de este trabajo consiste en el hecho que delecionar KR4, pero no ED5, sea esencial para mantener la integridad de la vía HOG1 (Figura 3.2.8). Como se ha comentado con anterioridad, es difícil encontrar un fenotipo en el mutante *Δhot1*. Sólo tras 24 horas de fuerte estrés salino, el mutante por deleción presenta una menor viabilidad (aproximadamente 5 veces inferior a la de la cepa

silvestre, Rep y col., 1999b; Gomar-Alba y col., 2013). Que ambos mutantes, *hot1ΔKR4* y *hot1ΔED5*, presenten viabilidades similares al *Δhot1* demuestra que dichos elementos, al ser esenciales para la funcionalidad de Hot1, son relevantes para las células de *S. cerevisiae* en la respuesta a estrés osmótico. El otro método de observar un fenotipo en *Δhot1* es indirecto, mediante la integridad de la vía HOG1, tal como se explica en el Capítulo 2 de esta Tesis. *Δhot1* revierte (aunque no tanto como *Δhog1*) el fenotipo deletéreo del mutante *ssk2Δnt* (en el que la ruta HOG1 está siempre activa). Que el mutante *hot1ΔKR4*, pero no el *hot1ΔED5*, también lo revierta, implica que el dominio básico es esencial para la funcionalidad de la ruta HOG1. Esto es coherente con las evidencias obtenidas en este trabajo acerca del papel de cada dominio, pues la interacción Hog1-Hot1 ocurre más arriba en la ruta que la interacción de Hot1 con el DNA (función afectada, entre otras posibles, en el mutante ED5), por lo que su ausencia tiene efectos más deletéreos en la respuesta a osmoestrés.

Durante el Capítulo 2 de esta Tesis (Gomar-Alba y col., 2012) se presenta un exhaustivo estudio sobre los elementos de Hot1 necesarios para su funcionalidad en la respuesta a estrés, que queda completado, como se detallará a continuación, durante el Capítulo 3.

Módulos del factor transcripcional Hot1

En el Capítulo 2 se inicia el estudio de los elementos de Hot1 necesarios para su unión al DNA. El dominio de unión al DNA (DBD), es, tal como se ha descrito en la Introducción de esta Tesis, uno de los módulos fundamentales de los factores de transcripción. Hasta el momento no se había definido el DBD de Hot1, aunque se sabía que, tal como ya se ha comentado, la región carboxi-terminal de la proteína (610-719) presenta homología de secuencia con el dominio de unión al DNA de otros factores transcripcionales, como Msn1 y Gcr1 (Figura 3.3.1) y que, estructuralmente, podría tratarse de un dominio hélice-vuelta-helice (Krantz y col., 2006). Esta región no incluye el ED5, (situado en posición 541-546), por lo que el primer análisis a realizar era ver si el dominio de unión de Hot1 lo comprendía la región 610-719 o una mayor, que incluiría ED5, de 534-719. Por ese motivo, se construyeron dos truncados de ambas regiones fusionados al

dominio de activación de Gal4 y se realizaron ensayos de Un-Híbrido, utilizando como genes reporteros *lacZ* (Figura 3.3.1) e *HIS3* (Figura 3.3.3). El uso de este último presenta un grado de exigencia mayor para la especificidad de la interacción, al realizarse ensayos de crecimiento en placas de SC-his conteniendo 3-AT 1mM (Figura 3.3.3). Los resultados de ambos experimentos demuestran que la región comprendida entre los aminoácidos 610-719 es el Dominio de Unión al DNA (DBD) de Hot1. Estos resultados se ven reforzados, además, por los ensayos *in vitro* de retardo en gel, todos ellos realizados sólo con esta región de Hot1 fusionada a GST. Es importante destacar, además, que al delecionar esta región (610-719), Hot1 no puede unir, a pesar de tener ED5, el promotor de sus genes diana (Figura 3.3.1), por lo que la región ácida, como se ha discutido anteriormente, no es en absoluto lo único necesario para la unión del factor transcripcional al DNA, aunque sigamos sin poder explicar porque su deleción la impide.

Otro módulo esencial en los factores de transcripción es el dominio de transactivación. A pesar de que éste no fue su objetivo directo, el estudio de Un-Híbrido realizado con varios truncados de Hot1 fusionados al dominio de unión al DNA de LexA (Figura 3.2.5) nos permite obtener algunas conclusiones sobre este aspecto. Ya se ha comentado que es difícil descartar la implicación del dominio ácido ED5 en la activación transcripcional, aunque dado que los truncados HOT.1 y HOT.3, que carecen de él, activan la transcripción (en las cepas silvestre y *Δhot1*) de forma similar a HOT.0 que sí lo posee, es aun más difícil poder afirmarlo. Dejando a un lado el conflictivo ED5, el resultado más evidente de este estudio es que Hot1 necesita a Hog1 para poder activar el gen reportero en condiciones de estrés. Por eso, en el mutante *Δhog1* la capacidad transactivadora de Hot1 es totalmente nula e idéntica a la observada en ausencia de estrés. Por ese mismo motivo, todos los mutantes que carecen de (o tienen mutada) la región KR4 son incapaces de activar la transcripción. Este es, pues, el primer requisito: como ya se ha demostrado, Hot1 necesita interaccionar con Hog1 para activar la expresión de genes diana. Obviando el efecto enmascarador de Hog1, y centrándonos en los truncados que tienen, por tanto, KR4, se observa que el que presenta una menor capacidad transactivadora es, con la excepción de *hot1ΔED5*, HOT.4. Este mutante carece de la región 420-719, con lo que presumiblemente el dominio transactivador de Hot1 debe estar situado dentro de esta amplia región. Hay que tener en cuenta, además, que tal como se muestra en el *western-blot* de la Figura 3.2.1,

HOT.4 se expresa mucho más que HOT.0, HOT.1 y HOT.3, por lo que si se aplicara una normalización haciendo referencia a la cantidad de proteína, las diferencias de niveles de actividad β-galactosidasa con respecto a éstos serían considerablemente mayores.

En este sentido, es muy importante destacar que la actividad enzimática que observamos en la cepa silvestre (Figura 3.2.5 Panel A) es mucho mayor que la observada en la cepa *Δhot1* (Panel B). Esto conduce a dos conclusiones importantes. La primera, y más evidente, es que Hot1 se comporta como un factor de transcripción poco usual, ya que no es capaz de mantener altos niveles de activación transcripcional en ausencia de la copia genómica, lo que nos remite a la segunda, y es si la necesidad de esa copia genómica para sostener altos niveles transactivadores se podría explicar por el hecho de que haya de organizarse en forma de dímero para ser completamente funcional. En caso de ser esa hipótesis cierta, habría que tener en cuenta si en los truncados se está delecionando el módulo de dimerización, lo que impediría a la proteína formar dímeros. En la cepa con la copia genómica siempre se tendría un Hot1 completo y funcional, con su dominio transactivador, y capaz de unir otro Hot1 que mantenga el módulo de dimerización, lo que aumentaría considerablemente la capacidad activadora del factor transcripcional.

Además de los análisis obtenidos en el experimento de Un-Híbrido, la hipótesis de que Hot1 pueda formar dímeros se ve reforzada potentemente por los resultados de la coinmunoprecipitación mostrada en la Figura 3.2.9, donde se demuestra que, tanto en condiciones de estrés osmótico como en ausencia del mismo, Hot1 es capaz de interaccionar consigo mismo, y con altos niveles. La evidencia de la capacidad formadora de dímeros del factor transcripcional se obtuvo mediante un experimento de entrecruzamiento. En nuestros resultados (Figura 3.2.9) se ve claramente la banda correspondiente al dímero (de 200 kDa) pero aunque el experimento se realizó con varios agentes entrecruzadores distintos, nunca conseguimos ver la banda del dímero incrementarse al disminuir el monómero, lo que supondría una confirmación definitiva.

Estos resultados nos conducen a otra pregunta interesante, ¿cuál sería el dominio de dimerización de Hot1? Una hipótesis posible es que el mismo dominio de unión al DNA actúe también como dominio de dimerización (como ocurre con algunos factores transcripcionales, Murre y col., 1989) y otra es que el elemento ácido ED5 sea necesario

para la formación del dímero (de hecho, si fuera así y Hot1 necesita ser un dímero para unir el DNA, eso explicaría algunos de los resultados obtenidos con el mutante ED5 discutidos anteriormente). Con el objetivo de testar estas posibilidades se realizó un experimento de coinmunoprecipitación con las mismas condiciones que el descrito en la Figura 3.2.9. En estos ensayos se utilizaron cepas que expresaban Hot1-TAP con los mismos niveles a partir de la copia genómica, así como variantes de Hot1 etiquetadas con HA codificadas por copias presentes en plásmidos centroméricos. La inmunoprecipitación Hot1-TAP (Figura 4.9) permitió observar de nuevo que la ausencia de ED5 o del dominio de unión repercute en mayores niveles proteicos. Pero el resultado realmente sorprendente es que Hot1 puede interaccionar consigo mismo, tanto en ausencia de ED5, como del dominio de unión al DNA (IP carreras 3 y 4 respectivamente), lo que significa que estas regiones no son esenciales para la dimerización.
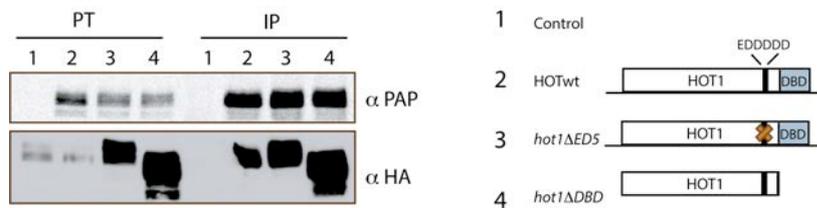

Figura 4.9. Hot1 puede interaccionar consigo mismo en ausencia de su dominio C-terminal (610-719) y de ED5, sugiriendo que no necesita ser un dímero para unir el DNA. Se llevaron a cabo experimentos de coinmunoprecipitación con cepas derivadas de la FY86 que expresan la copia genómica de *HOT1* etiquetada con TAP y diversas variantes del plásmido pRS313-HOT1 (wt (2), ΔDBD (3), ΔED5(4)) etiquetado con HA. Como control del experimento se utilizó una cepa sin la copia genómica etiquetada y con el plásmido pRS313-HOTwt (1). El anticuerpo α-PAP fue utilizado para detectar el Hot1-TAP precipitado y el anticuerpo α- HA peroxidasa para detectar el Hot1-HA coinmuniprecipitado. PT hace referencia a extractos totales de proteína (misma cantidad de proteína total) mientras que IP se refiere a la fracción retenida después de la elución de la fracción inmunoprecipitada.

En cualquier caso, la pregunta realmente interesante sería si Hot1 necesita ser un dímero para ser funcional. En relación con esto, y a la vista de la información recopilada, sólo podemos responder que no necesita estar activado para formar dímeros (Figura 3.2.9), ya que en ausencia de estrés, y por tanto, sin interaccionar con Hog1, es capaz de interaccionar consigo mismo (puede que incluso más que en condiciones de estrés). También podemos deducir, aunque de un modo más indirecto, en base a los resultados de la Figura 4.9 y al carácter necesario y suficiente de la región entre 610-719 para la unión al

DNA de Hot1, que este factor puede unir el DNA en forma de monómero. Este hecho, sin embargo, no implica en absoluto descartar que también pueda hacerlo como dímero. De hecho, sería interesante realizar experimentos de CoIP sólo con el fragmento de unión al DNA (610-719), para ver si es suficiente para dimerizar o no, ya que, como muestra la Figura 4.9, no es imprescindible Hot1 completo para formar dichas asociaciones. También se podrían llevar a cabo experimentos de entrecruzamiento con DNA utilizando diferentes versiones de Hot1 (proteína completa, diferentes truncados y dominio de unión al DNA). Este tipo de ensayo permitiría, en caso de confirmar la unión del factor transcripcional al DNA en forma de dímero, establecer la región responsable de la adopción de dicha estructura. En cualquier caso, el estudio de la posible dimerización entraña una notable comlejidad, y sería un trabajo aparte de los Capítulos presentados en esta Tesis, que aportaría nuevas perspectivas al estudio de la funcionalidad del factor transcripcional.

Con todo, y como queda resumido en la Figura 4.10, los resultados presentados en esta Tesis en los Capítulos 2 y 3 permiten hacer un análisis exhaustivo sobre los distintos módulos del factor transcripcional Hot1, demuestran por primera vez la región responsable de unión el DNA, y descubren nuevos elementos de secuencia necesarios para su activación y funcionalidad en la respuesta a estrés.
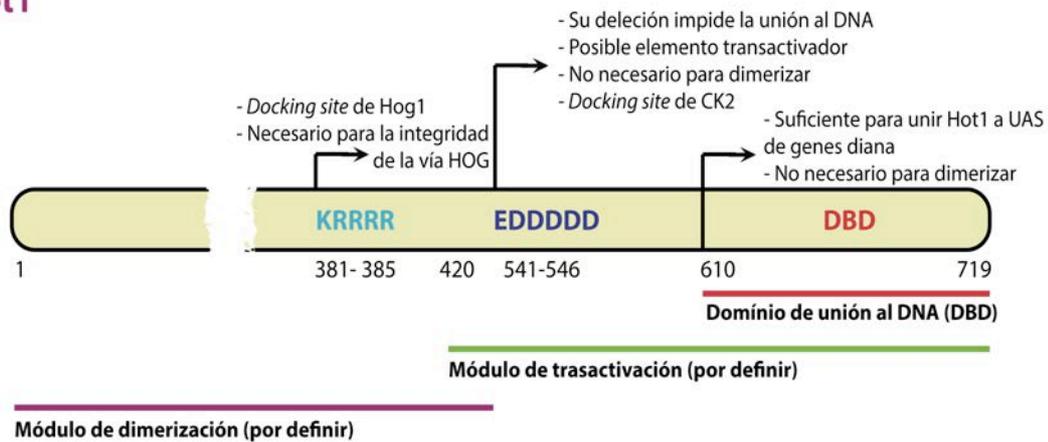
Figura 4.10. Representación esquemática de los elementos de secuencia esenciales para la funcionalidad de Hot1 y de los distintos módulos del factor transcripcional descritos en este trabajo. Se indica la posición de los mismos dentro de la proteína y la información más relevante descrita en esta Tesis acerca los mismos.

Estudio de la secuencia de unión al DNA reconocida por Hot1

En el Capítulo 3 se realiza un análisis *in silico* de los promotores de los genes regulados por Hot1 para hallar posibles secuencias consenso (Tabla 3.3.2). Se demuestra, mediante ensayos *in vitro* (EMSA) e *in vivo* (Un-Híbrido haciendo uso del gen reportero *HIS3* en ensayos de crecimiento en placa con 3AT 1mM) que Hot1 puede unir la secuencia GGGACAAA, situada en posición -173 en el promotor del gen *STL1*. Esta secuencia no se encuentra en todos los otros genes regulados por el factor transcripcional, pero sí en *GPD1* (-247) y *GPP2/HOR2* (-450). Curiosamente, estos son los genes (denominados del Gupo 1, Tabla 3.3.3 y Figura 3.3.2) en los que mediante ChIP se detecta mayor unión de Hot1, que además, puede reconocer sus promotores también en ausencia de estrés osmótico. Otro de los genes controlados por Hot1 y ampliamente analizado en este trabajo, el gen *HGI1/YHR087W,* no presenta en su promotor esta secuencia, pero, en posición -318 contiene una muy similar, GGGACAA. Ensayos de competencia en retardo en gel llevados a cabo *in vitro* demuestran que Hot1 también es capaz de unir este elemento (Figura 3.3.3), pero no otra posible secuencia consenso (de acuerdo con los análisis bioinformáticos), AGAATAAA, situada en una posición cercana en este promotor. El hecho de que, *in vivo*, mediante los ensayos de Un-Híbrido no se observe unión a la secuencia GGGACAA (Figura 3.3.3), que además está incluida en la presente en *STL1*, GGGACAAA, puede ser debido a diferencias de afinidad. Si, tal como se demuestra en este capítulo, Hot1 puede unir la secuencia de *STL1* con mayor afinidad que la de *HGI1* (Figura 3.3.5) es posible que las condiciones del ensayo de Un-Híbrido con gen reportero *HIS3* sean demasiado estrictas para detectar esta unión, incluyendo la presencia de estrés osmótico requerida para que Hot1 se una al promotor de *HGI1*.

Una de las aportaciones más interesantes de este trabajo es ver cómo las variaciones en la secuencia UAS de Hot1 GGGACAAA determinan las particularidades de unión del factor transcripcional al promotor de cada gen en los ensayos *in vivo* de inmunoprecipitación de la cromatina. Hot1 sólo puede unirse en ausencia de estrés en los genes que poseen la secuencia completa y la unión a estos genes, clasificados como Grupo1, es siempre mayor que al resto. En ese sentido, los genes del Grupo 2, que tienen la secuencia GGGACAA, incluida en la anterior, pero que Hot1 une con menos afinidad, presentan niveles de unión inferiores a los del Grupo 1, pero significativamente aún

bastante altos (aunque Hot1 sólo los puede unir en presencia de estrés) con respecto a los genes del Grupo 3, que presentan dos cambios de nucleótidos con respecto a la original. Este tercer grupo de genes, aunque estén regulados por Hot1 según los resultados de las micromatrices realizadas para estos experimentos (Tabla 3.3.1), presentan niveles muy bajos de unión del factor transcripcional (Figura 3.3.2 Panel B). Es importante señalar, sin embargo, que estamos analizando la capacidad de unión de Hot1 en función de la secuencia consenso que reconoce, y que esto, no está inexorablemente unido al nivel de expresión de cada gen, que puede estar influido por la unión de otros factores transcripcionales a otros elementos del promotor. Así, entre los genes regulados total o parcialmente por Hot1 y Hog1, *STL1* y *HGI1* son los que presentan mayores niveles de expresión (Capaldi y col., 2008; Cook and O'Shea 2012; Bai y col., 2015). Pero hay que tener en cuenta que en todos los genes considerados, a excepción de *STL1*, otros factores transcripcionales (Msn1, Sko1, Msn2/4, etc.) participan en el control de su expresión. En cualquier caso, los resultados mostrados en la Figura 3.3.5, en donde se compara la expresión del gen *lacZ* bajo el control del promotor completo de *HGI1* y de su variante mutada por introducción de "A" a su secuencia GGGACAA para tener la UAS completa de *STL1* en el contexto de dicho promotor (*Stl1-Like*), demuestran que, efectivamente, la secuencia determina las peculiaridades de unión de Hot1 a los genes que regula.

Es también interesante comentar que hay varias evidencias que apoyan la propuesta de la secuencia GGGACAAA como UAS de Hot1. En primer lugar, este elemento presenta cierta similitud al reconocido por el factor transcripcional Msn1 (Fordyce y col., 2010), y el dominio de unión al DNA de Hot1 también presenta homología de secuencia al de este factor transcricional (Figura 3.3.1). Estas similitudes en las propiedades relacionadas con la unión al DNA explicarían la redundancia funcional propuesta entre Hot1 y Msn1 (Rep y col., 1999b). El hecho que la secuencia descrita en este trabajo sea consistente con la propuesta por Cook and O'Shea en 2012 refuerza también nuestros resultados.

Por todo ello quizás, el mayor punto de discusión radica en el recientemente publicado trabajo de Bai y col. (2015) en el que identifican un nuevo elemento de secuencia (HoRe, *Hog1-Responsive Element*) en el promotor de *STL1* entre las posiciones -654 y -626, que es necesario para la activación del gen en condiciones de osmoestrés de forma dependiente de Hot1 y Hog1. Este elemento sería reconocido por Hot1 *in vitro*, y contiene

la secuencia CATTTGGC repetida dos veces en su interior. Es importante mencionar que el cebador utilizado por estos investigadores para el ensayo de retardo en gel (Figura 4.11), contiene la secuencia TTTGGCCC, cuya complementaria sólo difiere de un nucleótido de la sugerida en este trabajo como UAS del factor transcripcional.


Figura 4.11. Secuencia del oligonucleótido utilizado en el trabajo de Bai y col. (2015) para los ensayos de EMSA. Se indica en color rosa la secuencia que plantean como candidata a ser reconocida por Hot1, repetida dos veces y una tercera vez con la variación de dos nucleótidos (azul). Dentro de este oligo se encuentra la secuencia planteada en este trabajo como UAS de Hot1, con una sola discrepancia (rojo), lo que explicaría que observe union in *vitro* y que se pierda dicha unión al mutar las posiciones indicadas con puntos en la Figura, lo que supondría un total de 3 diferencias con la secuencia consenso propuesta en este trabajo. Adaptado de Bai y col. (2015).

Como el elemento HoRe sólo se encuentra en *STL1*, y la transcripción de este gen es nula en un mutante *Δhot1*, los autores de este artículo proponen que este es el único gen que estaría críticamente regulado por Hot1, siendo muy poca la influencia del factor transcripcional sobre el resto de sus genes diana. Sin embargo, como se ha discutido anteriormente, son numerosos los estudios que muestran un efecto de dicho factor transcripcional en la expresión de otros genes (Figura 4.6, Figura 3.1.2) identificados en estudios de diversos autores y también es un hecho repetidamente demostrado que Hot1 puede además unirse al promotor de algunos de ellos (Rep y col., 1999b; Alepuz y col., 2001; Alepuz y col., 2003; Capaldi y col., 2008; Gomar-Alba 2012; Cook and O'Shea 2012; Gomar-Alba y col., 2013 Figura 3.3.2). Ensayos de ChIP realizados en estos trabajos demuestran que Hot1 puede unir *CTT1*, *GPD1*, *HSP12* y *HGI1*, y también *STL1*, en regiones situadas entre -100 y -400, es decir, no tan alejadas como las que incluyen el elemento HoRe (-600 a -800), por lo que es esperable pensar que reconozca alguna secuencia concreta y común, como la planteada en este trabajo, para su unión. Por ello, quizás el resultado más contradictorio de nuestros resultados con el publicado por Bai y col. (2015) sea que no detecten mediante ensayos de β-galactosidasa ninguna unión en la región entre -583 y -1 del promotor de *STL1*.

Nos parecio de fundamental importancia demostrar, por tanto, que el elemento GGGACAAA era necesario para la unión de Hot1 y la expresión de sus genes diana, por lo

que se utilizó un plásmido multicopia (YEp358R) que contiene el gen *lacZ* bajo el control del promotor completo (-825 a +4) de *STL1* (Alepuz y col., 2003) y su correspondiente versión mutada (*STL1ΔUAS*) en la que se delecciona mediante mutagénesis dirigida la secuencia GGGACAAA (presente en -173) para realizar ensayos β-galactosidasa. El resultado obtenido (Figura 4.12) demuestra que sin los 8 nucleótidos de la UAS de Hot1, la expresión del gen reportero *lacZ* se reduce al menos a la mitad, en los distintos tiempos tras aplicar estrés osmótico (NaCl 0.9M).
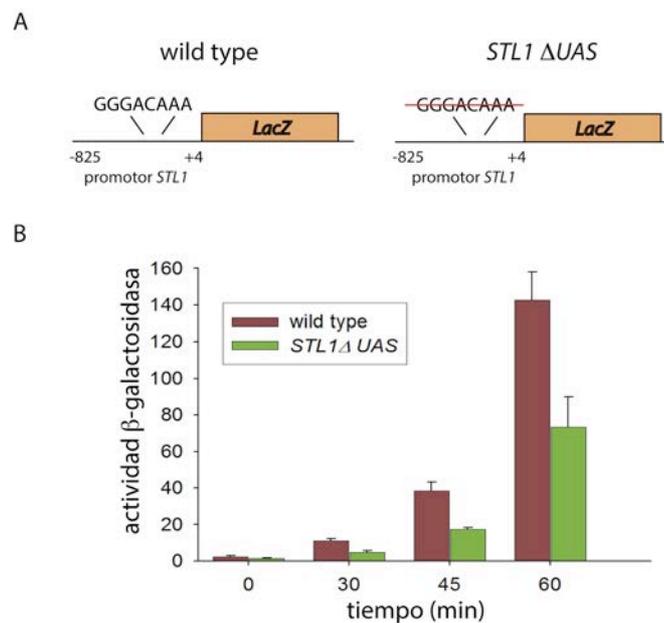
Figura 4.12. La secuencia GGGACAAA es necesaria para la activación transcripcional mediada por Hot1. Una cepa que expresa la copia silvestre de Hot1 fue transformada con el plásmido multicopia YEP358R, que contiene el gen reportero *lacZ* bajo el control del promotor completo (-825 a +4) de *STL1* (wild type) o su correspondiente versión mutada en la que se delecionó la secuencia GGGACAAA (*STL1ΔUAS*). Células en fase exponencial ($OD_{600} = 1$) fueron sometidas a estrés osmótico (NaCl 0.9M) y se recogieron a los tiempos indicados en la Figura. Se realizaron ensayos β-galactosidasa, tal y como se describe en *Materiales y Métodos* de Gomar-Alba y col. (2013). Los experimentos se llevaron a cabo, al menos, por triplicado; se muestra el rango de desviación estándar de los datos.

Este resultado es de fundamental importancia, ya que se aporta la evidencia directa de que solamente delecionando esa pequeña secuencia, se reduce a la mitad la expresión del gen reportero. El hecho de que no se anule completamente, sugiere que Hot1 se puede estar uniendo a otras regiones, presumiblemente a secuencias presentes en el promotor que sólo presentan un cambio con respecto a la consenso (Figura 3.3.4), entre las cuales estaría la ya

comentada presente en el HoRE. Actualmente tratamos de realizar un ensayo de ChIP, similar al mostrado en la Figura 3.3.5, haciendo uso de estos plásmidos para demostrar que delecionando GGGACAAA se afecta directamente a la unión de Hot1.

Finalmente hay otro punto interesante a comentar sobre la secuencia de reconocimiento de Hot1, y es que, tal como se muestra en la Figura 3.3.4, muchas veces se encuentra repetida (la misma o con un cambio de nucleótido) en el promotor de los genes diana en posiciones cercanas. Esto nos remite otra vez a la estructura del dominio de unión al DNA de Hot1, y a la posibilidad de que forme dímeros que reconozcan estas secuencias cercanas. Por otro lado, el dominio de unión de Gcr1 podría contener un dominio hélice-vuelta-hélice en posición -784 a -803. (Baker, 1986). Un estudio más reciente (Krantz y col., 2006) de la región homóloga de Hot1, es decir, del ahora descrito como dominio de unión al DNA, revela que se podría plegar en cuatro hélices de nueve residuos (hélices 1-3) y quince residuos (hélice 4). Esto podría permitir la configuración en dos motivos hélice-vuelta-hélice que estarían separados por una región de aminoácidos básicos y también podría explicar que, aun uniéndose como monómero, Hot1 pudiera reconocer repeticiones en secuencias cercanas.

Genes regulados por Hot1

Del análisis de las micromatrices realizadas con la cepa *Δhot1* en el Capítulo 3, se obtiene una serie de genes que están regulados, al menos parcialmente, por el factor transcripcional Hot1. Como ya se ha comentado en esta discusión, es realmente interesante observar las particularidades de regulación que presentan cada uno, determinadas, por ejemplo por la secuencia consenso presente en sus promotores, y las peculiaridades de unión de Hot1 que esto implica. Entre ellos, están los genes utilizados como modelo a lo largo de este trabajo, *HGI1* y *STL1*, de los que ya se ha comentado también las características que presentan en cuanto a su regulación.

Uno de los aspectos más interesantes observados, y del cual ya existía bibliografía previa, es que Hot1 puede unir los promotores de *STL1*, *GPD1* y *GPP2* en ausencia de estrés (genes del Grupo1). Esto implica que Hot1, sin ser activado por osmoestrés, y por

tanto, de forma independiente de Hog1, puede unir el DNA, y en niveles elevados (Figura 3.3.2). En estos genes (al menos en *GPD1* y *STL1*, no hay datos publicados sobre *GPP2*), en una cepa *Δhog1*, Hot1 está situado en el promotor, mientras que en una cepa *Δhot1* Hog1 no puede ser reclutado al mismo (Alepuz y col., 2001; Alepuz y col., 2003; Gomar-Alba y col., 2013). Estas observaciones nos llevan a concluir que en los genes del Grupo 1, Hot1 previamente unido dirige y recluta Hog1 al promotor en respuesta a estrés.

En los genes de los Grupos 2 y 3 (*CTT1*, *HSP12*, *HGI1*, entre otros), por el contrario, Hot1 no puede unirse en ausencia de estrés, y necesita la presencia de Hog1 para ser reclutado. Tal como se muestra en el Capítulo 1 (Gomar-Alba y col., 2012), en una cepa *Δhog1*, Hot1 no puede reconocer el promotor de *HGI1*, y lo mismo ocurre con *CTT1* y *HSP12* (Alepuz y col., 2001). Consistentemente con todo ello, en una cepa *Δhot1* Hog1 puede unirse al promotor de estos genes, aunque en niveles más bajos que en una cepa silvestre, lo que sugiere que Hot1 no es imprescindible, aunque participa, en el reclutamiento de Hog1 a los promotores de los genes del Grupo 2 y 3 (Alepuz y col., 2001; Gomar-Alba y col., 2013). En el caso de *CTT1* y *HSP12*, el reclutamiento de Hog1 se pierde en el mutante *Δmsn2/4* (Alepuz y col., 2001).

Estas consideraciones tienen gran interés porque, por primera vez, se puede observar una relación entre estas series de datos, pudiendo clasificar los genes regulados por Hot1 como más o menos dependientes de la interacción (y por tanto activación) mediada por Hog1. Por eso, en el caso de *STL1*, en el mutante *hot1ΔKR4* aun se observa un cierto nivel de expresión, y por tanto de transcritos, sobre todo a tiempos cortos, debido a la asociación de Hot1 en el promotor independientemente de la presencia de Hog1, mientras que en *HGI1*, el perfil de dicho mutante es idéntico al del *Δhot1*, pues es completamente dependiente de la interacción con Hog1 para además de ser activado, reclutarlo (Figura 3.2.6). Con todo, no hay que olvidar que, finalmente, siempre es necesaria la presencia de la MAPK para que se expresen estos genes (incluido *STL1*), pues participa, entre otros aspectos, en el reclutamiento de la RNApolII y la maquinaria de inicio de la transcripción (Alepuz y col., 2003; Cook y O'Shea, 2012; Nadal-Ribelles y col., 2012), y tal como se ha explicado con anterioridad, la actividad transactivadora de Hot1 es dependiente de la activación por estrés y la interacción con Hog1.

En el Capítulo 3 de esta Tesis se aportan, por tanto, importantes resultados sobre la secuencia reconocida por Hot1 y las particularidades de unión y regulación a sus genes diana, todos ellos fundamentales en la respuesta adaptativa a la situación adversa de osmoestrés.

Estudio de las interacciones físicas del factor transcripcional Hot1

En el último Capítulo referente al estudio de Hot1 de esta Tesis (Capítulo 4), se analizan las interacciones físicas en las que participa el factor transcripcional, más allá de las tan importantes, ya descritas, interacciones Hog1-Hot1 y Hot1-Hot1.

Este estudio está focalizado en las interacciones en que participa Hot1 principalmente después de su activación por estrés osmótico causado por glucosa (10 minutos en presencia de concentraciones de este azúcar de 20%, p/v). Se eligió esta condición particular porque, tal como se discute en el Capítulo 5 de esta Tesis, diversas evidencias sugieren que bajo esta condición de estrés hay una reprogramación de la expresión génica dependiente de Hog1 hacia los genes regulados por Hot1 y Sko1 (Capaldi y col., 2008 y diversos resultados presentados en este estudio, Gomar-Alba y col., 2015, Figuras 3.5.2 y 3.5.3) debido al menor papel de Msn2/4, causado por la disponibilidad de nutrientes en el medio de crecimiento y la activación de la vía PKA. El estudio se ha centrado en las interacciones que tienen lugar tras la activación de Hot1 debido a que aquéllas que suceden anteriormente (entre ellas la interacción con Hog1) y están relacionadas con quinasas o fosfatasas que regulen su actividad son muy rápidas y transitorias y difíciles de detectar en una purificación a gran escala como la llevada a cabo en el método TAP. Por otro lado, el mismo análisis se realizó en condiciones de no estrés, en un cultivo con células en crecimiento exponencial, y los resultados obtenidos (Tabla 3.4.1) muestran que, sin estrés, Hot1 interacciona con muy pocas proteínas y que, una vez activado, se incrementa el número de interacciones en las que participa, aunque, seguramente debido al papel de Hot1 como factor transcripcional, siguen sin aparecer demasiadas proteínas candidatas a interaccionar con dicha proteína.

Los resultados del TAP muestran que Hot1 interaccionaría con alguna proteínas

implicadas en la elongación transcripcional, como Rpb6, Sub1 y Spt5. La interacción con las dos primeras ha sido confirmada por CoIP (Figura 3.4.1) y son coherentes con algunos estudios previos. Así, Constanzo y col. (2010) habían sugerido una interacción de Hot1 con la subunidad grande de la polimerasa. Por otro lado, el trabajo realizado por García y col. (2012) demostró la existencia de interacción entre el co-activador transcripcional Sub1 y el factor de elongación Spt5, lo que sugiere que las tres proteínas, Sub1-Hot1-Spt5 pueden interaccionar entre ellas, en genes de respuesta a estrés. El hecho de que Hot1 presente, además, interacciones genéticas con Sub1, Spt4 y Spt5 (Figura 3.4.5) en ensayos de crecimiento en placa con 6-AU refuerza la vinculación del factor transcripcional con el complejo de elongación Spt4/5.

Como se ha comentado anteriormente, aunque ambos procesos son dependientes de Hog1, la presencia de Hot1 en los promotores de genes de respuesta a estrés es necesaria para el reclutamiento tanto de la maquinaria transcripcional como de la RNApolII (Alepuz y col., 2003). Si bien en los genes no completamente dependientes de Hot1 para su expresión es posible detectar polimerasa unida en el mutante *Δhot1*, en aquellos que, como *STL1*, son totalmente dependientes del factor, en dicho mutante, Rpb1 no es reclutada al promotor (Figura 3.2.7). Todo esto sugiere que es coherente pensar que Hot1 interaccione con la polimerasa y con algunos elementos de la maquinaria de inicio y elongación transcripcional. Esta posibilidad aparece reforzada por los resultados de los análisis de inmunoprecipitación de la cromatina que indican que Hot1 se puede localizar dentro de la ORF más allá de la región promotora de los genes que regula (Figura 3.4.4). No obstante, tal como muestra la Figura 3.4.4 Panel D, el posicionamiento en la región 5' y central de la ORF disminuye mucho y se reduce ya a un 20% en la región 5' de la ORF cuando se compara con el promotor. Todos estos datos sugieren que, aunque Hot1 pueda tener un papel más allá del inicio de la transcripción, no parece probable que viaje junto a la RNApolII y el resto de maquinaria de elongación a lo largo de genes de respuesta a estrés, como ha sido descrito, por ejemplo, para la MAPK Hog1 (Proft y col., 2006; de Nadal y Posas, 2011).

Respecto a las interacciones detectadas en este trabajo, probablemente la que presenta un mayor interés es la vinculada a Sub1. Se trata de un activador transcripcional que facilita la elongación y procesamiento en 3' del mRNA a través de factores que

modifican la polimerasa (Henry y col., 1996; Knaus y col., 1996; Calvo y Manley, 2001; He y col., 2003; Sikorski y col., 2011; García y col., 2012). En este trabajo se demuestra, en el caso de *STL1*, que la entrada de Sub1 a los promotores es totalmente dependiente de la presencia de Hot1. Con respecto a *HGI1*, en el mutante *Δhot1* disminuye mucho el reclutamiento de Sub1 a la región promotora, aunque sigue habiendo proteína unida, debido seguramente a otros factores transcripcionales que también ejercen la función de direccionamiento de la RNA polimerasa II, y mantienen, aunque en menores niveles, la expresión del gen. Es importante destacar que esta dependencia Sub1-Hot1 se mantiene a lo largo de toda la ORF de *STL1* (Figura 4.13) aunque no tiene sentido indagar en las repercusiones que esto tiene en la expresión génica, debido a que en ausencia de Hot1 no hay reclutamiento de la polimerasa ni transcripción de *STL1*.
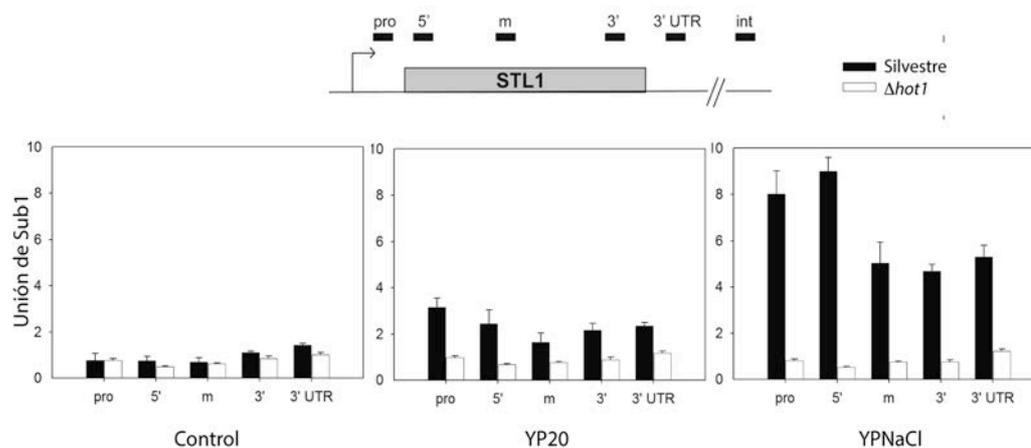
Figura 4.13. Asociación de Sub1 a diferentes regiones de *STL1* en una cepa silvestre (FY86) y en el mutante *Δhot1*, en condiciones de osmoestrés. La Figura muestra la ocupación del coactivador Sub1 en las regiones del gen *STL1* indicadas en el esquema, determinada mediante experimentos de inmunoprecipitación de cromatina (ChIP). En el mutante *Δhot1,* Sub1 no puede unir ninguna de las regiones consideradas. El experimento se realizó con células en fase exponencial que fueron transferidas a YPD (control), YP20 (osmoestrés por glucosa al 20% (p/v)) o YPNaCl (osmoestrés por NaCl 0.6M) durante 20 minutos. La técnica de ChIP se realizó como se describe en *Materiales y Métodos* del Capítulo 4 de esta Tesis, y para la inmunoprecipitación, se usó el anticuerpo α-HA 3F10 de *Roche*.

Contrariamente, la ausencia de Sub1 sólo afecta muy ligeramente a la disminución de unión de Hot1 (Figura 3.4.3) lo que implica una menor, aunque relevante, expresión del gen. Esto hecho evidencia, de nuevo, el papel clave de Hot1 como factor principal en el reclutamiento del resto de elementos de la maquinaria transcripcional en la respuesta a

estrés hiperosmótico causado por elevadas concentraciones de glucosa, independientemente del papel que pueda tener posteriormente, en la fase de elongación temprana de la transcripción. Con todo, los resultados de los análisis de ChIP apoyan la interacción descrita en el Capítulo 4 entre Sub1-Hot1, también reforzada por las interacciones genéticas detectadas entre ambas proteínas (Figura 3.4.5) y por su patrón de asociación similar a la cromatina a través de la región codificante de los genes hasta 3' (Figura 3.4.4), aunque la asociación de Hot1 sea mucho mayor en las regiones promotoras y de inicio de la ORF.

Es también interesante destacar que se ha sugerido (Rosonina y col., 2009) un papel de Sub1 en la regulación de genes en condiciones de osmoestrés, aunque, los niveles de mRNA de los genes que controlaría en estas condiciones (*ALD3, STL1, GPD1, CTT1, HSP12* y *HSP26*) no se ven muy afectados en el mutante *Δsub1*. Los resultados presentados en este trabajo, con respecto a los genes *STL1* y *HGI1*, apuntan en la misma dirección, puesto que sólo se observa una ligera disminución en sus niveles de transcrito con respecto a la silvestre y una reducción del 20% aproximadamente en el reclutamiento de la RNApolII. La implicación de la interacción Hot1-Sub1 parece estar, por tanto, explicada por la necesidad de la presencia previa en el promotor de Hot1 para reclutar a Sub1, siendo el coactivador necesario para el total reclutamiento de Hot1 a esta región, y por tanto, de la RNApolII, para asegurar niveles óptimos de transcrito.

Los resultados del TAP realizado en este trabajo también sugieren que Hot1 podría interaccionar en respuesta a estrés con proteínas relacionadas con el proceso de exportación, como Sub2, Dbp5, Yrb1, Pbp1, Pbp4, Yra1 y Pab1. Dada la distribución de ocupación del factor transcripcional a lo largo de la ORF, con una presencia casi inexistente en 3', es difícil aportar pruebas que puedan explicar estas interacciones, más allá de la interacción entre Hot1 y Pab1, confirmada por coinmunoprecipitación, y de la conocida simultaneidad de las distintas etapas del proceso de expresión génica.

Finalmente, hay otro tipo de interacciones que son foco de estudio en este trabajo, interacciones muy rápidas y transitorias que no pueden ser detectadas por la metodología del *Tandem Affinity Purification*, pero que pueden ser importantes en la regulación del factor transcripcional. Son las ejercidas por quinasas y fosfatasas, aunque el estudio de estas últimas no se aborda directamente en este trabajo. A lo largo de esta Tesis se ha

discutido ampliamente sobre la importancia de la interacción Hog1-Hot1, para poder tener un Hot1 activado y funcional, a pesar de que la fosforilación ejercida por la MAPK no es necesaria para el reclutamiento de la RNApolII ni tampoco, por tanto, para la expresión de los genes diana. Otra quinasa que parece regular a Hot1 es CK2. Varios autores han descrito interacciones físicas entre Hot1 y las cuatro subunidades de la quinasa CK2 (Ho y col., 2002; Gavin y col., 2006; Collins y col., 2007; Breitkrentz y col., 2010). Además, recientemente, se ha descrito que esta quinasa participaría en la regulación de Hot1 (Burns y Wente, 2014), a través de su fosforilación e inhibición en condiciones de osmoestrés.

Los resultados obtenidos en el Capítulo 4 referentes a CK2 son coherentes, y hasta cierto punto complementarios a los presentados por estos autores. Nosotros no detectamos en los mutantes *Δcka1* y *Δcka2* (Figura 3.4.2) la reducción en la fosforilación de Hot1 observada por Burns y Wente (2014), aunque hay que destacar que ellos sólo la encuentran en el doble mutante *Δhog1 Δcka2*, y no en el simple *Δcka2*. En este sentido, en la Figura 3.4.2 se muestra claramente que en condiciones de no estrés Hot1 se encuentra fosforilado en los mutantes *Δcka1* y *Δcka2*, por lo que, de algún modo, CK2 debe estar implicada en la desfosforilación del factor transcripcional en ausencia de estrés. Este hecho no es detectado por dichos autores, pero sí es coherente con sus resultados, pues en el mutante *Δcka2* observan mayor unión de Hot1 al promotor de los genes que regula (*STL1* y *GPD1*) en estas condiciones, sugiriendo que CK2 reprime Hot1 en condiciones de no estrés. Nuestros datos sugieren que esta regulación en condiciones fisiológicas podría ser mediada por alguna fosfatasa que estuviera controlada por CK2.

Algunos análisis globales han permitido encontrar otras quinasas candidatas a interaccionar con Hot1: Rim11, Kcs1 (Bandyopadhyay y col., 2010) y Ssk2 (Rep y col., 1999b). Pero en los resultados mostrados en la Figura 3.4.2 no parece que afecten a la fosforilación del factor transcripcional. Lo mismo sucede con las fosfatasas Gpp1/2 que, según los resultados del TAP (Tabla 3.4.1), podrían interaccionar con Hot1 tanto en condiciones de estrés como en ausencia del mismo. Estos resultados no descartan directamente que puedan tener algún papel en las modificaciones post-transcripcionales de Hot1, pues, tal como ocurría en CK2 en presencia de estrés, puede que sea necesario realizar el ensayo con dobles mutantes para observar cambios en la fosforilación de Hot1.

Consideraciones finales

Durante el desarrollo de esta Tesis Doctoral hemos profundizado en algunos aspectos relacionados con la respuesta a estrés hiperosmótico, particularmente al causado por elevadas concentraciones de glucosa. De este modo hemos podido avanzar en el conocimiento de la función, relacionada con la traducción de transcritos particulares, de uno de los genes cuya expresión experimenta un fuerte incremento en dichas condiciones, *YHR087W/HGI1*. Por otro lado hemos profundizando en el conocimiento del factor transcripcional Hot1 entendiendo mejor cómo interacciona con la MAPK Hog1 y con el DNA. Además se han identificado interacciones que no se habían descrito previamente con el factor Sub1 y con el complejo de elongación Spt4/5. Aunque todavía existen muchos aspectos desconocidos, en la Figura 4.14 se intenta plasmar la visión que este trabajo aporta acerca de cómo, en diferentes condiciones de estrés osmótico, pero concretamente en el causado por glucosa, Hot1 contribuiría al control de la expresión de los genes diana contemplados en este trabajo: *STL1* y *HGI1*.
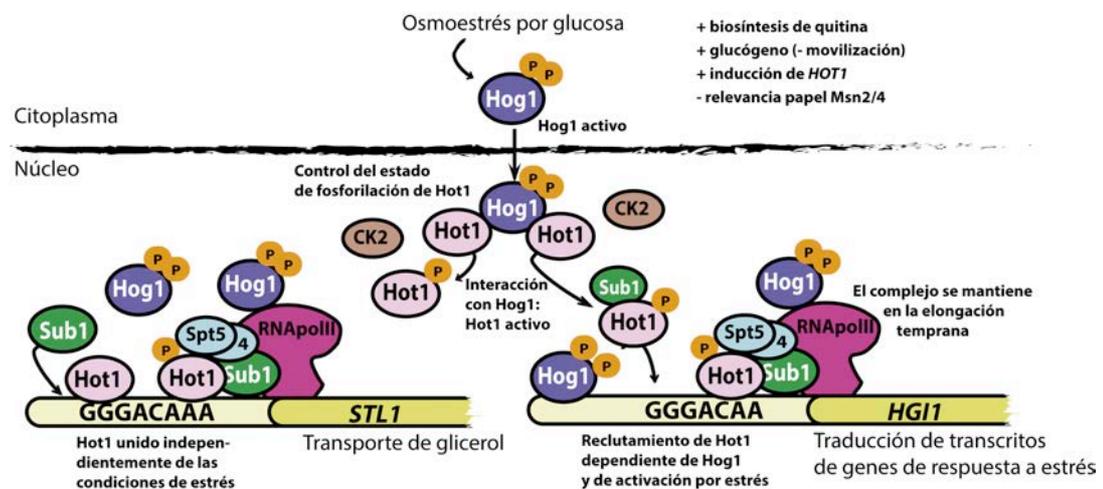

Figura 4.14. Modelización de algunos de los principales resultados aportados en los distintos estudios realizados en esta Tesis acerca del control de Hot1 sobre la expresión de sus genes diana en respuesta a estrés por altas concentraciones de glucosa.

5. Conclusiones

En los anteriores apartados, se han presentado y discutido los resultados obtenidos a lo largo de esta Tesis Doctoral. A continuación, se exponen las principales conclusiones que derivan de este trabajo.

1.- El gen *YHR087W/HGI1* se induce en condiciones de estrés causado por choque térmico y, más significativamente, por estrés osmótico provocado por altas concentraciones de glucosa. La regulación de la expresión del gen es distinta en cada caso, siendo Hot1 y Sko1 los efectores principales en caso de osmoestrés, y Msn2/4 en choque térmico. También presenta interacciones genéticas diferenciales en una u otra condición, habiéndose demostrado una supresión parcial del fenotipo deletéreo del mutante *Δbcy1* en el doble mutante *Δbcy1Δhgi1* en condiciones de estrés debido a altas temperaturas.

2.- En condiciones de osmoestrés por alta glucosa, la unión de Hot1 al promotor de *HGI1* es dependiente de la presencia de Hog1, tal como sucede en algunos otros genes regulados por Hot1, como *CTT1* y *HSP12*. La alta y mantenida expresión de *HGI1* (de 20 minutos a más de 2 horas) en estas condiciones hace de este gen un buen modelo para el estudio de otros regulados parcialmente por el factor transcripcional de respuesta a osmoestrés Hot1, siendo el complemento perfecto del gen *STL1,* controlado exclusivamente por dicho factor transcripcional.

3.- Hgi1 interacciona físicamente con proteínas implicadas en el proceso de (inicio) de la traducción, como Cdc33, Tif32 y Tif4631, y además se purifica con la fracción ribosomal, indicando que también se asocia (al menos parcialmente) con los ribosomas. El mutante *Δhgi1* presenta sensibilidad a cicloheximida y de forma débil a higromicina, al igual que otros muchos mutantes implicados en distintas etapas del proceso de traducción. En esta última condición también interacciona genéticamente con Cdc33.

4.- El mutante *Δhgi1* presenta una recuperación de la traducción general en respuesta a estrés osmótico más lenta que la cepa silvestre, debido a los defectos en la traducción de mensajeros concretos de respuesta a estrés, como *GPD1*, *HSP104* y *HSP78*, lo que explica que el mutante presente niveles inferiores de proteína, al menos en los dos últimos casos. Se atribuye, por primera vez, una funcionalidad a Hgi1 relacionada con la respuesta a estrés en la traducción de mensajeros específicos.

5.- La interacción con la MAPK Hog1 (y no la fosforilación por la misma) es el punto clave para activar al factor transcripcional de respuesta a osmoestrés Hot1. El *docking site* de la quinasa está situado en las posiciones 381-385 y comprende los residuos básicos KRRRR. Otro elemento clave para que Hot1 sea funcional son los aminoácidos ácidos EDDDDD situados en posición 541-546, cuya deleción impide la unión de Hot1 a los promotores de los genes que regula. No se puede descartar que el dominio ED5 esté también implicado en la actividad transactivadora del factor transcripcional. Los elementos KR4 y ED5 de Hot1 son necesarios para la funcionalidad de la proteína y, sin ellos, la expresión de los genes diana del factor transcripcional es la misma que en el mutante por deleción *Δhot1*. Ambos elementos se requieren para la adquisición de osmotolerancia dependiente de Hot1 y KR4, además, es necesario para la integridad de la ruta HOG.

6.- Aunque sin ED5 Hot1 no puede unir sus promotores diana, el motivo de unión al DNA de Hot1 está situado en la parte carboxi-terminal de la proteína, abarcando los residuos 610-710. Esta región, que no incluyen ED5, es capaz de unir por si misma *in vitro* e *in vivo* secuencias diana en el DNA.

7.- Hot1 regula la expresión de los genes de respuesta a estrés osmótico *GPD1, DIA3, STL1, GPP2, SPI1, NQM1, HGI1, GRE1, CTT1* y *HSP12*. El análisis *in silico* de las secuencias de sus promotores revela una serie de posibles secuencias consenso candidatas a ser reconocidas por Hot1. De entre ellas, la secuencia GGGACAAA puede ser unida *in vitro* e *in vivo* por el factor transcripcional, por lo que se propone como UAS de Hot1. La

unión diferencial de Hot1 a los promotores de los genes que regula viene determinada por las particularidades y variaciones de esta secuencia UAS consenso, de modo que cuando esta presente en forma íntegra, Hot1 se une en altos niveles y en ausencia de estrés al promotor (*STL1, GPD1 y GPP2*). Cuando está presente GGGACAA (*HGI1, HSP12 y NQM1*) sólo puede unirse en presencia de estrés, y en niveles más bajos que en la original. En promotores que contienen secuencias que se diferencian en dos posiciones de la UAS la capacidad de interacción de Hot1 es mucho menor.

8.- Las secuencias GGGACAAA y GGGACAA se encuentran (considerando un cambio) repetidas dos veces y en posiciones cercanas dentro de los promotores de los genes diana de Hot1. Esto sugiere que Hot1 podría unirlas, bien mediante dos motivos hélice-vuelta-hélice que serían la supuesta estructura (4 hélices) del dominio de unión al DNA o bien en forma de dímero. Hot1 puede interaccionar consigo mismo y formar dímeros, y ni el dominio ED5 ni el dominio de unión al DNA son imprescindibles para dimerizar. El hecho que sin la copia genómica los truncados de Hot1 posean mucha menos actividad transactivadora sugiere que Hot1 es un factor de transcripción atípico que no presenta una gran capacidad transactivadora, y refuerza la posibilidad que el dímero sea la forma funcional del factor transcripcional, aunque no se puede descartar que se una al DNA también como monómero.

9.- Hot1 no solamente interacciona consigo mismo y con la MAPK Hog1, sino también con una serie de proteínas relacionadas con la elongación transcripcional, como Sub1, Spt5 y Rpb6. Hot1 también presenta interacciones genéticas con Sub1, Spt4 y Spt5, siendo en todos los casos el doble mutante más sensible a 6-AU. El reclutamiento de Sub1 a los promotores de respuesta a estrés es dependiente de Hot1, y esta dependencia se mantiene también a lo largo de toda la ORF. La distribución de Hot1 y Sub1 a lo largo del gen *STL1* es también parecida. Este dato, unido a las interacciones físicas y genéticas con el complejo Spt4/5, sugiere un papel para Hot1 más allá del inicio transcripcional, al menos en la fase de elongación temprana.

10.- La quinasa CK2, que une y fosforila Hot1, tiene también un papel en su regulación en ausencia de estrés, pues en el mutante *Δcka1* y *Δcka2* Hot1 se muestra fosforilado en condiciones fisiológicas, lo que sugiere que CK2 determinaría, mediante su acción sobre una o varias fosfatasas, el estado no fosforilado del factor transcripcional en ausencia de estrés.

11.- La respuesta a estrés osmótico específica causada por elevadas concentraciones de glucosa implica una mayor expresión del factor transcripcional Hot1, así como una regulación diferencial del mismo (orquestrada por Msn2/4 y Skn7) cuando se compara con el estrés equivalente causado por sal o sorbitol. Aunque, al igual que en las otras condiciones, Msn2/4 sigue siendo el factor transcripcional que regula la expresión de la mayoría de genes, lo hace de manera más parcial en osmoestrés por glucosa, condición en la que muestra una activación (entendida como fosforilación y localización nuclear) más transitoria.

12.- La levadura *S. cerevisiae* sobreexpresa diferencialmente en condiciones de osmoestrés por alta glucosa genes implicados en la biosíntesis de la pared celular, lo que a nivel fisiológico repercute en un aumento de la presencia de quitina en este compartimiento y una menor resistencia a *calcofluor white*. Por el contrario, en presencia de sorbitol como osmolito aparecen diferencialmente sobreexpresados genes implicados en la biosíntesis y movilización de glucogéno, lo que se traduce en una mayor resistencia de las levaduras a la aplicación posterior de otra condición de estrés.

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Anexos

Anexo 1: Capítulo 3, Tabla Suplementaria 3.3.S4. Datos completos del análisis bioinformático presentado en este trabajo para determinar las secuencias putativas de unión al DNA de Hot1. Archivo: "Supplementary Table 3.3.S4.xls"

Anexo 2. Capítulo 1, Respuestas a los Revisores durante el proceso de corrección del artículo "The *Saccharomyces cerevisiae* Hot1p regulated gene *YHR087W* (*HGI1*) has a role in translation upon high glucose concentrations stress". Archivo: "Response to Reviewers BMC.doc"

Anexo 3. Capítulo 2, Respuesta a los Revisores durante el proceso de corrección del artículo "Dissection of the elements of osmotic stress response transcription factor Hot1 involved in the interaction with MAPK Hog1 and in the activation of transcription". Archivo: "Response to Reviewers BBA.doc"

Anexo 4. Capítulo 5, Respuesta a los Revisores durante el proceso de corrección del artículo "Response of yeast cells to high glucose involves molecular and physiological differences when compared to other osmostress conditions". Archivo: "Response to Reviewers FEMS.doc"