Universidad de Oviedo

Departamento de Bioquímica y Biología Molecular

Programa de doctorado: Biología Funcional y Molecular

The nuclear hexokinase 2 acts as an intracellular glucose sensor in *Saccharomyces cerevisiae*

TESIS DOCTORAL

Montserrat Vega López

Oviedo, 2016

This work was performed at the University of Oviedo (Spain) and the University of Osnabrück (Germany) as part of the International Ph.D. interchange program (IPID) funded by Deutscher Akademischer Austauschdienst (DAAD)

Supervisors:

Prof. Dr. Fernando Moreno

Prof. Dr. Pilar Herrero

Prof. Dr. Jürgen J. Heinisch

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Inglés: The nuclear hexokinase 2 acts as an intracellular glucose sensor in Saccharomyces cerevisiae	Español/Otro Idioma: La hexoquinasa 2 nuclear actúa como un sensor intracelular de glucosa en *Saccharomyces cerevisiae*
Alemán:	

2.- Autor	
Nombre: MONTSERRAT VEGA LOPEZ	DNI/Pasaporte/NIE:
Programa de Doctorado: Biología Funcional y Molecular	
Órgano responsable: BIOQUIMICA Y BIOLOGIA MOLECULAR	

RESUMEN (en español)

Para Saccharomyces cerevisiae, la glucosa no solo es el combustible preferido para su metabolismo energético, sino que también funciona como una señal reguladora de las vías centrales del metabolismo de carbohidratos. En condiciones de alta glucosa, el represor Mig1 es el principal factor transcripcional responsable de la represión de los genes implicados en la utilización de fuentes de carbono alternativas a la glucosa, como es el caso del gen *SUC2*. Recientemente se ha demostrado que en la mayoría de los genes regulados por Mig1, el proceso también requiere la presencia de la proteína hexoquinasa 2 (Hxk2). En S. cerevisiae, la Hxk2 es la principal quinasa de glucosa para las células que crecen en condiciones de alta glucosa. Esta proteína tiene una doble actividad, asociada a una doble localización subcelular. Por un lado tiene una función catalítica en el citoplasma, donde actúa como una enzima glucolítica esencial para el metabolismo energético de la célula, mientras que en el núcleo tiene una actividad reguladora reprimiendo la expresión génica.

A pesar de que esta ruta de represión por glucosa ha sido extensivamente estudiada y que recientemente se han obtenido importantes avances, poco se sabe acerca del mecanismo que regula la asociación de la Hxk2 nuclear con la cromatina del promotor del gen *SUC2* y como esta asociación es necesaria para reprimir su expresión. Nuestros datos demuestran que en el contexto del promotor *SUC2*, la Hxk2 actúa coordinadamente con diversos factores no relacionados estructuralmente. Entre ellos se pueden destacar los represores transcripcionales Mig1 y Mig2, capaces de unirse directamente al DNA y los factores reguladores Snf1 y Reg1. La Hxk2 actuaría estabilizando la estructura del complejo represor y manteniendo por tanto el estado de represión del gen *SUC2*. Utilizando experimentos de co-inmunoprecipitación de la cromatina, hemos descubierto que la Hxk2 en su conformación abierta (condiciones de baja glucosa), abandona el complejo represor lo que induce la disociación de este y promueve la expresión del gen *SUC2*. En condiciones de alta glucosa, Hxk2 adopta una conformación cerrada que favorece su unión con la proteína Mig1 y el ensamblaje del complejo represor. La formación y disociación de este complejo represor implica un transporte nucleocitoplásmico de los factores implicados en esta vía. Nuestros experimentos sugieren que tanto los factores reguladores Snf1 y Reg1, como las proteínas Snf4 y Gal83, de la misma forma que Hxk2, son transportados al núcleo a través de la vía clásica de importación mediada por las carioferinas Kap95 y Kap60, y su exportación nuclear se lleva a cabo a través de la exportina Xpo1.

Recientemente se ha descrito que la expresión del gen *SUC2* también está regulada por su localización subcelular. Se ha demostrado que el gen *SUC2* está asociado a los complejos de los poros nucleares tanto en condiciones de alta como de baja glucosa.

Nuestros datos indican que las proteínas nucleares Nup84 y Nup120 están asociadas con el promotor del gen *SUC2*, formando parte del complejo represor. Además, la nucleoporina Nup120 parece estar implicada en la represión del gen *SUC2*. Nuestros resultados también indican que estas dos nucleoporinas, Nup84 y Nup120 son importantes para regular la asociación de Hxk2 con el promotor del gen *SUC2*.

Resultados adicionales resaltan la posibilidad de que Hxk2 es un sensor de glucosa intracelular, que operaría cambiando su conformación en función de los niveles de glucosa citoplasmática, el cambio conformacional regularía su interacción con Mig1 y por lo tanto su asociación con el complejo represor del gen *SUC2*. Por todo ello, nuestros datos sugieren que la proteína Hxk2 está más íntimamente relacionada con la regulación génica de lo que previamente se había pensado.

RESUMEN (en Inglés)

Glucose is not only a fuel that serves as a preferential substrate for energy yielding metabolism in the yeast *Saccharomyces cerevisiae*, but also functions as a signalling molecule that regulates the central pathways of carbohydrate metabolism. Under high glucose conditions, Mig1 is the main transcriptional factor responsible for the repression of genes needed for the utilization of alternative fermentable carbon sources, such as the *SUC2* gene. It has been demonstrated that in several of the Mig1-regulated genes, this process requires the presence of the Hxk2 protein. In *S. cerevisiae*, Hxk2 is the predominant glucose-kinase in cells growing in high-glucose conditions and has dual functions. It is a glycolytic enzyme, essential for cell energy metabolism in the cytoplasm, but also acts as a regulator of gene transcription in the nucleus.

Despite considerable effort in this field and recent progress in the last few years, little is known about the regulatory mechanism that controls nuclear Hxk2 association with the *SUC2* promoter chromatin and how this association is necessary for *SUC2* gene repression. Our data indicate that in the *SUC2* promoter context, Hxk2 functions through a variety of structurally unrelated factors, mainly the DNA-binding Mig1 and Mig2 repressors and the regulatory Snf1 and Reg1 factors. Hxk2 sustains the repressor complex architecture maintaining transcriptional repression at the *SUC2* gene. Using chromatin immunoprecipitation assays, we discovered that the Hxk2 in its open configuration, in low glucose conditions, leaves the repressor complex which induces its dissociation and promotes *SUC2* gene expression. In high glucose conditions, Hxk2 adopts a close conformation that promotes Hxk2 binding to Mig1 protein and the reassembly of the *SUC2* repressor complex. The formation and dissociation of the repression complex implicates the nucleocytoplasmic transport of the factors involved in this pathway. Our experiments suggest that the two regulatory factors Snf1 and Reg1, and the Snf4 and Gal83 proteins, in the same way as Hxk2, are transported into the nucleus by the classic import system Kap95/Kap60, and exported from the nucleus to the cytoplasm through the exportin Xpo1.

Another mechanism that regulates the expression of the *SUC2* gene is its subnuclear location. It has been demonstrated that the *SUC2* gene is recruited to the nuclear pore complexes under high and low glucose conditions. Our data indicates that the nucleoporin Nup84 and Nup120 also form part of the repressor complex of the *SUC2* promoter, and that Nup120 is involved in glucose repression. In addition, our results

also suggest that Nup84 and Nup120 are also important in regulating Hxk2 recruitment to the *SUC2* promoter.

Additional findings highlight the possibility that Hxk2 constitutes an intracellular glucose sensor, which operates by changing its conformation in response to cytoplasmic glucose levels that regulate its interaction with Mig1 and thus its recruitment to the repressor complex of the *SUC2* promoter. Thus, our data indicate that Hxk2 is more intimately involved in gene regulation than previously thought.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOLOGÍA FUNCIONAL Y MOLECULAR

A mis padres y hermano,

por vuestra generosidad, constante ayuda y por darme la oportunidad de llegar hasta aquí.

A David,

por tu optimismo, tu interés y por tu apoyo incondicional.

AGRADECIMIENTOS

En primer lugar, agradecer a mis directores de tesis, el Dr. Fernando Moreno y la Dra. Pilar Herrero, la oportunidad de hacer el doctorado en su laboratorio. Gracias por todo el apoyo recibido durante estos años, la confianza, el cariño y los ánimos cuando las cosas no salían. Pero sobretodo daros las gracias por todo lo que me habéis enseñado durante estos años.

Al Dr. Jürgen Heinish y la Dra. Rosaura Rodicio, gracias por vuestra ayuda y disponibilidad, pero sobre todo gracias por darme la oportunidad de ir Alemania. A la Dr. Eulalia Valle por su disposición y cercanía.

Al ministerio de ciencia e innovación que me concedió la beca FPI que me ha permitido desarrollar este trabajo.

Agradecer a mis compañeros de laboratorio, Ale, Rafa, Poly y Lucía, lo que he aprendido de cada uno de vosotros y por los buenos momentos compartidos. En especial agradecer a Rafa y a Poly por vuestra ayuda continua y por vuestro apoyo, gracias por responder siempre a mis dudas, pero sobre todo daros las gracias por todos los buenos momentos tanto fuera como dentro del laboratorio. Esperemos que “Pikachu ya no siga necesitando vuestra ayuda”. A Lucía, por tu ayuda, consejos y en especial por nuestras conversaciones.

A mis amigos de toda la vida, Ana, Sara, Lydia y Diego, gracias por estar siempre ahí. A Bea, David, Estela, Natalia, Juan y Silvia, por todos nuestros momentos de risas y dramas, gracias por preocuparos, escucharme y animarme siempre. En especial gracias a ti Natalia por aguantar seis tesis a la vez y nunca quejarte.

También agradecer a todos mis compañeros de departamento, en especial a Ana por su ayuda cuando las cosas no funcionaban, y a Clea por tu ayuda y por todos los buenos momentos.

To Sharon and Caroline, thank you for teaching me english, your interest and your support, without your help this would not have been possible.

To my german colleagues, Christian, Carol, Doris, Dorthe, Dette, Severin, Katja, Sascha, Bettina, Fred, Pedro and Johannes thank you for make me feel like at home, for your help, my daily German lessons, coffee breaks and the good moments together.

Thanks to Sascha and Katja, thanks for your care, for the nice time we spent in Spain and in Germany, and our trips together.

Special acknowledgment to Anne, thank you for your continuous help, support, for always take care of me, and for all our great moments, you made my time in Germany unforgettable.

A mis padres, hermano, abuelos, tíos y primos, gracias por todos los valores que me habéis transmitido, vuestra generosidad, paciencia y por estar siempre ahí.

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English summary

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Spanish summary

Para *Saccharomyces cerevisiae*, la glucosa no solo es el combustible preferido para su metabolismo energético, sino que también funciona como una señal reguladora de las vías centrales del metabolismo de carbohidratos. En condiciones de alta glucosa, el represor Mig1 es el principal factor transcripcional responsable de la represión de los genes implicados en la utilización de fuentes de carbono alternativas a la glucosa, como es el caso del gen *SUC2*. Recientemente se ha demostrado que en la mayoría de los genes regulados por Mig1, el proceso también requiere la presencia de la proteína hexoquinasa 2 (Hxk2). En *S. cerevisiae*, la Hxk2 es la principal quinasa de glucosa para las células que crecen en condiciones de alta glucosa. Esta proteína tiene una doble actividad, asociada a una doble localización subcelular. Por un lado tiene una función catalítica en el citoplasma, donde actúa como una enzima glucolítica esencial para el metabolismo energético de la célula, mientras que en el núcleo tiene una actividad reguladora reprimiendo la expresión génica.

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los factores reguladores Snf1 y Reg1. La Hxk2 actuaría estabilizando la estructura del complejo represor y manteniendo por tanto el estado de represión del gen *SUC2*. Utilizando experimentos de co-inmunoprecipitación de la cromatina, hemos descubierto que la Hxk2 en su conformación abierta (condiciones de baja glucosa), abandona el complejo represor lo que induce la disociación de este y promueve la expresión del gen *SUC2*. En condiciones de alta glucosa, Hxk2 adopta una conformación cerrada que favorece su unión con la proteína Mig1 y el ensamblaje del complejo represor. La formación y disociación de este complejo represor implica un transporte nucleocitoplásmico de los factores implicados en esta vía. Nuestros experimentos sugieren que tanto los factores reguladores Snf1 y Reg1, como las proteínas Snf4 y Gal83, de la misma forma que Hxk2, son transportados al núcleo a través de la vía clásica de importación mediada por las carioferinas Kap95 y Kap60, y su exportación nuclear se lleva a cabo a través de la exportina Xpo1.

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Resultados adicionales resaltan la posibilidad de que Hxk2 es un sensor de glucosa intracelular, que operaría cambiando su conformación en función de los niveles de glucosa citoplasmática, el cambio conformacional regularía su interacción con Mig1 y por lo tanto su asociación con el complejo represor del gen *SUC2*. Por todo ello, nuestros datos sugieren que la proteína Hxk2 está más íntimamente relacionada con la regulación génica de lo que previamente se había pensado.

German summary

Glukose ist nicht nur die bevorzugte Energiequelle für die Hefe *Saccharomyces cerevisiae*, auch dient es als Signalmolekül, das den zentralen Kohlenstoffmetabolismus reguliert. Bei hohen Glukoseleveln ist Mig1 der hauptsächliche Transkriptionsfaktor zuständig für die Repression von Genen, die für die Nutzung alternative Kohlenstoffquellen verantwortlich sind, wie das *SUC2* Gen. Es wurde gezeigt, dass in der Regulation einiger Mig1-regulierter Gene, die Präsenz des Hxk2 Proteins notwendig ist. In *S. cerevisiae* Zellen, die unter hohen Glukosebedingungen gewachsen sind, ist Hxk2 die vorherrschende Glukose-Kinase. Hxk2 hat eine duale Funktion. Zum Einen ist es ein glykolytisches Enzym, das essenziell ist für den Energiemetabolismus der Zelle und zum Anderen agiert es als Regulator der Transkription im Nukleus.

Neben umfangreichen Bemühungen und neuesten Fortschritten in den letzten Jahren, ist nur wenig bekannt über den regulatorischen Mechanismus, der die Asssoziation von Hxk2 mit dem Chromatin des *SUC2* Promotors im Nukleus kontrolliert, und darüber warum diese Assoziation notwendig ist für die *SUC2* Expression. Unsere Daten deuten darauf hin, dass im Kontext des *SUC2* Promotors die Glukose-Kinase Hxk2 durch eine Vielzahl verschiedener, strukturell unabhängiger Faktoren agiert. Hier sind vor allem die DNA-bindenden Repressoren Mig1 und Mig2 sowie die regulatorischen Faktoren Snf1 und Reg1. Hxk2 stabilisiert die Architektur des Repressorkomplexes und halt so die Repression des *SUC2* Gens aufrecht. Mit Hilfe von Chromatin-Immunpräzipitationsversuchen haben wir festgestellt, dass Hxk2 in

seiner offenen Konfiguration bei niedrigem Glukoselevel den Repressorkomplex entlässt. Daraufhin wird dessen Dissozitation induziert und die Expression des *SUC2* Gens wird begünstigt.

Erhöhte Glukoselevel führen zu einer geschlossenen Hxk2 Konformation, welche die Bindung zu Mig1 unterstützt sowie die Reassemblierung des *SUC2* Repressorkomplexes. Die Assoziation und die Dissoziation des Repressorkomplexes hat den nukleo-cytoplasmatischen Transport der Faktoren, die in diesem Weg involviert sind, zur Folge. Unsere Untersuchungen zeigen, dass die beiden regulatorischen Faktoren Snf1 und Reg1 sowie Snf4 und Gal83 auf dem gleichen Weg wie Hxk2 in den Nukleus transportiert werden. Dies erfolgt durch das klassische Importsystem Kap95/Kap60 und der Export aus dem Nukleus in das Cytoplasma durch das Exportin Xpo1.

Ein weiterer Mechanismus, der die Expression von *SUC2* reguliert, ist seine subnukleare Lokalisierung. Es wurde gezeigt, dass das *SUC2* Gen zu den Komplexen der Zellkernporen bei hohen und niedrigen Glukoseleveln rekrutiert wird. Unsere Daten zeigen, dass die Nukleoporine Nup84 and Nup120 auch einen Teil des Repressorkomplexes des *SUC2* Promotors bilden und dass Nup120 in der Glukoserepression involviert ist. Außerdem weisen unsere Daten auch daraufhin, dass Nup84 and Nup120 ebenfalls für die Regulation der Hxk2 Rekrutierung zu dem *SUC2* Promotor wichtig sind.

Weitere Ergebnisse heben die Möglichkeit hervor, dass Hxk2 einen intrazellulären Glukosesensor darstellt, der durch seine Konformationsänderungen in Antwort auf cytoplasmatische Glukoselevel seine Interaktion mit Mig1 reguliert und folglich seine

Rekrutierung zu dem Repressorkomplex am *SUC2* Promotor. Letztlich zeigen unsere Daten, dass Hxk2 sehr viel enger in diese Genregulation involviert ist als bislang angenommen.

ABBREVIATIONS

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Å: Armstrong

ADP: Adenosine 5´-diphosphate

AMPc: 3',5'-cyclic adenosine monophosphate

Amp$_r$: Ampicillin resistance

ATP: Adenosine 5´-triphosphate

ºC: Celsius degree

ChIP: Chromatin immunoprecipitation

C-terminal: Carboxyl-terminus

d: forward

DAPI: 4',6-diamidino-2-phenylindole

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide triphosphate

D.O.: Optical density

EDTA: Ethylenediamenetetraacetic acid

GFP: Green Fluorescent Protein

GST: Glutatione S Transferase

Glucose-6P: Glucosa 6 phosphate

GDP: guanosine 5´-diphosphate

GTP: guanosine 5´-triphosphate

HA: Hemagglutinin

IPTG: Isopropy β-D-thiogalactopyranoside

kDa: KiloDalton

l: liter

LB: Luria-Bertani medium

M: Molar

mg: milligram

ml: milliliter

mM: millimolar

MOPS: Ácido 3-(N-Formilo)-propanosulfónico

mRNA: Messenger RNA

N: Normal

$NADP_+$: Nicotinamide adenine dinucleotide phosphate

NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen

NES: Nuclear Export Sequence

ng: nanogram

NLS: Nuclear Localization Sequence

nM: nanoMolar

nm: nanometer

NPC: Nuclear Pore Complex

N-terminal: Amino terminal

ONPG: Ortho-nitrophenyl-beta-d-galactopyranoside

Ori: Replication origin

P: Phosphate

PAGE: Polyacrylamide gel electrophoresis

pb: base pair

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PMSF: Phenylmethysulfonyl fluoride

PVDF: Polyvinylidene fluoride

r: reverse

RNA: Ribonucleic acid

rpm: revolutions per minute

SC: Sintetic Culture

SCD: Sintetic Culture Dextrose

SCE: Sintetic Culture Ethanol

SDS: Sodium dodecyl sulfate

µg: microgram

µl: microliter

TBE: Tris borate EDTA buffer

TBS: Tris-buffered saline

TE: Tris EDTA

TRIS: Tris (hydroxymethyl) aminomethane

Tris-HCl: Hidroclururo de Tris (hidroximetil) aminometano

tRNA: Transfer RNA

U: Enzyme unit

YEP: Yeast Extract Peptone

YEPD: Yeast Extract Peptone Dextrose

YEPE: Yeast Extract Peptone Ethanol

YNB: Yeast Nitrogen Base

Amino acids

A (Ala) Alanine

C (Cys) Cysteine

D (Asp) Aspartate

E (Glu) Glutamate

F (Phe) Phenylalanine

G (Gly) Glycine

H (His) Histidine

I (Ile) Isoleucine

K (Lys) Lysine

L (Leu) Leucine

M (Met) Methionine

N (Asn) Asparagine

P (Pro) Proline

Q (Gln) Glutamine

R (Arg) Arginine

S (Ser) Serine

T (Thr) Threonine

V (Val) Valine

W (Trp) Tryptophan

Y (Tyr) Tyrosine

Nitrogenous base

A Adenine

C Cytosine

G Guanine

T Thymine

INTRODUCTION

INTRODUCTION

The capability to respond and adapt to environmental fluctuations determines the successful survival of every organism, from bacteria to eukaryotes. Cells have developed a large variety of mechanism to control gene expression in order to achieve the optimal adaptation in the different situations.

An interesting example of the response of a cell to an environmental change is the response of *Saccharomyces cerevisiae* to the presence of glucose. Nutrients are used in a hierarchical system, its preferential use is related with the energetic cost of the synthesis of the metabolic machinery required for using each one. For *S. cerevisiae*, like for almost every organism, glucose is the preferential source of carbon and energy. Due to its central role in yeast metabolism, cells have evolved mechanisms to sense glucose levels in the environment and to adapt the expression of their genetic information to glucose availability.

Glucose presence affects transcription of many different genes, for example, genes which encode for respiration enzymes or enzymes required for the use of alternative carbon sources are repressed, while transcription of genes encoding glycolytic enzymes or glucose transporters are induced. In addition, glucose can also affect yeast physiology at many different levels, for example: there are modifications and activation of some enzymes (Portela and Moreno, 2006), alterations in the stability of some mRNAs (Cereghino and Scheffler, 1996; Mercado et al., 1994; Yin et al., 2003), and changes in the concentration of intracellular metabolites (Kresnowati et al., 2006).

1. Glucose sensing

Maintaining glucose homeostasis is for great importance to yeast and many other organisms. Cells have develop different mechanisms to detect extracellular glucose levels, transport it to the cell inside and trigger different transduction pathways which coordinate various metabolic and cellular responses.

1.1. Glucose transport

S. cerevisiae have at least 20 genes which encode for glucose transporters (*HXT1* to *HXT17*, *GAL2*, *SNF3* and *RGT2*). These Hxt proteins belong to the major facilitator superfamily (MFS) of transporters, which transport their substrates by passive, energy-independent facilitated diffusion (Pao et al., 1998). Glucose transporters have different glucose affinities, for example, Hxt1 has a low glucose affinity and high transport capacity, while Hxt2 and Hxt4 have high affinity and low transport capacity. This allows cells to adjust its response with regard to glucose levels (Ozcan and Johnston, 1999).

1.2. Snf3 and Rgt2 glucose sensors

Most of Hxt proteins are very similar, sharing between 50-100% amino acid sequence identity. The most divergent members of the family are Snf3 and Rgt2 which are only 25% similar. Snf3 and Rgt2 are plasma membrane proteins, they act as a receptor of the external glucose but

they are unable to transport it. As Hxt transporters they consist of 12 transmembrane domains, but unlike them, they have long C-terminal tails in the cytoplasm. This cytoplasmic tails play an important role in glucose signalling, although there is not an absolute requirement because it was observed that an over-expressed tail-less Rgt2 can be functional (Moriya and Johnston, 2004). The sequences of the Snf3 and Rgt2 tails are dissimilar except for a stretch of 25 amino acids, Snf3 contains two repetitions of these 25-amino-acid sequences, while Rgt2 contains only one (Ozcan and Johnston, 1996).

Snf3 and Rgt2 trigger the induction of *HXT* gene expression. Snf3 is a sensor of low levels of glucose and it activates the expression of *HXT2* and *HXT4*, and Rgt2 is required for the complete induction of *HXT1* by high glucose levels (Ozcan et al., 1998). Glucose induction of *HXT* genes is mediated by a repression mechanism involving the zinc-finger-containing protein Rgt1. Under low glucose conditions, Rgt1-mediated repression of the *HXT* genes occurs by a mechanism that requires the paralogous proteins Mth1 and Std1 and recruiting the general corepressors Ssn6 and Tup1 (Belinchon and Gancedo, 2007; Lakshmanan et al., 2003; Schmidt et al., 1999). When Snf3 and Rgt2 detect glucose in the media, Mth1 and Std1 are degraded by proteasome, resulting in the disruption of the repressor complex and thereby derepression of *HXT* expression (Flick et al., 2003; Pasula et al., 2007).

The ability of Rgt1 to bind to *HXT* promoters is correlated with its phosphorylation state: Rgt1 is phosphorylated at a basal level and binds to the promoters in the absence of glucose. When glucose levels are high, Rgt1 is hyperphosphorylated and dissociated from the *HXT* promoters (Kim et al., 2003).

Apart from regulating *HXT* gene expression, Rgt1 also regulates *HXK2* gene expression. This gene presents a domain for the union of Rgt1 and another one for Med8. The two kinases involved in regulating Rgt1 activity by its phosphorylation are Snf1 and Tpk3. During the growth in low glucose levels, Snf1 activate the repression function of Rgt1. Under high glucose conditions Rgt1 is hyperphosphorilated by Tpk3 and in this situation, Rgt1 is release from the repressor complex allowing the expression of *HXK2* (Palomino et al., 2006).

1.3. The cAMP/PKA pathway and its stimulation through Grp1 and Ras1/2

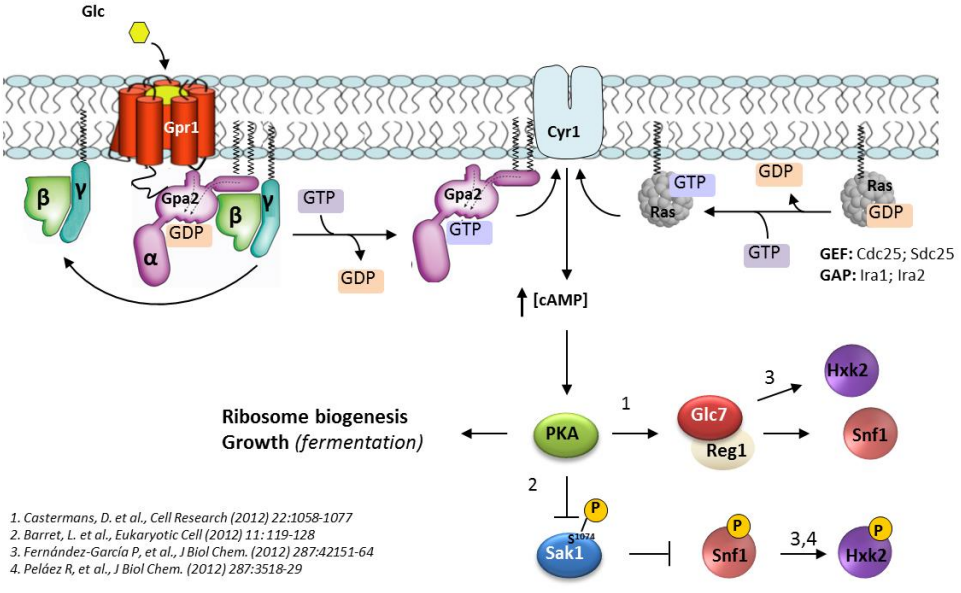


Figure 1. ***Diagram representing the cAMP/PKA pathway and its stimulation through Grp1 and Ras1/2.***

The cyclic AMP-dependent protein kinase A (PKA) has an important role in controlling mechanism of stress resistance, division and cell growth. It is a fast response pathway, in which, levels of cAMP can

increase between 5 to 50 times after the addition of glucose in the media, and basal levels are recover after 20 minutes (Santangelo, 2006).

PKA is a heterotetrameric protein formed by two catalytic subunits encoded by TPK1-3 genes, and two regulatory subunits encoded by BCY1 gene. PKA is activated by the binding of cAMP to the regulatory subunits, which cause the dissociation from the catalytic subunits. Glucose is a potent activator of cAMP synthesis by Cyr activation through a G protein coupled receptor (GPCR) system (Rolland et al., 2002).

Thus, the mechanism that activates PKA is by sensing the extracellular glucose through a G-protein coupled receptor (GPCR), Gpr1. The heterotrimeric G protein comprises three subunits: α (Gpa2), β and γ subunits. Glucose binding to the Gpr1 receptor triggers a conformational change which promote a GDP-GTP exchange on the Gpa2 subunit, and release the Gβγ complex. GTP-Gpa2, the active form, binds to adenylate cyclase, Cyr1, and activates the synthesis of cAMP, causing PKA activation (Peeters et al., 2006; Yun et al., 1997).

The other pathway that activate PKA is through the Ras1/2 proteins. It is not certain how glucose stimulates Ras1/2, but uptake and conversion of glucose into glucose 6-phosphate have been claimed to be required. Ras protein is a monomeric GTPase which activity depends on GDP/GTP exchange by a guanine nucleotide-exchange factor (GEF), Cdc25 and Sdc25 (Jones et al., 1991) and regulation of its intrinsic GTPase activity by a GTPase activating factor (GAP), Ira1 and Ira2 (Tanaka et al., 1990). When Ras is activated, it activates the adenylate cyclase, Cyr1, which catalyze the conversion of ATP into cAMP. cAMP acts as an

intracellular second messenger, which cause PKA activation by dissociating the regulatory subunit from the catalytic subunit.

Activated PKA mediate different effects. One effect, already mentioned, is the phosphorylation of Rgt1 by Tpk3, which cause its dissociation from the promoters which is repressing (Kim et al., 2003; Palomino et al., 2006). In addition, PKA contributes to negatively regulate the AMPK, Snf1, by two indirect mechanisms. Snf1 protein kinase activation requires phosphorylation by three partially redundant upstream kinases: Sak1, Tos3, and Elm1, being Sak1 the major activating kinase, and Snf1 is turned off by the protein phosphatase Glc7-Reg1. PKA activation is required for the activation of protein phosphatase Glc7-Reg1 (Castermans et al., 2012), and on the other hand, PKA phosphorylates the kinase Sak1 inactivating it (Barrett et al., 2012). Moreover PKA also influences the Hxk2 phosphorylation state, on one hand Glc7-Reg1 maintains Hxk2 dephosphorylated under high glucose conditions, and on the other Snf1 is also inactive in this circumstances so it cannot phosphorylate Hxk2.

1.4. Repression pathway

Glucose also triggers the repression of many target genes, such as *SUC2, MAL, GAL1* (Nehlin et al., 1991; Nehlin and Ronne, 1990). The repression pathway involves the formation of a repression complex constituted by different elements: Mig1, Mig2, Hxk2 and the SNF1 complex.

Mig1 and Mig2 are the transcriptional repressors. Mig1 is a zinc-finger-containing protein of the Cys_2His_2 type which bind to GC boxes, $^{5'}$(G/C)(C/T)GGGG$^{3'}$, which is flanked by a AT-rich sequence in 5' (Lundin et al., 1994), this sequence is present in the promoters of many of the glucose repressed genes (Klein et al., 1998; Nehlin and Ronne, 1990). Thus, Mig1 binds to DNA and inhibits transcription of approximately 350 genes (Gertz et al., 2009; Treitel and Carlson, 1995).

Mig1 activity is regulated by phosphorylation of the residues Ser^{222}, Ser^{278}, Ser^{310} and Ser^{311} (Smith et al., 1999). During the growth in high glucose conditions Mig1 is dephosphorylated and is located in the nucleus of the cells where it acts as a transcriptional repressor. However, when glucose becomes limiting SNF1 complex is activated and then it phosphorylates Mig1 (Treitel et al., 1998) which cause it exit from the nucleus (De Vit et al., 1997). It is possible that Snf1 phosphorylates Mig1 in a key residue of nuclear export sequence (NES) which allows the karyopherin Msn5 to recognise Mig1 and transport it to the cytoplasm (DeVit and Johnston, 1999). Ser^{311} seems to be the essential residue because its phosphorylation cause Mig1 nuclear export and therefore the derepression of the Mig1-regulated genes by it. If Ser^{311} is replaced by Ala, Mig1 remains nuclear acting as a repressor at high and low glucose conditions (Ahuatzi et al., 2007). Mig1 dephosphorylation is taking place through two different mechanisms: the direct mechanism is its dephosphorylation by the protein phosphatase Glc7-Reg1, and the indirect mechanism is through the dephosphorylation and therefore inactivation of the Snf1 kinase.

Mig2 was identified several years ago as a repressor that collaborates with Mig1 to cause glucose-induced repression of the *SUC2* gene

(Lutfiyya and Johnston, 1996). A genome wide expression profiling survey of the yeast genome revealed that all the genes regulated by Mig1 are also regulated by Mig2 (Lutfiyya et al., 1998). Thus, it was postulated that Mig2 always works in conjunction with Mig1. However, Mig1 appears to be the essential component, since its presence is necessary and sufficient to cause full repression of most genes even in the absence of Mig2.

The third element of the repression pathway is the protein Hxk2. Hxk2 is a bifunctional protein which plays an important role in glucose metabolism. There are three enzymes in charge of glucose phosphorylation at C_6 in the first step of the glycolysis: Hxk1, Hxk2 and Glk1. However, only *HXK2* is highly express during the growth in high glucose conditions. *HXK1* and *GLK1* are important when other non-fermentable carbon sources or galactose are present in the media (Herrero et al., 1995).

Expression of *HXK2* gene is controlled by glucose availability and is mediated by the Rgt1 and Med8 transcription factors, which repress *HXK2* expression in low-glucose containing media (Palomino et al., 2005; Palomino et al., 2006). Transcriptional analysis of a *Δhxk2* mutant strain showed significant upregulation of genes with binding sites for Mig1 and/or Cat8 (Rodriguez et al., 2001; Schuurmans et al., 2008). In addition, Hxk2 also participates in the control of genes encoding sugar transporters (Ozcan and Johnston, 1995) and genes modulating mitochondrial cytochrome content and respiratory activity (Noubhani et al., 2009). This is also supported by a diminished Crabtree effect in *Δhxk2* mutants, resulting in a nearly complete respiratory metabolism at high glucose concentration (Schuurmans et al., 2008). Thus, Hxk2 protein can

be regarded as a global regulator of carbon metabolism that is essential for mediating the glucose repression signal.

Due to its bifunctionality, Hexokinase 2 is classified as a moonlighting protein (Gancedo and Flores, 2008; Pelaez et al., 2010). It has a double subcellular localization related with its double function. In the cytoplasm it functions as a glycolytic enzyme, and in the nucleus act as regulator protein of the gene transcription of several Mig1-regulated genes. Both functions create a connection between nutritional signals and energy metabolism (Schuurmans et al., 2008). The domains in charge of carrying out each function of Hxk2, catalytic and regulatory, have been identified. The last eight amino acids of Hxk2 have been demonstrated to play an essential role in the catalytic activity of Hxk2, and amino acids from Lys^{6} to Met^{15} (Hxk2 is numbered from residues 1 to 485; residue 1 is a valine because the initiator methionine is cleaved off from the primary translation product) are essential for the regulatory role of Hxk2. Mutant strains of each of these domains retain single functions, as a transcriptional factor or as an enzyme with hexose-phosphorylating activity, but have lost the original bifunctionality of Hxk2 (Pelaez et al., 2010).

Hxk2 is composed of two lobes, one bigger than the other, and in the middle of both lobes is where the active site of the enzyme reside. The active site is the most conserved region in the hexokinase family. Different residues of the active site are implied in the associated with glucose union: Thr^{172}, Lys^{173}, Asp^{211}, Asn^{237}, Glu^{269} and Ser^{302} (Kuser et al., 2008). Another region highly conserved is the domain located between Gly^{229} and Glu^{248}, which is enriched in Ser, Thr, Gly and Asp, and it has been associated with ATP union (Kuser et al., 2000).

During the first reaction of glycolysis, Hxk2 catalyses the transfer of the γ-phosphoryl group of an ATP molecule to the oxygen at the C-6 of glucose. The magnesium ion is required as the reactive form of ATP is the complex with Mg^{2+}. This step is a nucleophilic attack of the hydroxyl group by the terminal phosphoryl group of the ATP molecule. This reaction produces glucose-6-phosphate and ADP.

Hxk2 has two different conformational states, an open state which occurs prior to glucose binding, and in this conditions ATP is bound to the large lobe, but far away from the glucose binding site. When the glucose entry in the active site of hexokinase, a large conformation change occurs, and the two lobes close around the glucose substrate, this state is referred as the closed state. The closed state favours the interaction between the substrates and the enzyme, and therefore facilitates the reaction.

Hxk2 present two different forms, homodimeric and monomeric. During the growth in a low glucose conditions Hxk2 is mainly monomeric, while in the presence of glucose the protein is in dimeric state. Thus, there is an equilibrium of both forms which depends directly on the glucose present in the media. In addition, Hxk2 is phosphorylated in the residue Ser^{14} during the growth in low glucose medium (Randez-Gil et al., 1998), and under this circumstances the enzyme is more sensible to ATP levels (Golbik et al., 2001; Moreno et al., 1986). This mechanism allows cells to correlate intracellular ATP concentration and controlling glucose phosphorylation.

It is proposed that exists a correlation between phosphorylation of Ser^{14} and the processes of homodimerization and monomerization. Under low glucose conditions, Hxk2 is phosphorylated and in the monomeric state.

Glucose addition causes its dephosphorylation by Reg1/Glc7 phosphatase, and in these conditions Hxk2 is in the homodimeric form (Randez-Gil et al., 1998).

Moreover, it was also observed that Ser^{14} is adjacent to the Hxk2 nuclear localization sequence (NLS), and its phosphorylation by Snf1 promotes an increase in binding affinity to Xpo1 and a decrease in binding affinity to Kap60, producing the exit of nuclear Hxk2 to the cytoplasm. These changes in Hxk2 binding affinity for its karyopherins correlate with important structural changes in the N-terminal region of the protein. Ser^{14} phosphorylation forms an acidic patch in the NLS region that induces binding of Hxk2 to Xpo1 and consequently allows nuclear export through the nuclear pore. Hxk2 dephosphorylation at Ser^{14} by Glc7-Reg1 protein phosphatase form an extended hydrophobic patch in the NLS motif region that induces binding of Hxk2 to Kap60 and consequently allows nuclear import (Fernandez-Garcia et al., 2012; Pelaez et al., 2012; Pelaez et al., 2009) .

Apart from Ser^{14}, Hxk2 has another phosphorylatable residue, Ser^{157}, which is phosphorylated in the presence of pentoses, and its phosphorylation produce the enzyme inactivation (DelaFuente, 1970; Fernandez et al., 1986; Fernandez et al., 1985; Heidrich et al., 1997). It was considered that this mechanism could be an autophosphorylation consequence of the entry in the active site of an unsuitable monosaccharide to which the phosphate cannot be transferred and this inactivates the enzyme (Moreno and Herrero, 2002).

The other member of the repressor pathway is the SNF1 complex. SNF1 is a serin-threonin-kinase belonging to the AMPK family. It is required for the yeast cell to adapt to glucose limitation and to utilize

alternative carbon sources which are less preferred than glucose, such as sucrose, galactose and ethanol (Gancedo, 1998; Honigberg and Lee, 1998). SNF1 also has roles in various nutrient-responsive, cellular developmental processes, including meiosis and sporulation (Carlson et al., 1981; Honigberg and Lee, 1998), aging (Ashrafi et al., 2000), haploid invasive growth (Cullen and Sprague, 2000), and diploid pseudohyphal growth (Kuchin et al., 2002). In addition to its primary role in responses to nutrient stress, SNF1 is involved in the cellular responses to other environmental stresses, including sodium ion stress, heat shock, alkaline pH, oxidative stress, and genotoxic stress (Alepuz et al., 1997; Dubacq et al., 2004; Hong and Carlson, 2007; McCartney and Schmidt, 2001; Portillo et al., 2005; Thompson-Jaeger et al., 1991). SNF1 affects cellular regulatory processes through a variety of mechanism, including a major role in control of the genomic transcriptional program and direct effects on the activity of metabolic enzymes.

Like the human AMPK, SNF1 kinase is a heterotrimeric protein, and *S. cerevisiae* encode for three subunits: α subunit (Snf1) with a catalytic function, γ (Snf4) with a regulatory role and three alternative β subunits (Sip1, Sip2 and Gal83) which function is related with the subcellular localization of the complex. Therefore, there are three alternative SNF1 complexes (Smith et al., 1999; Vincent et al., 2001).

Snf1 catalytic subunit has two different domains; a kinase domain near the N-terminus and a C-terminal regulatory domain. This regulatory domain modulates the kinase activity through an autoinhibitory sequence (AIS) located between residues 381 to 414. During the growth in high glucose media, the regulatory domain is interacting with the catalytic domain through the AIS, and Snf1 acquires a close

conformation. When the glucose becomes limiting, Snf4 subunit interacts with the AIS and prevents AIS binding to the kinase domain, then Snf1 subunit acquires an open conformation. The C-terminal region also interacts with the beta subunits (Hedbacker and Carlson, 2008).

Another mechanism controlling the SNF1 complex is the phosphorylation of Snf1 Thr^{210}, located in the N-terminal domain. Phosphorylation of this residue does not require the Snf1 C-terminal domain, Snf4, or the beta subunits.

Snf1 phosphorylation state is regulated by three upstream kinases: Sak1, Elm1 and Tos3 (Elbing et al., 2006), and the protein phosphatase Reg1-Glc7 (Sanz et al., 2000). Snf1 kinases are highly similar and exhibit overlapping function as all three must be deleted to abolish SNF1 activity *in vivo*. Sak1 is the major activating kinase and it associates with SNF1 to form a stable complex, whereas Elm1 and Tos3 associate with SNF1 more transiently (Elbing et al., 2006).

Snf1 catalytic activity is negatively regulated by the Reg1-Glc7 protein phosphatase. Glc7 is the protein phosphatase and Reg1 its regulatory subunit. During the growth in high glucose conditions Reg1 is phosphorylated, and therefore activated, by PKA. Thus, in glucose conditions Snf1 remains inactivated (Barrett et al., 2012; Castermans et al., 2012).

Another regulatory protein involved in this repression pathway, is the phosphatase Reg1-Glc7. Glc7 is the catalytic subunit, and it has many different targets but is specifically directed toward glucose by its regulatory subunit, Reg1(Rubenstein et al., 2008; Tu and Carlson, 1995). Under high glucose conditions, Glc7-Reg1 is activated by PKA, and in this

state Glc7-Reg1 maintains dephosphorylated the proteins Hxk2, Mig1 and Snf1 (Fernandez-Garcia et al., 2012; Randez-Gil et al., 1998).

1.4.1. Role of Hxk2/SNF1/Mig1 in glucose repression of the *SUC2* gen.

SUC2 gene expression is regulated almost exclusively by glucose. Thus, *SUC2* gene was traditionally used as a model to study the glucose repression mechanisms in yeast.

The *SUC2* gene of *S. cerevisiae* encodes two different forms of the enzyme invertase, external and internal. The two forms of invertase are translated from two distinct, differentially regulated mRNAs, which differ only in their 5'-ends. The 1.9 kb mRNA encodes the external form and specifies a leader peptide which directs the protein product into the secretory pathway. This signal sequence is missing in the 1.8 kb mRNA encoding the internal invertase (Carlson and Botstein, 1983; Carlson et al., 1983). External invertase is a highly-glycosylated homodimer that is excreted into the periplasmic space, where the sucrose hydrolysis occurs. Only the monosaccharide products of the reaction, glucose and fructose, are transported into the cell. Internal invertase also forms a homodimer, but is not glycosylated and is localized to the cytoplasm (Gascon et al., 1968; Trimble and Maley, 1977).

Under high glucose conditions, the DNA-binding proteins, Mig1 and Mig2, generate a repression state of the *SUC2* gene by recruiting the general corepressors Tup1-Ssn6. Tup1 represses the expression of genes via distinct mechanisms: by establishing a repressive chromatin structure around the target gene promoter, by recruiting histone deacetylases,

and by directly interfering with the general transcription machinery. However, the precise molecular mechanism of action of Tup1 family proteins in gene repression induced by high glucose levels has not been fully resolved. This is probably because the mechanism depends on the kinetics of formation and dissociation of the repressor complex, which in the case of glucose repression signalling is not well characterized. In addition, the presence of Hxk2 protein is also essential to maintain SUC2 repression (Moreno et al., 2005; Moreno and Herrero, 2002; Rodriguez et al., 2001).

When glucose becomes limiting, Snf1 is activated by phosphorylation at threonine-120 by one of the three kinases, Sak1, Tos3 or Elm1 (Hong et al., 2003; Nath et al., 2003; Sutherland et al., 2003). ADP also appears to play a significant role in Snf1 activation in response to glucose limitation by protecting the enzyme against dephosphorylation by the Glc7-Reg1 phosphatase (Mayer et al., 2011). Snf1 kinase forms a complex with an activating subunit Snf4 and one of the three Snf1-interacting proteins Sip1, Sip2 or Gal83, which target Snf1 to distinct subcellular locations (Vincent et al., 2001). Gal83 subunit direct Snf1 to the nucleus, where Snf1 kinase phosphorylates Mig1 at the serine-311 (Ahuatzi et al., 2007) and Hxk2 at the serine-14 (Fernandez-Garcia et al., 2012), respectively. Phosphorylation of Hxk2 and Mig1 results in the export of these proteins from nucleus to cytoplasm and the disassembly of the repressor complex at the *SUC2* promoter.

Although the glucose repression pathway has been extensively studied, there still a lack of detailed information about how glucose level is detected and how this information is transmitted to the nucleus to generate a coordinated cellular response.

2. Nucleocytoplasmic transport

Eukaryotic chromosomes are housed within the nucleus. It is an organelle delimited by two parallel membranes named the nuclear envelope (NE). In between of these two membranes there is a space of 20-40 nm called the perinuclear space.

Nuclear compartmentalization allows cells to strictly coordinate numerous key cellular processes, separating the genetic material and the transcriptional machinery from the rest of the cytoplasm components, such as the ones involved in metabolism or protein translation machinery. Both compartments are connected thanks to the existence of macrostructures called the nuclear pore complexes (NCPs), which allow to have a selective and bidirectional transport of proteins and RNA between both compartments.

2.1. Structure and function of nuclear pore complexes.

NPCs are macromolecular structures which share its overall architectural features among the eukaryotes. In yeast they have a molecular mass of 66 MDa (Rout and Blobel, 1993) and they have around 200 NPCs per nucleus (Winey et al., 1997) . They act as a selective barriers that facilitate the transport of molecules through the two membranes composing the NE. NPCs are composed of approximately 30 different proteins termed nucleoporins (Nups) (Cronshaw et al., 2002; Rout et al., 2000). Each Nup is repeated 8-, 16-, 32- or 48-fold to form this

multiprotein complex. Nups have different molecular masses that vary between 50 and 358 kDa.

The core of the NPC consist of an octogonally symmetrical cylinder, the axis of which lies perpendicular to the plane of the NE. This core is made of coaxial inner, outer, and membrane rings surrounding a central channel of 40 nm of diameter through which the nuclear trafficking occurs. A part from this central channel, 8 peripheral channels of 5 to 10 nm in diameter have been revealed by electron microscopy. These channels may be in charge of the passive diffusion of ions, small soluble proteins, and membrane proteins (Grossman et al., 2012). NPC project eight short filaments from the core into the cytoplasm, and, similarly, eight filaments extend 50 nm into the nucleoplasm, where they conjoin distally to form a structure said to resemble a “basket” on the nuclear face of the NPC. (Kiseleva et al., 2004)

Although most Nups have a low sequence similarity, they are conserved in terms of structure and/or function (Suntharalingam and Wente, 2003). On the basis of structure, amino acid sequence motifs, and location within the NPC, the different Nups can be sorted into: transmembrane Nups (TM), scaffold Nups, and barrier Nups.

-Transmembrane Nups anchor the NPC to the pore membrane and bind the assembled complex to the nuclear envelope.

-Scaffold Nups connect membrane Nups to barrier Nups, they are the skeleton of the NPC. On the basis of their structure and location in the NPC, scaffold Nups can be divided in three subgroups: outer ring Nups, inner ring Nups, and linker Nups. There are two outer rings, one is localized in the cytoplasmic side and the other in the nuclear side. The

inner ring consist of two identical rings that are closely associated with each other and form connections with all three integral membrane proteins in the membrane rings thereby anchoring the NPC scaffold to the pore membranes.

-Barrier Nups act as selective gatekeepers that regulate the nuclear transport. They are formed by repetitions of phenylalanine and glycine in multiples of 4 to 48 in the following combinations; GLFG, FxFG, PxFG, or SxFG. Barrier Nups represent about one-third of the total pore proteins, and they are symmetrically distributed. They are anchored mainly to the inner ring or to the linker Nups (only a few Nups bind directly to the outer rings), extending into the cytoplasmic and nucleoplasmic sides. This FG repetitions have a great importance because they are able to interact directly with transport receptors which is essential for the specific transport through the nuclear pore. Collectively they also provide a diffusion barrier of the NPC.

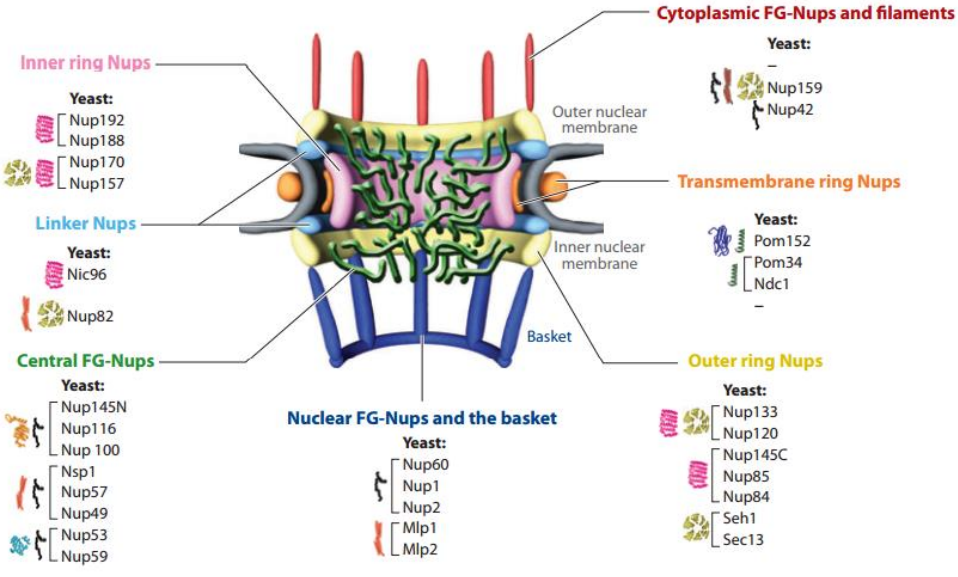


Figure 2. ***Diagram representing the nuclear pore complex architecture (Grossman et al., 2012).***

Since NPCs are the only gateway into and out of the nucleus, all molecules must pass through this barrier. NPCs are freely diffusible for molecules smaller than 40 kDa or 5nm, such as ions or metabolites. Nevertheless, a mediated energy-dependent passage of larger molecules, such as proteins, RNA, ribosomal subunits, and viral particles is required.

2.2. Karyopherins

The import or export macromolecules need to have signal transport, the import signals are termed as NLS (nuclear localization sequence) and the export signal NES (nuclear export signal). Such signals are recognized by soluble transport receptors, and actively transport these molecules across the pores. Most nuclear transport receptors belong to the family of β-karyopherins (Kaps), which depending on their function are termed importins or exportins.

Different models have been proposed to describe the molecular transport mechanism through the NPC. One model suggest that FG repeat from a dense meshwork in and around the central tube, stablishing an entropic potential that would exclude macromolecules from their vicinity, while permitting small macromolecules approach. Karyopherins will overcome this barrier interacting with FG Nups until they diffuse through the NPC. This concept has been termed “virtual gating” (Rout et al., 2003; Rout et al., 2000).

The ‘hydrophobic gel’ model, also called ‘saturated model’, proposed that the phenylalanines in the FG repeat regions are crosslinked with

each other and form a dense gel of FG repeat filaments. Transport factors bind to these FG repeats, dissolve the crosslinks and facilitate passage through the nuclear pore (Ribbeck and Gorlich, 2002).

The "reduction in dimensionality" model posits that the FG repeat regions form a layer coating the inner walls of the central tube. Although transport factors enter this layer through binding giving them full access to the tube's volume, nonbinding molecules can only pass through the narrow FG Nup free middle (Peters, 2005).

All these theories are not exclusive from each other, in fact there are hybrid models which combine some of these theories (Krishnan et al., 2008; Patel et al., 2007; Strawn et al., 2004)

In each of these models, the transport of molecules which are not capable of crossing the nuclear envelope by passive diffusion, such as RNAs or proteins, require an active transport through the nuclear pore complexes. This is mediated by transport factors, the karyopherin superfamily which is evolutionarily conserved. They play an important role in several cellular processes, such as gene expression, signal transduction, immune response, viral propagation, oncogenesis or cell cycle control (Taberner and Igual, 2010). In addition it has been demonstrated that karyopherins would also act enhancing selectivity of the NPC by competing away non-specific macromolecules (Jovanovic-Talisman et al., 2009).

In *S. cerevisiae* this superfamily comprises 17 proteins which are divided in four groups: α importin (α-Kap), the family of β-Kaps, the heterotrimer Mmex6-Mtr2 and the karyopherin Ntf2.

In yeast this β-karyopherin family comprises 14 members (while in human cells contain at least 20). There are 10 import karyopherins, 3 export karyopherins and one import/export karyopherin (Mosammaparast and Pemberton, 2004). They share similar molecular weight, which varies from 95 to 150 kDa, and isoelectric point 4 to 5. Although they have low sequence identity (10-20%), all members of this β-family contain the helical HEAT repeats. The HEAT motif was first observed in four proteins that they give rise to the acronym HEAT (the Huntingtin protein, the Elongation factor required for protein synthesis, the PR65/A subunit of protein phosphatase 2A and the lipid kinase TOR). It consist of a repetition of approximately 40 amino acids which form two antiparallel α helices (A and B) connected by a short turn. Each karyopherin is composed by 18-20 of these HEAT repeats (Matsuura and Stewart, 2004), and they stack together forming two C-shaped arches that together generate a helical molecule (Chook and Blobel, 1999; Groves and Barford, 1999). The relative orientation of the arches differs among the different β-karyopherin and is also influenced by the interaction of different partners. Thus, despite being based on a similar overall architecture of HEAT repeats, β-karyopherin superhelices show considerable variability in the pitch of the helicoid into which they coil. This structural flexibility allows β-karyopherin to transport different substrates and also different β-karyopherin can transport the same cargo (Conti et al., 2006).

Each β-karyopherin is able to transport a unique set of proteins or RNAs, thus creating multiple transport pathways across the nuclear pore complex. Cargo recognition is taking place through the C-terminal domain of the karyopherin, and N-terminal is the Ran-binding domain

(Gsp1 in yeast). β-karyopherin also bind weakly to the FG-Nups and this target the β-kap-cargo complexes to the nuclear pore complex for its translocation (Chook and Blobel, 2001; Chook and Suel, 2011).

The localization of a specific protein is predominantly determined by the presence of an NLS (*nuclear localization sequence*) or NES (*nuclear export signal*), which are the signals recognized by the importins or exportins, respectively. Most of the β-kaps bind the NLS of their cargos directly, but a significant exception of this is the Importin-β pathway, which recognizes the classical NLSs through the adaptor protein Importin-α (Gorlich et al., 1995; Vetter et al., 1999).

Directionality across the NCP is determined by the asymmetrical distribution of Ran-GTP and Ran-GDP between the nucleus and the cytoplasm. Thus, mediating assembly and disassembly of import and export complexes through interactions with the nuclear import machinery.

Ran (***RA****s-related* ***N****uclear protein*) (Gsp1 in yeast) is a protein of 25 kDa, which is very abundant and is among the most highly conserved proteins in eukaryotes. It belongs to the Ras superfamily of small GTPases, which are hydrolase enzymes that can bind and hydrolyze GTP. RanGTPase shifts between a GTP-bound and a GDP-bound form, which are regarded as the active and inactive states, respectively. In the nucleus there is approximately 100-fold greater concentration of RanGTP than in the cytoplasm, due to the presence of Ran´s guanine-exchange factor (RanGEF), which is restricted to the nucleus and bound to chromatin. RanGEF (Prp20 in yeast) triggers the exchange of GDP to GTP. In the cytoplasm the majority of Ran is bound to GDP, due to the cytoplasmic

localization of the GTPase activating protein, RanGAP (Rna1 in yeast) (Bischoff et al., 1994; Gorlich and Kutay, 1999; Lui and Huang, 2009).

The interaction between RanGTP and nuclear transport receptors dictates in which compartment a given receptor will bind or release its cargo. β-Kaps interacts with Ran through its N-terminal domain with a K_D in the range of mM for RanGTP, and 10μM for RanGDP bound. During nuclear import, interaction between β-importins and RanGTP results in the dissociation of the β-importin-α-importin-cargo complex, and cargo would be released in the nucleus. Whereas the interaction between exportins, RanGTP and cargo, seem to be cooperative. This export complex crosses the NPC and while in the cytoplasm, cargo would be released by the hydrolysis of GTP to GDP.

2.3. Import system

Importin-β are able to bind cargoes directly or through the adaptor proteins, such as importin-α or snurportin (Strom and Weis, 2001). The best characterized import pathway is named "classic pathway" and is mediated by the importin-β (Kap95 in yeast) and the adaptor protein, importin-α (Kap60 in yeast).

Proteins that undergo nuclear import contain an NLS. The first discovered or also named classical NLS, is a small, lysine rich sequence (K(K/R)X_{1-3}(K/R)) and is recognized by importin-α. Many proteins carry a more complex bipartite NLS consisting of two clusters of basic amino acids, separated by a spacer of 10 to 12 amino acids (K/R)(K/R)X_{1-3}(K/R)X_{10-12}(K/R)$_{3-5}$ (Dingwall et al., 1982; Kosugi et al., 2009) . However,

most other proteins imported into the nucleus do not utilize the importin-α adaptor protein and bind directly to a β-Kap. Characterized NLSs that bind directly β-Kaps are diverse, encompassing both structural domains and linear epitopes, it makes extremely difficult the identification of common features which define the NLS.

Like importin-β, importin-α is also constructed from a tandem repetitions of α-helical repeats, termed Armadillo repeats (ARM). Importin-α comprises two domains which function and structure are different. The N-terminal domain is involved in the interaction with importin-β, while C-terminal is the cargo recognition domain. The basic importin-α structure is a cylindrical superhelix consisting of ten ARM repeats (Conti et al., 1998). An individual ARM repeat contains approximately 40 amino acids, which form three helices (H1, H2 and H3) arrange in an antiparallel way, generating a superhelix with the inner concave surface formed by the H3 helices. There are structural and topological similarities of importin α and β, which support the suggestion that these proteins are evolutionarily related.

Monopartite and bipartite NLSs bind to an extended conformation to the ARM-repeat domain of the importin-α. This interaction is mediated by a set of conserved residues that are solvent exposed on the concave surface of importin-α. In particular, several tryptophan residues which form hydrophobic pockets along the concave face of importin-α. The presence of multiple pockets along the concave face allows specific NLS recognition despite the lack of a strict consensus sequence (Conti and Izaurralde, 2001).

The recognition of NLS-containing protein by importin-α initiates the process of nuclear import. After which, importin-β associates with the importin-β-binding (IBB) domain of the importin-α (located in the N-terminal domain) forming a ternary complex. The ternary complex formed by α/β-importins and cargo is translocated across the nuclear pore complex into the nucleus thanks to the interaction between importin-β and FG-nups. Translocation through the NPC is mediated by importin-β which has been shown to contain two binding sites for FG-rich motifs. Once in the nucleus, dissociation of the import complex is stimulated by RanGTP which interact with importin-β inducing a conformation change that liberates the cargo and importin-α (Chook et al., 2002).

Importin-α is recycled back to the cytoplasm through the nuclear exporter Cse1, whereas importin-β is separately transported back to the cytoplasm together with RanGTP. RanGAP facilitated GTP hydrolysis of Ran on the cytoplasmic side, causes the release of importin-β for the next cycle (Hood and Silver, 1998).

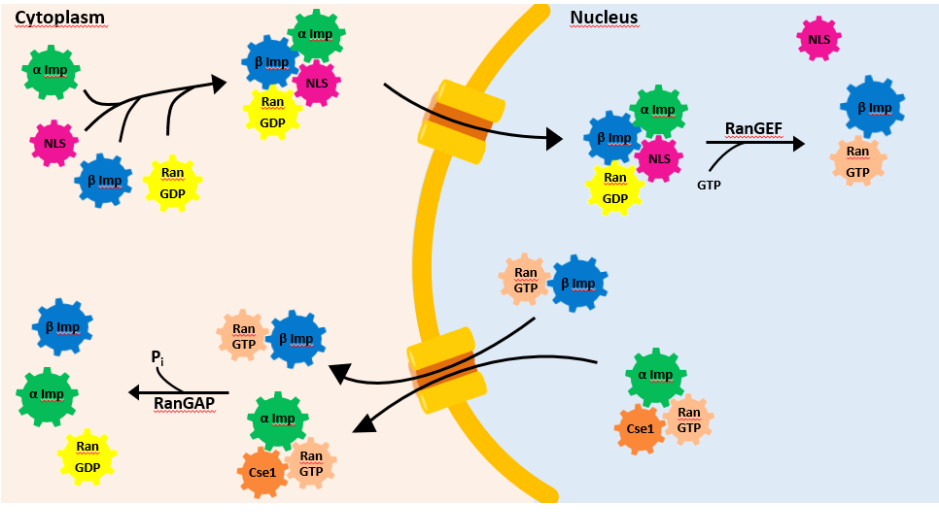


Figure 3. ***Diagram representing the classic nuclear import pathway.***

2.4. Export pathway

Export karyopherins also recognize specific signals, referred to as NESs. The NESs of several cargoes have been defined, and the most well characterized is the hydrophobic NES, also called LR-NES (leucine-rich NES), which is a loosely conserved motif containing three to four hydrophobic residues. The consensus sequence for NES is ϕ1-$X_{(2–3)}$-ϕ2-$X_{(2–3)}$-ϕ3-X-ϕ4 (where ϕ represents one of the hydrophobic residues L, V, I, F or M, and X can be any amino acid but preferentially is charged, polar or a small amino acid). The hydrophobic NES is utilized in all eukaryotes, and is recognized by the karyopherin Crm1 (Xpo1 in yeast) (Wen et al., 1995). However, this patter is abundant but does not even describe the NES satisfactorily, because not every NES contain this pattern. It has been observed that apart from these hydrophobic residues, glutamate, aspartate and serine seen to be important for NESs, since they are over-represented in certain regions near the conserved hydrophobic residues. In addition, sequence features, secondary structure, flexibility and/or surface exposure are characteristics that also define a NES (Henderson and Eleftheriou, 2000; la Cour et al., 2004).

In *S. cerevisiae* there are four exportins: Los1, Msn5, Crm1/Xpo1 and Cse1. Los1 is involved in tRNA transport (Arts et al., 1998). Mns5 karyopherin is unusual in that it can mediate both nuclear import and nuclear export and is responsible for the transport of several transcription factors such as Mig1 (DeVit and Johnston, 1999; Yoshida and Blobel, 2001). Interestingly, most Msn5 export cargoes identified thus far have been shown to be positively regulated by phosphorylation, suggesting the phosphosite is either part of the NES, or phosphorylation

indirectly affects NES recognition by the receptor (Pemberton and Paschal, 2005). The best characterized exportins are Crm1 and Cse1. Crm1 is in charge of LR-NES-containing proteins transport from the nucleus to the cytoplasm, and the exporter Cse1 participates in α-importin recycling.

Crm1 is a member of the β-karyopherin protein family. It contains 20 HEAT domains that allow RanGTP to bind. Cargo binding takes place outside of this HEAT domain ring in a hydrophobic cleft producing a generic NES docking site. Crm1 recognized NESs that are present in a large range of proteins that are structurally unrelated. It can also be recruited by adaptor molecules in situations where it does not bind directly to a protein that is to be exported from the nucleus (Dong et al., 2009).

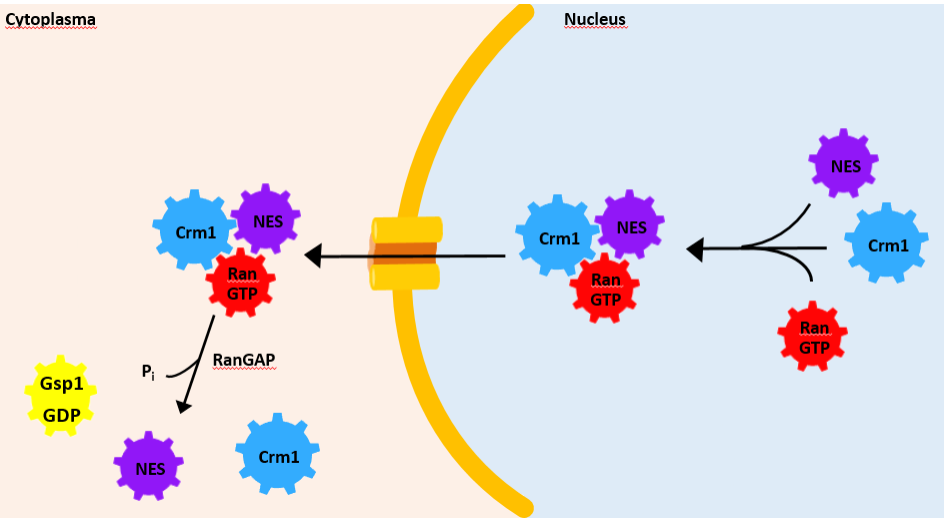


Figure 4. ***Diagram representing the nuclear export pathway.***

The process of nuclear export is carried out according to principles which are analogous to those of nuclear import. The export factor Crm1 recognized the NES-containing protein and they form a ternary complex

together with RanGTP. This complex moves thorugh the NPC via the interaction with FG-nups. Within the cytoplasm, RanGAP cause the hydrolysis of GTP which promotes the dissociation of the export complex, releasing the cargo and freeing Crm1 for recycling to the nucleus (Pemberton et al., 1998).

2.5. Regulation of the nucleocytoplasmic transport

The nucleocytoplasmic transport processes are regulated by cellular signalling systems via cargo protein modifications and the transport machinery, including the receptors, the NPC and the Ran system.

Importin-NLS/exportin-NES interactions can be modulated by conformational changes in both NLS/NES regions and in the substrate binding site of karyopherins. In some cases the three-dimensional structure of a protein masks its own transport signal as in the case of NF-κβ (Craig et al., 2002). The precursor p50 subunit of NF-κβ is phosphorylated, the C-terminal part of the protein is degraded and the NLS becomes accessible allowing the p50 to be recognized by the importin-α/importin-β complex (Riviere et al., 1991).

Furthermore, post-translational modifications such as phosphorylation, ubiquitination and methylation, can also regulate the affinity of importins/exportins and its cargos. The most common case is phosphorylation, and it can be within or close to the NLS/NES. One example is the Hxk2 phosphorylation, in this case, phosphorylation of Ser^{14} promotes Xpo1 recognition (Pelaez et al., 2009). Another examples

are phosphorylation of SV40 virus (Hubner et al., 1997) large T antigen and Pho4 (Komeili and O'Shea, 1999), but in these two cases phosphorylation increases the affinity of their NLS/NES signal to the corresponding karyopherin receptors.

Another regulatory mechanism is controlling the expression of transport machinery components. Cargo transport depends directly of the presence of karyopherin and Nups, and its translocation can be affected in its absence. As an example of this, is the factor dHSF (Drosophila Heat-Shock Factor) which is transported to the nucleus by Imp-3α. Imp-3α is absent during the early stages of Drosophila development and dHSF does not entry in the nucleus. But from cycle 13 onward the transport factor is present and the dHSF is localized within the nucleus thus allowing the embryo to respond to heat shock (Fang et al., 2001).

2.6. Multifunctional nuclear pore complex.

In addition to their established roles in nucleocytoplasmic transport, recently it has been demonstrated that nuclear pore complex are also involved in another processes, such as transcription, mRNA export, genomic organization and transcriptional memory.

Chromatin is not randomly distributed within the nucleus. Chromosomes occupy a defined nuclear place, and chromatin localizes to specific spatial domains that are dependent upon distinct structural and functional states, including heterochromatin, which is highly

compact and transcriptionally silent, and euchromatin, which is loosely packed and contains transcriptionally active loci. The spatial arrangement of chromatin and the genome as a whole dramatically affects the DNA function. Furthermore, many transcription factors, chromatin proteins, and RNA-processing factors are also compartmentalized and accumulate in distinct nuclear domains (Misteli, 2007).

Many silenced chromatin regions such as telomeric and subtelomeric chromatin, centromeres and silenced mating loci, are localized in the nuclear periphery or associated with the NPC (Niepel et al., 2013). Furthermore, recent studies in yeast have implicated the nuclear basket as a binding platform for numerous transcribed housekeeping genes and genes strongly induced by changes in the environmental conditions. These active genes interact with Nups present in the nuclear basket, the core scaffold, and the central channel (Dieppois and Stutz, 2010).

Activated genes recruitment to NPCs may facilitate mRNA export, but it could also have additional purposes. Genes involved in galactose metabolism (*GAL* genes) are among the most studied inducible genes. These genes are derepressed and actively transcribed when the carbon source switches from glucose to galactose, and its derepression is accompanied by their translocation to the NPC nuclear basket. Nuclear translocation is independent of active transcription, which suggest that this phenomenon may not be a consequence of mRNA export and transcription. One study propose that the initial interaction of *GAL* with the NPC involves the upstream activating sequence (UAS) union with the nuclear basket in a Gal4p transcriptional activator dependent manner (Schmid et al., 2006). Another study showed that *GAL* recruitment to the

NPC is dependent upon desumoylacion of the Ssn6 corepressor, and possible another transcriptional associated factors. It is proposed that there is a translocation of promoters to the basket and there occurs the desumoylation of the associated proteins by Ulp1p, which is a SUMO-isopeptidase that associates with the nuclear basket. Desumoylation may uncover binding sites on these proteins and promote the association of transcriptional activators and the assembly of chromatin remodelers on the promoter (Texari et al., 2013; Zhao et al., 2004).

Another example is the case of *INO1*, which is also an inducible gene activated when the cells grow in media lacking inositol. Under these conditions, *INO1* gene associates with the NPC. It was found that this gene contains a gene recruitment sequence (GRSs), a cis-acting element, which together with the transcriptional activator Put3p mediate the INO1-NPC association (Ahmed et al., 2010; Brickner and Walter, 2004).

The role of NPC in transcriptional regulation does not seem to be restricted to activated genes, it also extents to repressed genes. It was observed, that components of the NPC interact with the *SUC2* promoter when it is both repressed and derepressed (Sarma et al., 2007). It was demonstrated that deletion of *NUP84* gene causes 40% decrease in invertase production when cells are growing under derepressing conditions (low glucose-containing media). In contrast, deletion of either *NUP120* or *NUP133* genes has a minor impairment in invertase production, but it results in severe defects in repression of *SUC2* gene, which in the case of *NUP133* deletion, the defect in repression is as severe as the elimination of Mig1 itself (Sarma et al., 2011).

NPC are also involved in mRNA synthesis, processing and export. In yeast many transcriptional complex are assembled to the NPC employing different regions of the nuclear basket, for example SAGA complex is bound to the nuclear basket through the Mlp1p protein, and TREX-2 interacts with Nup1. In addition, the RNA pol II elongation complex may also be linked to NPCs through the mRNA export factor Mex67 (Dieppois and Stutz, 2010).

The nuclear basket has been shown to associate with the 3' and 5' of some activated genes to form NPC tethered gene loops. This gene loops are associated with mRNA maduration and export, but they also may facilitate rapid reuse of RNA pol II post-transcription by placing 5' and 3' ends in close proximity so that polymerase can easily re-engage the promoter (Dieppois and Stutz, 2010).

FG Nups are also involved in what is called transcriptional memory. Transcriptional memory is defined as a process whereby genes that have been recently repressed are poised for a more rapid reactivation that when repressed for extended periods. It can be passed from mother to daughter cells. The phenomenon of transcriptional memory is also observed in higher eukaryotes (Brickner, 2009).

INO1 and *GAL1* remain at the nuclear periphery for generations after transcription is repressed. In the case of *GAL1* its retention at the nuclear periphery is very stable, and lasting greater than seven generations. This memory state is functionally different from the long-term repressed state, and indeed, it was found that reactivation of *GAL1* gene even after seven generations of repression, was much faster than its initial activation. This suggest that cells should have epigenetic mechanism to

mark previously expressed genes and to promote its reactivation (Brickner et al., 2007; Brickner, 2009).

It was shown that two chromatin-based mechanisms are involved in the rapid reactivation of these genes: the histone variant H2A.Z which is essential for the retention of *INO1* and *GAL1* genes at the nuclear periphery after repression and for its rapid reactivation (Brickner et al., 2007), and the SWI/SNF chromatin remodelling complex (Kundu et al., 2007).

AIMS

AIMS OF THIS THESIS

Saccharomyces cerevisiae is able to adapt to glucose availably by triggering different transduction pathways. One of them is the repression pathway which affect several genes, among which is the *SUC2* gene. It is known that the transcriptional repressors Mig1 and Mig2, and the protein Hxk2, together with other factors, control the expression of *SUC2* gene, however there is little know about how this process occurs. Additionally, it has been recently described that *SUC2* gene is recruited to the nuclear envelope under repression and derepression conditions, and this recruitment might be involved in regulating the *SUC2* gene expression.

Despite this repression pathway has been extensively studied over the last years, there is a lack of detailed information about how it occurs, and some questions need to be addressed, as for example: how the assembly and disassembly of the repressor complex at the *SUC2* gene occurs and which mechanism regulates its formation, how glucose level is detected and how this information is transmitted to the nucleus to generate a coordinate cellular response. All this questions led us to consider the following aims:

- Determine the composition of the repressor complex of the *SUC2* gene.
- Identify how is regulated the formation and dissociation of the repressor complex of the *SUC2* gene.
- Study the role of the *SUC2* gene association with the nuclear pore complexes.

-

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Microorganisms used in this work.

1.1. Bacterial strains.

The bacterial strains employed in this thesis were:

Strain	Genotype
BL21	*F^-, ompT, $hsdS_B$ (r_B^-, m_B^-), dcm, gal, λ(DE3), pLysS, Cm^r.*
DH5α	*F^- Ø80dlacZ ΔM15 recA1 endA1 gyrA96 thi-1 hsdR17 (r_k-r_k-) supE44 relA1 deoRΔ (lacZ YA-argF) U169.*

1.2. Yeast strains.

The *Saccharomyces cerevisiae* strains employed in this thesis were:

Name	Relevant genotype	Source/Ref
W303-1A	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100*	(Mercado et al., 1991)
DBY1315	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2*	(Ma and Botstein, 1986)
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf

DBY2052	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 hxk1::LEU2 hxk2-202*	(Ma and Botstein, 1986)
DBY2053	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 hxk1 ::LEU2*	(Ma and Botstein, 1986)
MAP24	*MATα can1-100 his3-11,15 leu2-3,112 trp1-1 ura 3-1 mig1::loxp mig2::loxp-KAN-lox*	(Mercado et al., 1991)
H174	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6J::LEU2*	(Nehlin and Ronne, 1990)
FMY084	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 Nup84-GFP*	This work
FMY085	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6J::LEU2 Nup84-GFP*	This work
FMY086	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 Nup84-GFP*	This work
FMY120	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 Nup120-GFP*	This work
FMY121	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6J::LEU2 Nup120-GFP*	This work
FMY122	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 Nup120-GFP*	This work
FMY301	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 mig1::LEU2 hxk2-202*	(Ahuatzi et al., 2004)
FMY303	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 SNF1-HA*	(Ahuatzi et al., 2007)
FMY350	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig1::kanMX4 SNF1-HA*	This work
FMY351	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 SNF1-HA*	This work
FMY403	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 SNF4-HA*	This work
FMY481	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 snf1::kanMX4 SNF4-HA*	This work
FMY833	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 GAL83-HA*	This work
FMY320	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 MIG1-GFP*	This work

FMY321	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 MIG1-GFP*	This work
FMY322	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 hxk1::LEU2 hxk2-202 MIG1-GFP*	This work
FMY501	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 MIG2-GFP*	(Fernandez-Cid et al., 2012)
FMY507	*MATα ade2-1 canJ-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6J::LEU2 MIG2-GFP*	This work
FMY509	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 MIG2-GFP*	This work
FMY901	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 REG1-GFP*	This work
FMY902	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig1::kanMX4 REG1-GFP*	This work
FMY903	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 REG1-GFP*	This work
JCY1407	*MATa; ade2-1; ura3-1; his3-11,15;trp1-1; leu2-3,112; can1-100; kap95::HIS3.*	(Taberner and Igual, 2010)
JCY1410	*MATa; ade2-1; ura3-1; his3-11,15; trp^63; leu2-3,112; can1-100; srp1-31.*	(Taberner and Igual, 2010)
Y03813	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YDL116w::kanMX4*	Euroscarf
Y04906	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YKL057c::kanMX4*	Euroscarf
Y04620	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4*	Euroscarf
Y14403	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig1::kanMX4*	Euroscarf
Y14575	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig2::kanMX4*	Euroscarf
Y14311	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 snf1::kanMX4*	Euroscarf
Y14482	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf4::kanMX4*	Euroscarf
Y16694	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gal83::kanMX4*	Euroscarf
Y13967	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 reg1::kanMX4*	Euroscarf

2. Media and culture conditions.

2.1. Media and culture used for bacteria.

LB media

1% tryptone.

0.5% yeast extract.

0.5% sodium chloride.

LB plates were prepared adding 2% agar.

The sterilization was done in the autoclave for 20 minutes.

For selective medium, antibiotics were added after sterilization. Ampicillin to the final concentration of 100mg/l and kanamycin to 50mg/l.

The growth of bacteria in solid medium was done in *Petri* dishes and incubated at 37^{o}C. Liquid medium growth was done in *Erlenmeyer* flasks at 37 ^{o}C and 200 rpm. The final volume in each flask was not more than 20% of the total volume of the flask, in order to provide good air conditions. Bacterial growth was determined by measuring the optical density (O.D.) at 600 nm.

2.3. Media and culture conditions used for yeast.

Rich media (YEP)

1% yeast extract.

2% peptone.

2% glucose (YEPD), 3% ethanol and 0.05% glucose (YEPE), 2% xylose (YEPX), were added as a carbon sources.

For the selection of *KanMX* expressing yeast cells, 200mg/l G418 were added to the medium.

In this work we will refer to high glucose for the glucose medium (YEPD), and low glucose for the ethanol (YEPE).

Synthetic medium (SC)

0.69% yeast nitrogen base without aminoacids (YNB).
2% glucose (SCD), 3% ethanol and 0.05% glucose (SCE) were added as a carbon sources.
Aminoacids were added as required, except for those ones used as markers.
In both, YEP and SC, solid medium were prepared adding 2% agar and the pH was adjusted to 5.8.
The sterilization was done in the autoclave for 20 minutes.
Yeast growth in solid medium was done in *Petri* dishes and incubated at 28^{o}C, except for the thermo-sensitive strains which were incubated at 25^{o}C. Yeast growth in liquid medium was done in a shaker at 200 r.p.m and 28 ^{o}C in *Erlenmeyer* flasks. The final volume in each flask was not more than 20% of the total volume of the flask, in order to provide good air conditions. Yeast growth was determined by measuring the optical density (O.D.) at 600 nm.

3. Cloning vectors, DNA constructions and oligonucleotides.

3.1. Cloning vectors.

The following cloning vectors were used in this work:

pFA6a-3HA-HIS3MX6

pFA6a-GFP-HIS3MX6

pFA6a-GFP-KanMX6

pUK78-pFA6a-3myeGFP-kanMX

pGEX-4T-1

pWS93

3.2. DNA constructions.

The following DNA construction were used in this thesis:

pGEX/HXK2	For the expression of GST-Hxk2	(Ahuatzi et al., 2004)
pGEX/KAP60	For the expression of GST-Kap60	Dr.M.P. Rout.
pGEX/KAP95	For the expression of GST-Kap95	Dr.M.P. Rout.
pGEX/GSP1	For the expression of GST-Gsp1	Dr. E. Hurt
pGEX/MIG1	For the expression of GST-Mig1	(Ahuatzi et al., 2004)

pGEX/SNF1	For the expression of GST-Snf1	Dr.Martin Schmith
pGEX/SNF4	For the expression of GST-Snf4	Dr.Martin Schmith
pGEX-3X/XPO1	For the expression of GST-Xpo1	(Hammell et al., 2002)
pWS93/Snf1-HA	For the expression of a Snf1 tagged with the triple HA epitope	Dr. Pascual Sanz
pWS93/Mig1-HA	For the expression of a Mig1 tagged with the triple HA epitope	Dr. Pascual Sanz
pJJH1599/Snf4-HA	For the expression of Snf4 tagged with three GFP	This work

3.3. Oligonucleotides.

The following nucleotides were used in this thesis:

Act-d	$^{5'}$GCCTTCTACGTTTCCATCCA$^{3'}$	ChIP experiments
Act1-r	$^{5'}$GGCCAAATCGATTCTCAAAA$^{3'}$	ChIP experiments
GAL83-pFA6a-d	$^{5'}$CGCATCCATTGTTAGGTATAAACAAAAATACGTGACCCAAATACTGTATACACCATTGCAACGGATCCCCGGGTTAATTAA$^{3'}$	Tagging cassette using as a template pFA6a cloning vectors
GAL83-pFA6a-r	$^{5'}$GAGCTATAATGGAAAAATCGCAGAAACATCTTCGCTTTTATCTAAAAA	Tagging cassette using as a

	GATGTAGCTATAGAATTCGAGCTCGTTTAAAC$^{3'}$	template pFA6a cloning vectors
HIS-S1-r	$^{5'}$ATCGATGAATTCGAGCTCG$^{3'}$	Test
MIG1-F2-d	$^{5'}$GGAAACATTACCACCCATAAGAAGTTTACCGTTGCCCTTCCCACACATGGACTCGGATCCCCGGGTTAATTAA$^{3'}$	Tagging cassette using as a template pFA6a cloning vectors
MIG1-R1-r	$^{5'}$TTAATTCTTGTCTATTGTCTTTTGATTTATCTGCACCGCCAAAAACTTGTCAGCGTAGAATTCGAGCTCGTTTAAAC$^{3'}$	Tagging cassette using as a template pFA6a cloning vectors
MIG2-pFA6a-d	$^{5'}$AACCTACTGAAACAAATTGATGTTTTCAACGGTCCCAAAAGAGTTCGGATCCCCGGGTTAATTAA$^{3'}$	Tagging cassette using as a template pFA6a cloning vectors
MIG2-pFA6a-r	$^{5'}$GACCATGCCGGCCGCAATTCGTATAGGTAAACGTGCTATTCATGACGATGAATTCGAGCTCGTTAAAC$^{3'}$	Tagging cassette using as a template pFA6a cloning vectors
NUP120-pFA6a-F2-d	$^{5'}$GGAAGTAAAGTGGTTACTTTAACTGATTTAAGAGATGAGTTACGAGGTCTACGGATCCCCGGGTTAATTAA$^{3'}$	Tagging cassette using as a template pFA6a cloning vectors

NUP120-pFA6a-R1-r	5´CCCGGATGCTTTGTTGACCAATAGTTACATTGAAATTTCTCTATTATCTCCGAATTCGAGCTCGTTTAAAC3´	Tagging cassette using as a template pFA6a cloning vectors
NUP120-test-d	5´CGGAGTGGTGATTGGGAGTG3´	Test
NUP84-pFA6a-F2-d	5´GAAAGTTAAAAGAGTATCTGGATCTCGTTGCTCGCACAGCAACCCTTTCGAACCGGATCCCCGGGTTAATTAA3´	Tagging cassette using as a template pFA6a cloning vectors
NUP84-pFA6a-R1-r	5´GTCAATTACTTCTGAAATAGATAGAAGAAATGCATAAAATTATTGCTGTTTACGAATTCGAGCTCGTTTAAAC3´	Tagging cassette using as a template pFA6a cloning vectors
NUP84-test-d	5´CGGAAGCCCTGGCATTTACC3´	Test
OL57-GFP-r	5´ATTGGGACAACTCGAGTG3’	Test
OL-Kan-MX5’-out	5´GGAATTTAATCGCGGCCTCG3´	Test

REG1-pF6a-F2-d	5´GAAAAAGAGTGACGTCAAGCCACAAGAAAATGGAAATGACAGCAGTCGGATCCCCGGGTTAATTAA3’	Tagging cassette using as a template pFA6a cloning vectors
REG1-pFA6a-R1-r	5´GATTACAGCTTACTTGGATCCTAAAGACGGCAATGATCCACACTACGAATTCGAGCTCGTTTAAAC3’	Tagging cassette using as a template pFA6a cloning vectors
REG1-test-d	5´GCGTCTTTGACGGATTCGAG3’	Test
SNF1-pFA6a-d	5´ACAACAAAACTAATTATGGAATTAGCCGTTAACAGTCAAAGCAATTCCGGTTCTGCTGCTAGT3´	Tagging cassette using as a template pFA6a cloning vectors
SNF1-pFA6a-r	5´TCATAGAGCGTGAAATTTGCTTTTCATCCGAAGAAATAATGCCAATCCTCGAGGCCAGAAGAC3´	Tagging cassette using as a template pFA6a cloning vectors
SNF1-test-d	5´TGAAGAATTTGGGTGCCGAA3´	Test
SNF4-pFA6a-d	5´TGACGTCGGACGGTTGGTTGGTGTCTTGACGTTAAGCGATATTCTCA	Tagging cassette using as a

	AATATATCCTTCTAGGTAGCAACCG GATCCCCGGGTTAATTAA$^{3'}$	template pFA6a cloning vectors
SNF4-pFA6a-r	$^{5'}$ATTCGGCGGAAATTCGGTTATA AAGCGAAGTGAAAAATCGATTAGTA ATGATACTGATGCGATCTCTGGGGA ATTCGAGCTCGTTTAAAC$^{3'}$	Tagging cassette using as a template pFA6a cloning vectors
SUC2-d-FMH	$^{5'}$AGCTCGAGTTATTACTCTGAACA GGA$^{3'}$	ChIP experiments
SUC2-r-FMH	$^{5'}$TAGTCGACAAGTCGTCAAATCTT TCT$^{3'}$	ChIP experiments

4. Methods.

4.1. Plasmidic bacteria DNA purification.

To purify plasmidic DNA from bacteria the *Miniprep* method was used. Cells are treated with STET buffer (8% sacarose, 5% X100 triton, 50 mM EDTA and 50 mM HCl-Tris pH 8.0) and lysozyme (10mg/ml).

4.2. Genomic DNA isolation from yeast.

To isolate genomic DNA from S.*cerevisiae*, we follow the method described in (Ciriacy and Williamson, 1981), which is based on the formation of protoplasts using zymolyase and followed by the lysis with SDS. Chromosomic DNA was obtained by ethanol precipitation in the presence of 5M ammonic acetate.

4.3. Restriction DNA analysis.

Restriction endonucleases from Roche Diagnostics and Fermentas were used, according to manufacturers´ instructions.

4.4. Agarose gel electrophoresis.

DNA was separated by electrophoresis in 0.8 or 2% gels in TBE Buffer (89 mM tris, 2mM EDTA, 89 Mm boric acid pH 8.3). Gels were stained using ethidiumbromide.

4.6. Plasmid construction.

Construction of recombinant plasmid was performed by subcloning the DNA fragments in the cloning vectors described previously. Cloning vector was digested with one or two endonucleases and later was

treated with alkaline phosphatase to prevent the vector recirculation. Fago T4 ligase was use to ligate the DNA fragment.

Purification of the DNA fragments was done using the kits: Matrix Gel Extraction System from Marling Bioscience Inc. and ATP^{TM} Gel/PCR Fragment DNA Extraction Kit from ATP Biotech Inc.

4.6. Bacteria transformation.

For transformation of chemically competent *Escherichia coli*, 50ml of LB medium were inoculated with 0.5 to 1 ml of cells from an overnight culture of DH5α or BL21 an incubated on a rotary shaker at 180 rpm until cells reached an OD_{600} of 0.4 – 0.6. The culture was harvested by centrifugation at 4^{o}at 3500 rpm Allegra 2IR centrifuge (Beckman coulter). The pellet was resuspended in 5ml of RF1 solution (100 mM RbCl, 50 mM $Mgcl_2$, 30 mM KAc, 10 mMm $CaCl_2$, 15% glycerol, pH 5.8) and incubated on ice for 15-20 minutes. The cells were precipitated again and resuspended in 4 ml of RF2 solution (10 Mm MOPS, 10Mm RbCl, 75mMm $CaCl_2$, 15 glycerol, pH 6.8). After incubation on ice for 20 minutes, the cells were aliquoted in 200 µl portions and stored at -80^{o}C until use. For transformation, 1 µl of plasmid DNA or 10 µl of a ligation mixture was added to a thawed aliquot of competent cells. After incubation on ice for 30 minutes, a heat shock at 42^{o}C for 2 minutes was performed. After a brief incubation of the sample on ice, 1 ml of LB medium was added and the cells were incubated for 1 hour at 37^{o}for phenotypic expression. Cells were collected by centrifugation in a microfuge at 1300 rpm for 1 minute,

resuspended in a drop of medium and plated on selective LB medium containing the required antibiotics.

4.7. Transformation of yeast cells according to the "freeze" protocol.

50 ml of full medium were inoculated with 0.5-1 ml of an overnight culture and incubated on a shaker until de OD_{600} was about 0.6. The yeast cells were harvested (5 min at 2500 rpm and 4ºV in a Allegra 2IR centrifuge (Beckman coulter) and washed in 5 ml of buffer A (1 M sorbitol, 10 mM bicine pH 8.35, 3% ethylene glycol) and again resuspended in 4 ml of buffer A. The cell suspension was aliquoted in 200 µl portions and stored at -80ºC until further use. For transformation, 5-10 µl of DNA and 5 µl of salmon sperm DNA (10mg/ml) were added. The samples were incubated for 5 min at 37ºC and mixed carefully several times. After that, 1 ml of buffer B (40% PEG 1000, 200 Mm bicine Ph8.35) was added and the samples were incubated at 28ºC for 1 h. The transformed yeast cells were spun down (6000 rpm for 5 min), resuspended in a small amount of buffer B and plated onto selective media. Cells, which were selected for resistance to G418, were incubated overnight in rich medium and plated onto rich medium plates containing G418.

4.8. Transformation of yeast cells with lithium acetate.

50 ml of full medium were inoculated with 1 ml of an overnight culture and incubated on a shaker until de OD_{600} was about 1. The cells

were harvested (3 min at 3000 rpm at 4ºC), washed with 25 ml of H_2O, resuspended in 1 ml of 100 mM lithium acetate and spun down again (3000 rpm for 3 min). The pellet was resuspended in 400 µl of 100 mMm lithium acetate and the suspension was split into 50 µl aliquots, which were spun down. To each pellet 240 µl 50% PEG 4000, 36 µl 1 m lithium acetate, 5 µl of salmon sperm DNA and 3 µl of a plasmid preparation or 25 PCR product were added. Afterwards, the pellet was resuspended and the samples were first incubated for 30 min at 28ºC followed by a heat shock at 42ºC for 20 min. The transformed cells were spun down and selection of transformants was done as described above.

4.9. Polymerase chain reaction.

The protocol we used was described by (Mullis et al., 1986). 1 µg of DNA was used as a template, 0.2 µg of the required oligosnucleotides, 2.5 U of High Fidelity Taq DNA polimesase (5 prime) and 0.2 mM of dNTPs (General Electric Healthcare), in a final volume of 50 µl.

For confirmation of correct cloning, MasterMix was employed.

The amplification program consists of 35 cycles (1´at 94ºC, 1´at 55ºC and 2´at 72º).

5. Proteins manipulation

5.1. Preparation of crude protein extracts.

Yeast cells were growing in 10-20 ml of the required medium, to an optical density at 600 nm of 0.8-1.0 at 28ºC. Cells were collected and washed with PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Ph7.5 Na_2HPO_4) and resuspended in 300 µl lysis buffer (PBS, 1x protease inhibitor (Complete Protease Inhibitor Tablets, Roche), 0.1% triton X100 and 1 mM DTT). Cells were broken in the presence of 400 µl of *ballotini* glass beads (0.50 mm diameter), using Speed mill homogenizer (Analytikjena). After centrifugation at 13000 rpm for 15 minutes at 4ºC, the supernatant was used as a crude protein extract in immunoprecipitation and GST pull-down experiments.

5.2. Protein quantification.

Quan-it ™ Protein Assay from Invitrogen was used to quantify protein according to manufacters´ instructions.

5.3. Enzime assay

Invertase activity was assayed in whole cells as it is described in {Gascon, 1968 #377}.

5.4. Denaturing polyacridamide gel electrophoresis (SDS-PAGE).

Protein extracts were separated in acrylamide gels in the presence of SDS and β-mercaptoethanol. Stacking gel was prepared with 3.5% acrylamid and N-N'-methylen-bisacrylamid, and running gel was prepared with the same solution but 12%.

SDS is an anionic detergent which causes proteins to denature and disassociate from each other. It also confers negative charge which is proportional to the protein weight, so at the end every molecule will have the same negative charge density. Under the influence of an electric field, proteins will migrate to the positive pole and the migration distance will be inversely proportional to its molecular weight logarithm.

5.5. Protein transfer; from polyacrilamid gels to PVDF membranes (Polyviylidene Fluoride).

Proteins separated in polyacrilamid gels were transfer to PVDF membranes as described as (Burnette, 1981), using BIO-RAD equipment. A buffer containing 25 Mm Tris-HCL pH 8.8, 192 Mm glycine and 20% methanol, was used. The transfer was done during an hour at 100V.

5.6. Bacteria expression and GST bound proteins purification.

Coding sequence of the protein was cloned in pGEX-4T vector, in the same reading frame as GST protein. *E.coli* BL21 was transformed with it and the expression of the fusion protein was induced by the addition of IPTG (isopropyl-β-Dtio-galactoside) at the final concentration of 0.5 mM, when bacteria reach DO_{600} 0.45. After three hours of incubation, bacteria were harvested, washed and resuspended in 1 ml of PBS pH 7.5 and 1 mg/ml lysozyme, they were incubated on ice, after this time cells were broken by sonification (5 pulses of 10 seconds at 200-300W). The extract resulted from this step was centrifuged two times at 14000 rpm during 20 minutes, in order to eliminate insoluble material. Soluble extract was incubated with 100 ml of glutathione sepharose 4B beads (which was equilibrated with 5ml of cold PBS pH 7.5) during at least one hour at 4^{o}C in a spinning wheel. After this period of time, the column was washed extensively with cold PBS pH7.5.

To purify the protein fused with GST, thrombin 2.5 U was used to release it from the GST.

5.7. Co-precipitation or GST pull-down

This technics are used to study interactions between proteins. Its base on the precipitation of a protein, which was purified or that comes from a protein extract, and it interaction with a protein fused with GST which is coupled to a glutathione sepharose beads.

The expression of this proteins was done as described above in 5.6, but the protein is not eluted from the sepharose. To this beads a protein extract (obtained as described in 5.1) or a purify protein was added and

this was incubated over night at 4ºC in a spinning wheel. Next day, beads were was 5 times with 1 ml of PBS pH 7.5 and were resuspended in 20 µl of dissociation buffer 4X (12.5 ml of Tris-HCl 1M pH 6.8, 4 g of SDS, 17.5 ml of glycerol, 12.5 ml of β-mercaptoethanol, 1.6 ml EDTA 250 mM, bromophenol blue and H_20 until 50 ml), and 60 µl of PBS. The samples were boiled during 10 minutes, after this time were centrifugated at 14000 rpm 10 minutes and a western blot was done as its indicated in the next section.

6. Immune techniques.

6.1. Western Blot

SDS loading buffer was added to crude extracts (obtained as described above 5.1), and were boiled during 10 minutes. This samples were loaded in a polyacrylamide gel and transfer to PVDF membrane.

PVDF membrane was incubated one hour at room temperature with a blocking solution TBS-Tween (20 mM HCl pH 7.5, 150 mM NaCl and 0.1% Tween 20) with 5% milk powder. Later, the membrane was incubated overnight with a blocking solution containing a specific antibody.

Next day, the membrane was washed twice with 15 ml of TBS-Tween during half an hour. After this time, blocking buffer containing the secondary antibody (horseradish peroxidase-conjugated protein A) was added and was incubated during one hour, after which the membrane

was washed again two times with 15ml of TBS-Tween. After the final wash, the membrane was dried and the Super-Signal West Pico Chemiluminiscent system (Pierce) was used as manufactures' instructions for detection.

6.2. Co-immunoprecipitation Assay

Immunoprecipitation experiments were performed using whole cell extracts. The extracts were incubated with 1.5 µl of a specific antibody during 4 hours at 4^{o}C in a spinning wheel. After this 100 µl of Protein A-sepharose beads were added and this was incubated during 4 hours at 4^{o}C. After extensive washing with PBS buffer, immunoprecipitated samples were boiled in 20 µl of SDS loading buffer and 60 µl of PBS. Samples were centrifuged and analyze in a Western blot (6.1 section).

6.3. Chromatin immunoprecipitation (ChIP)

Cells were grown in 50 ml overnight until they reach a DO_{600} 1.0, in this moment 500 µl of formaldehyde and it was incubated during 1 hour at room temperature and in a shaker, after this time glycine 2.5 M was added to a final concentration of 125 mM to stop the reaction, it was done at room temperature in a shaker and during 15 minutes.

Cells were washed 5 times with 25 ml of cold TBS (20 mM Tris-HCl Ph 7.5, 150 mM NaCl). The pellet was resuspended in 600 µl of lysis buffer (50 mM Hepes KOH pH 7.5, 140 mM NaCl, 1mM EDTA, 1% Triton X-100,

0.1% Sodium desoxicolate, 1 mM) and the cells were broken in SpeedMill using 400 µl of ballotini glass beads. After this the lysate was transferred to a new Eppendorf cups and the extracts were sonificated (periods of 10 seconds 20 times) to break the DNA in fragments of 200-500 bp, and after this the sample was centrifuged at 10.000 rpm during 15 minutes at 4ºC. The supernatant which contained the DNA-protein soluble was taken.15 µl of this was taken as a positive control (INPUT).

200 µl were taken and added 2 µl of a specific antibody which was incubated overnight in a spinning wheel. Another 200 µl were taken to make a negative control in which 2 µl were added of an unspecific antibody. Next day 200 µl of protein A-sepharose were added and was incubated during at least 4 hours at 4ºC, after this time the samples were washed 4 times with 1 ml of PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 pH 7.5), and the beads were resuspended in 50 µl of elution buffer (1% SDS, 100 Mm NaH_2CO_3) and was incubated at 65ºC, after 20 minutes the samples were centrifuged at 1.500 r.p.m. and the supernatant was taken and incubated overnight at 65ºC with 240 µl of elution buffer to revert the cross linking (positive control was also incubated overnight at 65ºC in elution buffer). Next day, 100 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), 5µl of proteinase K (10 mg/ml) and 6 µl of glycogen (10 mg/ml) were added to the samples and incubated them for 1 hour at 37ºC.

Finally, two DNA extractions with 300 µl of phenol/chloroform were done, and the DNA was precipitated with the same volume of isopropanol and 5M NaCl (4 µl per 100 µl of sample). The precipitated DNA was resuspended in 30 µl of TE and a PCR was done taking as a

template 1 µl of each sample (positive control, negative control and ChIP sample. PCR program was: 1’ at 94^{o}C, 1’ at 55^{o}C and 30’ at 72^{o}C, 35 cycles.

RESULS

RESULTS

1. The repressor complex.

SUC2 gene repression is mainly executed by Mig1, a zinc-finger DNA-binding protein (Nehlin and Ronne, 1990). Mig1 binds to two upstream repressing sequences (URS) of the *SUC2* promoter, called SUC2A and SUC2B (Figure 5), recruiting the general corepressors Tup1 and Ssn6 (Treitel and Carlson, 1995). Either of both sequences is sufficient to repress the *SUC2* gene, indicating that these two elements are functionally redundant with regard to Mig1 repressor function (Bu and Schmidt, 1998).

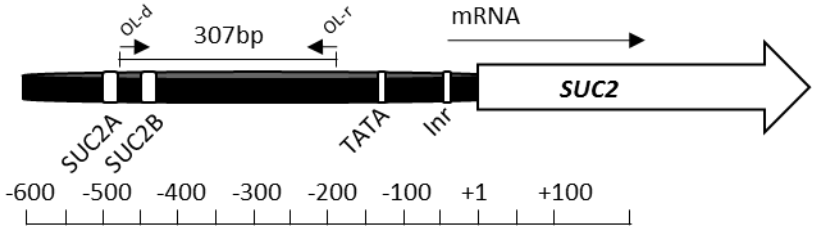


Figure 5. ***Schematic diagram of the SUC2 promoter*****.** It shows the location of the two Mig1 binding sites within the *SUC2* promoter, SUC2A (5'-A_{-499}ATAAAAATGCGGGGAA$_{-484}$-3') and SUC2B (5'-G_{-449}GAAATTATCCGGGGG C_{-431}-3'). The hybridization site of the primer pair used in the ChIP experiments is also represented, OL-d and OL-r.

A part from Mig1, there are other proteins which make a large contribution to glucose-induced repression of *SUC2* gene. If we indirectly measure the expression of *SUC2* gene by measuring the invertase

activity, it is observed that the proteins Mig1, Mig2, Hxk2, Reg1 and the SNF1 complex, influence its expression.

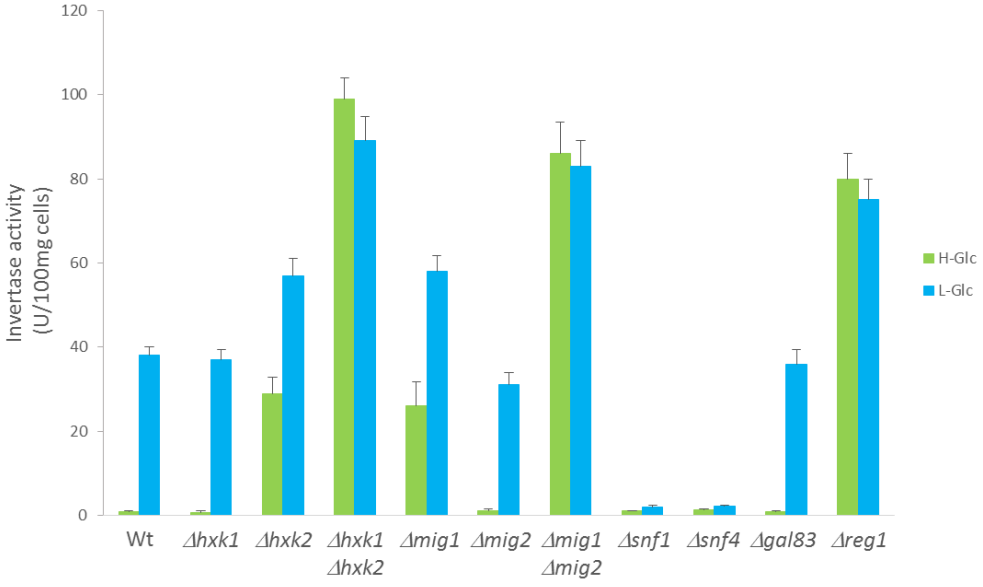


Figure 6. ***Quantitative invertase assays were performed on cells with the indicated mutations.*** Whole cells from the wild-type strain, W303-1A, and the mutant strains *Δhxk1, Δhxk2*, *Δhxk1Δhxk2, Δmig1, Δmig2, Δmig1Δmig2, Δsnf1, Δsnf4, Δgal83* and *Δreg1* were used for invertase activity determination. Invertase was assayed using cells grown in high-glucose medium (H-Glc, green bars) until an A_{600nm} of 0.8 was reached and then transferred to low-glucose medium for 60 min (L-Glc, blue bars). Error bars represent the standard error of the mean for three independent determinations using three colonies of each strain.

As the **¡Error! No se encuentra el origen de la referencia.** shows, ig1 and Hxk2 absence impair a loss of *SUC2* repression in high and low glucose conditions. Despite Mig2 and Hxk1 absence do not have any effect over *SUC2* expression, since invertase levels are the same as in a wild-type strain, the full derepresion of *SUC2* gene is only reached in the *Δmig1Δmig2* and in the *Δhxk1Δhxk2* double mutant strains.

SNF1 subunits also influence *SUC2* expression. Removal of either Snf1 or Snf4 proteins causes the non-derepression of the *SUC2* gene under low glucose conditions, and therefore in high and low glucose conditions there is no invertase synthesis. In contrast, the absence of the Gal83 subunit does not seen to have any influence in the *SUC2* gene expression.

In the absence of the regulatory subunit Reg1 of the phosphatase Glc7, there is a total derepression of *SUC2* gene in either high or low glucose-grown cells.

Even though that all this proteins affect the expression of the *SUC2*, there is a lack of detailed information with regard to the transmission mechanism of glucose signal from the environment to the Mig1 repressor complex, and the mechanism which regulates the assemble and dissemble of the repressor complex. In other to answer these questions, we decided to study the union of Mig1 and the other factors involved in glucose repression with the *SUC2* promoter elements.

1.1. Mig1 union with the *SUC2* promoter.

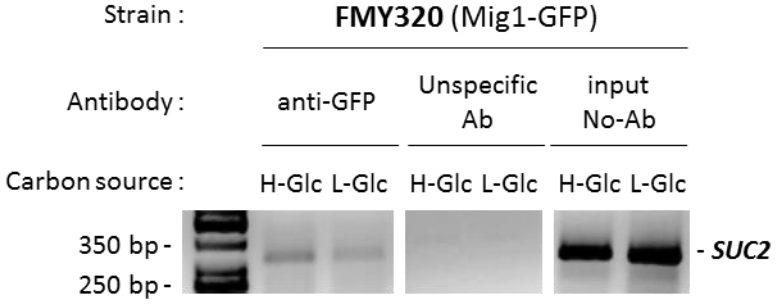
Firstly, we study the association of the transcriptional repressor Mig1 with the *SUC2* elements of the *SUC2* promoter. For this aim, cells expressing a functional GFP-tagged form of the Mig1 protein at its chromosomal locus, were used to perform a ChIP assay. Protein extracts from high glucose-grown cells and glucose-starved cells were immnunoprecipitated with an anti-GFP antibody, and after following the

protocol described in section 6.3 of materials and methods, a PCR and a qPCR were performed using the oligos SUC2-d and SUC2-r.

The results shown in Figure 7 , indicate that Mig1 binding to *SUC2* promoter elements occurs in a glucose-sensitive manner, showing the maximum binding level in cells grew in high glucose conditions. In contrast, glucose starvation decreases the association of Mig1 with the *SUC2*-URS region in a 68% (Figure 7B).

FMY320	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 MIG1-GFP*

A



B

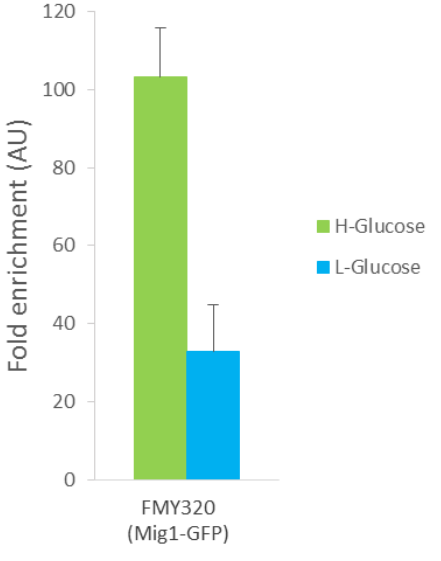


Figure 7. ***Association of Mig1 repressor with the SUC2 promoter*****.** Association of Mig1-GFP with the *SUC2* promoter measured by ChIP, using the strain FMY320 expressing a GFP-tagged Mig1 protein. **A** Represents the PCR analysis, the first two lanes are the ChIP results of the immunoprecipitation with anti-GFP antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and the last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **B** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

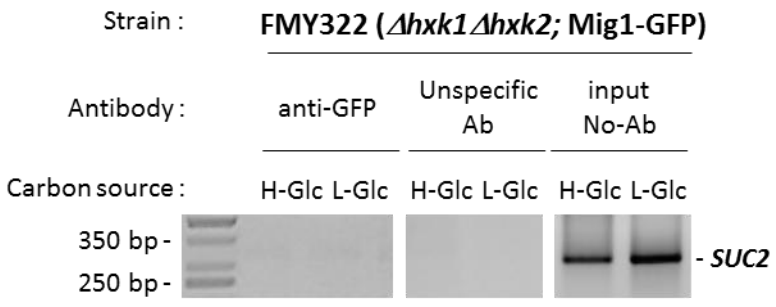
It was previously reported that Mig1 subcellular localization is regulated by glucose (Ahuatzi et al., 2007). Under high glucose conditions Mig1 is translocated to the nucleus, which is in agreement with our results, and glucose depletion produces its rapid translocation to the cytoplasm (De Vit et al., 1997). However, our results suggest that 32% of Mig1 remains associated with the *SUC2* promoter after glucose starvation Figure 7B. This indicate that the interaction of Mig1 with *SUC2* promoter might be regulated by two different mechanism: Mig1 phosphorylation state (McCartney and Schmidt, 2001) and an independent mechanism.

It is known *HXK2* gene is highly expressed in cells growing in high glucose medium (Herrero et al., 1995), and after a shift to low glucose medium *HXK2* is repressed and *HXK1* gene is rapidly expressed (Rodriguez et al., 2001). A possible explanation for the independent mechanism that regulates Mig1 interaction with the *SUC2* promoter, could be that under low glucose conditions Hxk1 mimics partially the function of Hxk2 in glucose repression signalling during high glucose-grown cells. In order to test this hypothesis, we performed a ChIP assay

using an *Δhxk1Δhxk2* double mutant strain, which contains a functional GFP-tagged form of Mig1 protein at its chromosomal locus.

FMY322	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 hxk1::LEU2 hxk2-202 MIG1-GFP*

A



B

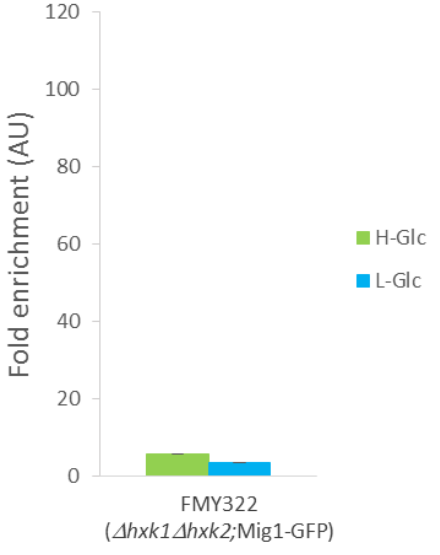


Figure 8. ***Association of Mig1 repressor with the SUC2 promoter in Δhxk1Δhxk2 double mutant strain.*** Association of Mig1-GFP with the *SUC2* promoter measured by ChIP, using the strain FMY322 (*Δhxk1Δhxk2*) expressing a GFP-tagged Mig1 protein. **A** Represents the PCR analysis, the first two lanes are the ChIP results of the immunoprecipitation with anti-GFP antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **B** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal

normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

The results show that less than 5% of Mig1 is bound to *SUC2* promoter elements in both high and low glucose conditions (Figure 8), a much lower degree of Mig1 union if compared with the wild-type strain at low glucose conditions (Figure 10).

Hxk2 absence produces a loss of 30% in the *SUC2* repression (Figure 5), and is also necessary for glucose-induced repression of the *HXK1* gene. These facts, make it likely that Hxk1 protein could mimics Hxk2 function. To address this possibility, we have also study Mig1 union with the *SUC2* promoter by using a *Δhxk2* mutant strain, which express a functional GFP-tagged form of the Mig1 protein at its chromosomal locus, to perform a ChIP assay.

These results show that in Hxk2 absence, the recruitment of Mig1 to the *SUC2* promoter is significantly decreased at high glucose conditions if its compared with its recruitment in the wild-tye strain. However, the recruitment of Mig1 to the *SUC2* promoter in low glucose conditions is identical to that found in the wild-type strain in glucose starved cells (Figure 9).

FMY321	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 MIG1-GFP*

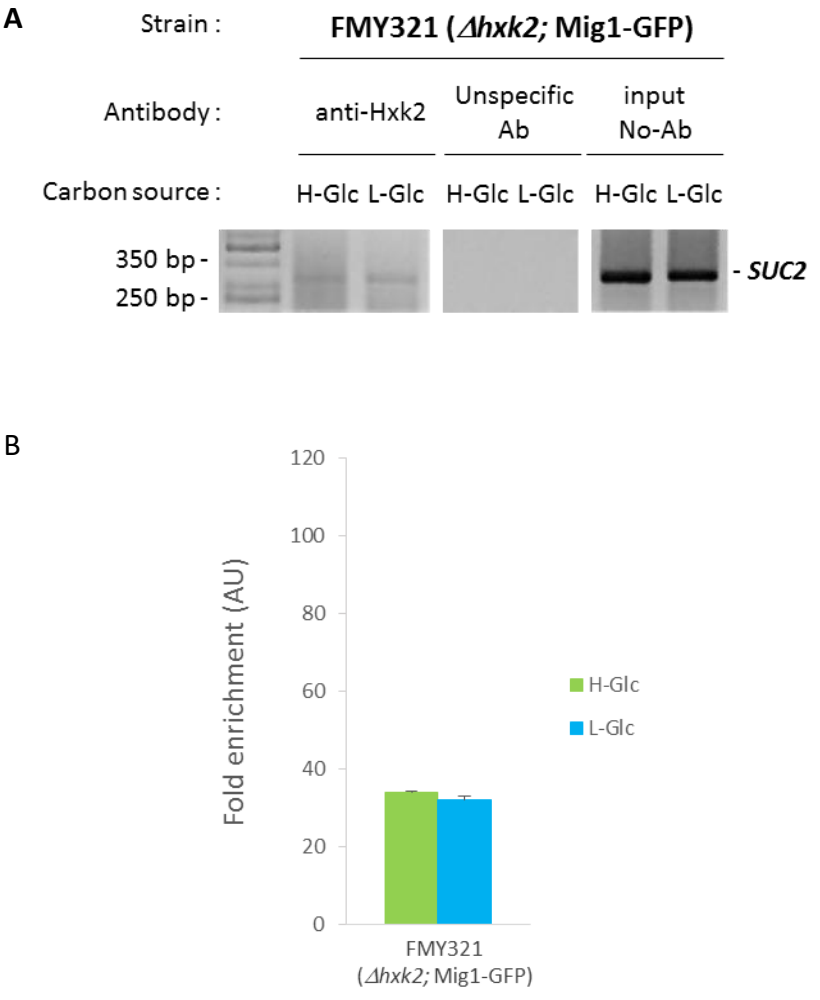


Figure 9. ***Association of Mig1 repressor with the SUC2 promoter in Δhxk2 mutant strain.*** Association of Mig1-GFP with the *SUC2* promoter measured by ChIP, using the strain FMY321 (*Δhxk2*) expressing a GFP-tagged Mig1 protein. **A** Represents the PCR analysis, the first two lanes are the ChIP results of the immunoprecipitation with anti-GFP antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **B** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

If we compare Mig1 association with the *SUC2* promoter in this three strains: wild-type, *Δhxk2Δhxk1* and *Δhxk2* (Figure 10), these results support that the idea that in high glucose conditions Mig1 recruitment to

SUC2-URS is Hxk2-dependent, and during the growth under low glucose conditions, Hxk1 is important to modulate Mig1 bound to the *SUC2* promoter elements.

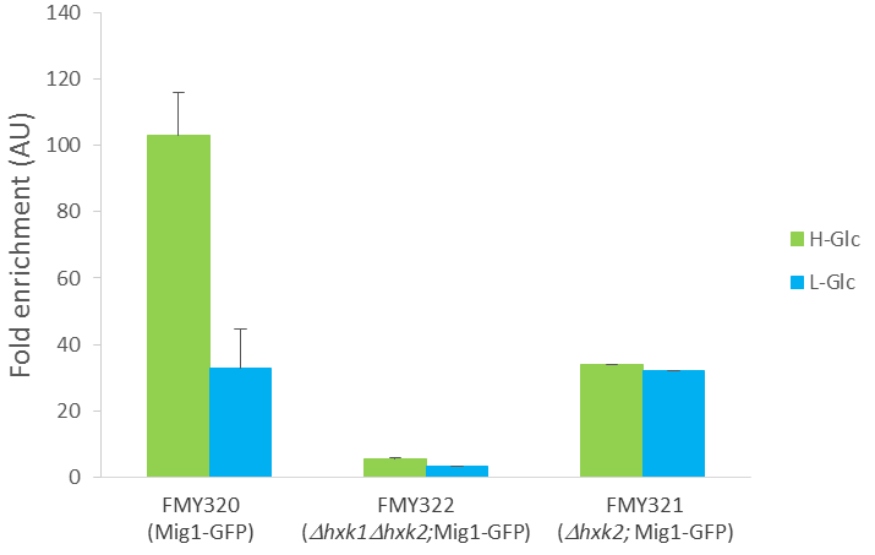


Figure 10. ***Comparison of Mig1 biding to the SUC2 promoter in different strains.*** It shows the association of Mig1 with the *SUC2* promoter in a wild-type, *Δhxk1Δhxk2* and *Δhxk2* strains, to compare the different values obtained in the RT-PCR of the ChIP experiments.

1.2. Hxk2 in the repressor complex.

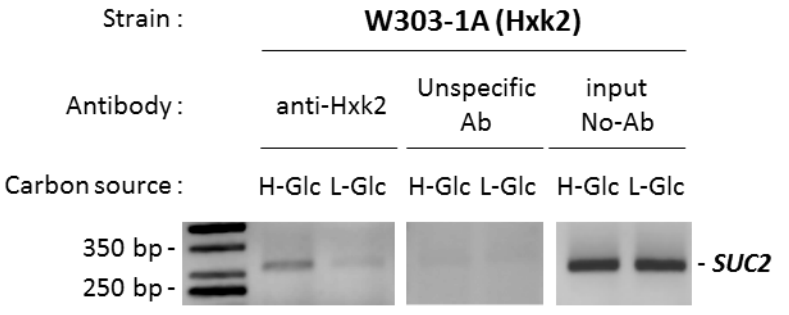
Since Hxk2 absence affects the repression of the *SUC2* gene (Figure 5), and it is known that Hxk2 interact directly with Mig1 *in vivo* and *in vitro* (Ahuatzi et al., 2004), we decided to investigated the association of Hxk2 with the *SUC2* promoter in high and low glucose conditions, and

how Mig1 affects this interaction. We tested the interaction *in vivo* by performing ChIP experiments using specific anti-Hxk2 antibody.

Our results reveal that in high glucose conditions Hxk2 is well engaged to the *SUC2* promoter, but under low glucose conditions only a small portion of Hxk2 (less than 8%) is bound to the *SUC2* elements (

Figure ***11***).

A



B

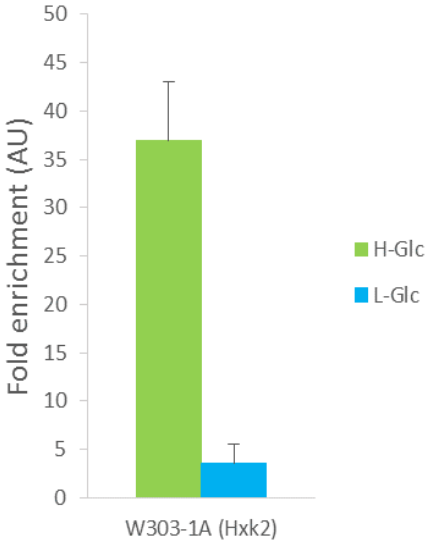
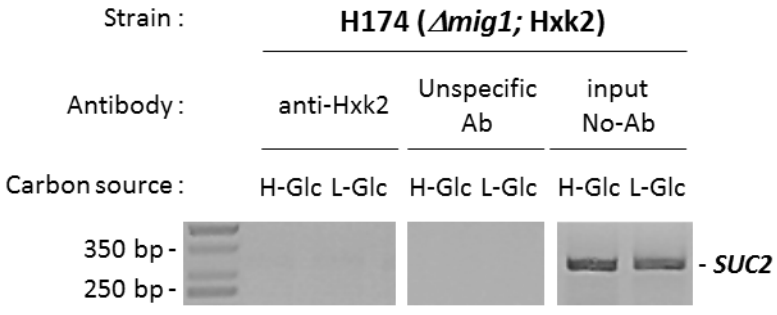


Figure 11. ***Association of Hxk2 protein with the SUC2 promoter.*** Association of Hxk2 with the *SUC2* promoter measured by ChIP, using the wild-type strain W3031-A. **A** Represents the PCR analysis, the first two lanes are the ChIP results, the immunoprecipitation with a specific anti-Hxk2 antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **B** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

In order to test if Hxk2 recruitment to the *SUC2* promoter is affected by Mig1 absence, we repeat the same ChIP experiment but in a *Δmig1* mutant strain.

A



B

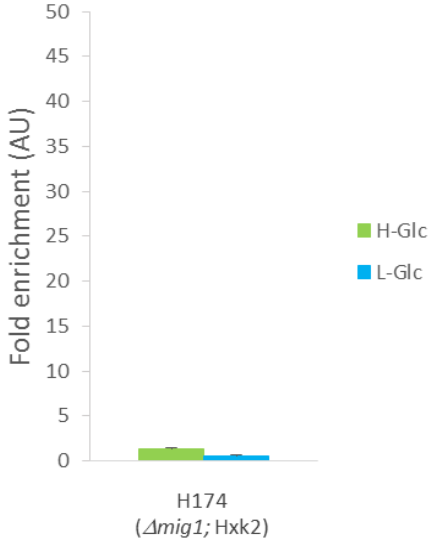


Figure 12. ***Association of Hxk2 with the SUC2 promoter in the absence of Mig1.*** Association of Hxk2 with the *SUC2* promoter measured by ChIP, using the H174 (*Δmig1*) strain. **A** Represents the PCR analysis, the first two lanes are the ChIP results, the immunoprecipitation with a specific anti-Hxk2 antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **B** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

What we can see in (Figure 12), is that in cells growing in high and low glucose conditions, the occupation rate of the *SUC2* promoter by Hxk2 is affected by the absence of the Mig1 repressor. In *Δmig1* less than 2% of Hxk2 is associated with the *SUC2* promoter, demonstrating that Hxk2 binding to the *SUC2* gene is Mig1-dependent. These results not only indicate that Mig1 is required to capture Hxk2 protein to the repressor complex of the *SUC2* promoter but also that Hxk2 do not interact directly with DNA.

1.3. Mig2 in the repressor complex.

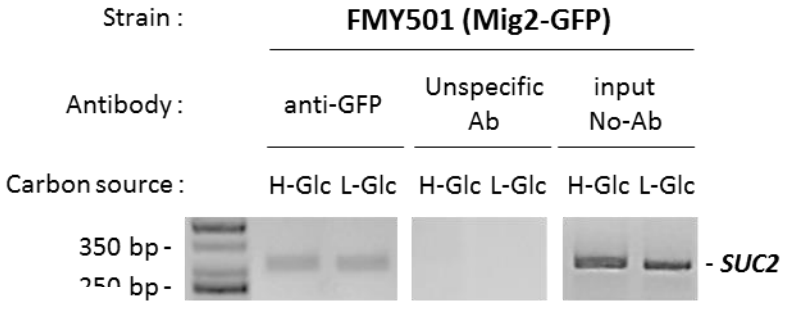
In *Δmig1* mutant cells growing in high glucose conditions, only 27% of *SUC2* repression is lost (Figure 5), so it seems likely that other binding proteins could mimic Mig1 function. Mig2, like Mig1, represses transcription in response to glucose through Tup1 and Ssn6 correpressors (Lutfiyya and Johnston, 1996), and only in the *Δmig1Δmig2* double mutant cells there is a complete derepression of *SUC2* gene. Considering all, we decided to investigate the role of Mig2 in the *SUC2*

repressor complex and how Mig1 and Hxk2 affect Mig2-*SUC2* promoter interaction.

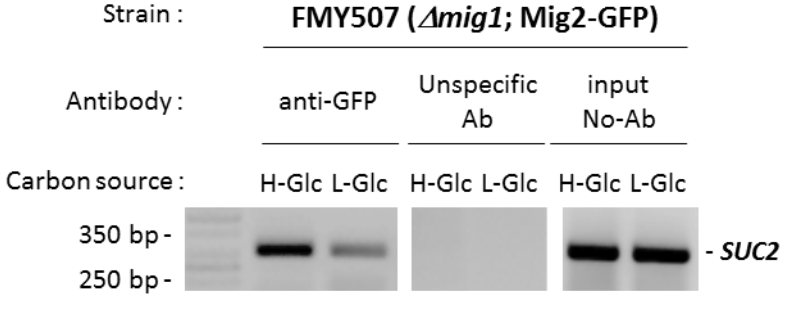
Three different strains: wild-type, *Δmig1* and *Δhxk2*, expressing a functional GFP-tagged form of the Mig2 protein at its chromosomal locus, respectively, were used to determine by ChIP the SUC2-URS localization of Mig2.

FMY501	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 MIG2-GFP*
FMY507	*MATα ade2-1 canJ-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6J::LEU2 MIG2-GFP*
FMY509	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 MIG2-GFP*

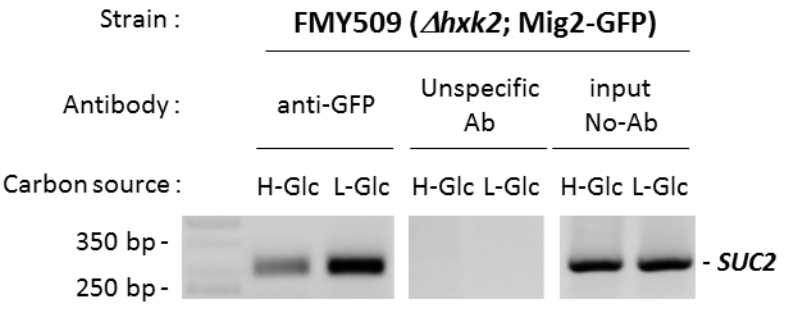
A



B



C



D

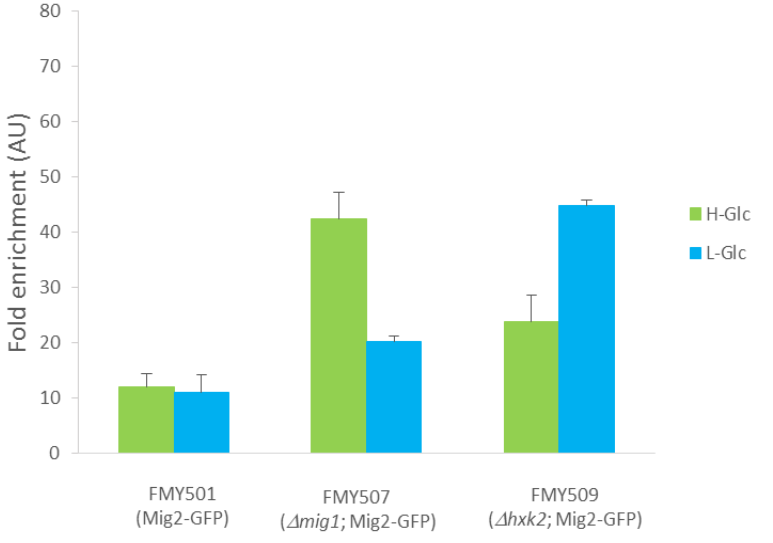


Figure 13. ***Association of Mig2 with the SUC2 promoter.*** Association of Mig2 with the *SUC2* promoter measured by ChIP in three different strains: **A** The FMY501 strain (wild-type) and the mutant strains FMY507 (Δmig1) (**B**) and FMY509 (Δhxk2) (**C**), expressing a GFP-tagged Mig2 protein. **A**, **B** and **C** Represent the PCR analysis, in each image the first two lanes are the ChIP results, the immunoprecipitation with an anti-GFP antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **D** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

In the wild-type strain grown in high or low glucose conditions, Mig2 is recruited to the SUC2 elements in about 26-28% (Figure 13A and 13D). The level of Mig2 associated with the *SUC2* promoter reaches a maximum at low glucose conditions in a *Δhxk2* mutant strain (100%) (Figure 13C). The level of Mig2 associated with the *SUC2* promoter also increases at high and low glucose conditions in a *Δmig1* mutant strain with respect to the wild-type (Figure 13B). Thus, in high glucose conditions in the absence of Mig1 protein, Mig2 is actively recruited to the *SUC2* promoter (95%) (Figure 13D) contributing to maintain 70% of *SUC2* repression (Figure 2). Although Mig2 appears to play no role in *SUC2* repression when Mig1 is present, because its deletion has no effect on *SUC2* expression in a *MIG1* strain (Figure 2), yeast cells are only fully desrepressed in a *Δmig1Δmig2* double mutant strain (Figure 2). These data support the idea that Mig2 may bind to the *SUC2* promoter mainly in the absence of Mig1 protein as happens in *Δmig1* or *Δhxk2* mutant strains. It also appears that the role of Mig2 in the yeast cell is as a redundant transcriptional repressor.

It is also tempting to speculate that Mig2 could function as a structural protein which, by interacting with other factors associated with the *SUC2* promoter, could stabilize the repressor complex structure. To address this possibility, we examined the interaction of Mig2 with Mig1, Hxk2, and Snf1 by performing an immunoprecipitation assays. The strain used in all the cases was wild-type FMY501 expressing a functional Mig2 tagged with GFP at its C-terminal region. Cells were grown overnight at high glucose conditions, and part of the culture was shifted to low glucose containing media.

In order to study the interaction of Mig2 and Mig1, the strain FMY501 was transformed with the plasmid pWS93-Mig1, which expresses a functional Mig1 protein tagged with the HA epitope. Protein extracts were immunoprecipitated with an anti-HA antibody, and the protein was detected by western-blot using an anti-GFP antibody.

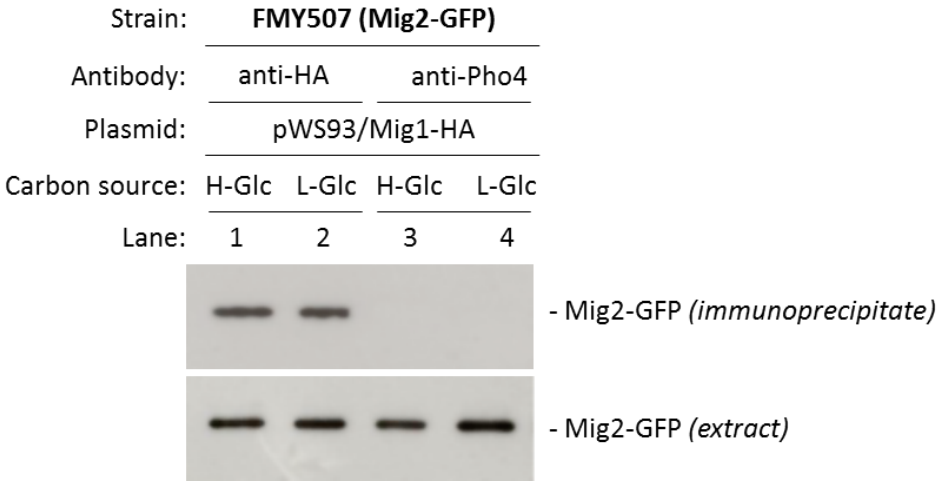


Figure 14. ***Interaction of Mig1 with Mig2.*** *In vivo* co-immunoprecipitation of Mig2-GFP with Mig1-HA. The FMY507 strain, expressing a Mig2 GFP-tagged fusion protein, was transformed with plasmids pWS93/Mig1 which encode an HA-tagged Mig1. Cell extracts were immunoprecipitated with a monoclonal anti-HA (lanes 1 and 2), and a polyclonal antibody to Pho4 (lanes 3 and 4). Immunoprecipitates were separated by 12% SDS-PAGE, and the level of immunoprecipitated Mig2-GFP in the blotted samples was determined by using anti-GFP antibody. The level of Mig2-GFP present in the different extracts was determined by western blot using anti-GFP antibody.

The results show that there is an *in vivo* interaction of Mig1 and Mig2 under both conditions: glucose-starved cells, and high glucose grown cells (Figure 14).

To study Mig2 interaction with Hxk2, we used protein extracts of the FMY501 (Mig2-GFP) wild-type strain. These extracts were

immunoprecipitated using a specific anti-Hxk2 antibody, and the levels of the retained Mig2 were detected in the blotted samples using a GFP antibody.

The result, shown in Figure 15, indicates that there is also an interaction *in vivo* conditions, between Hxk2 and Mig2, and this interaction is independent of the carbon source present in the media.

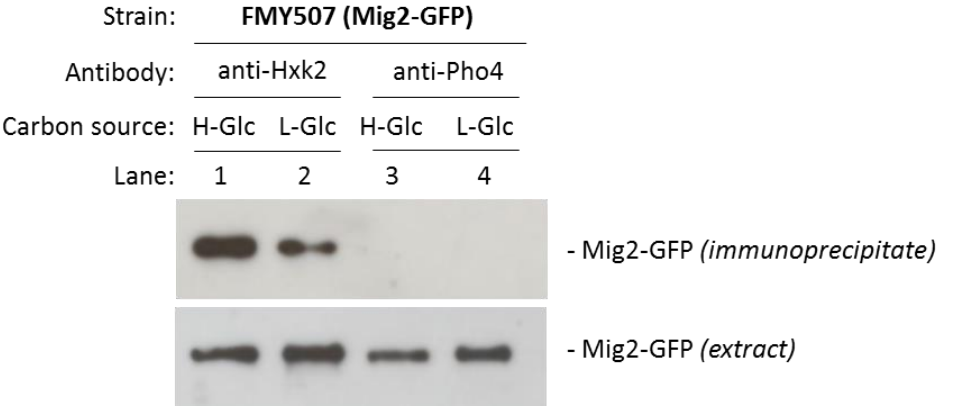


Figure 15. ***Interaction of Hxk2 with Mig2.*** In vivo co-immunoprecipitation of Mig2-GFP with Hxk2, using the FMY507 strain which express a Mig2 GFP-tagged fusion protein. Cell extracts were immunoprecipitated with a specific anti-Hxk2 antibody (lanes 1 and 2), and a polyclonal antibody to Pho4 (lanes 3 and 4). Immunoprecipitates were separated by 12% SDS-PAGE, and the level of immunoprecipitated Mig2-GFP in the blotted samples was determined by using anti-GFP antibody. The level of Mig2-GFP present in the different extracts was determined by western blot using anti-GFP antibody.

The interaction between Mig2 and Snf1 was also studied. For this aim, FMY501 wild-type was transformed with the plasmid pWS93/Snf1, which encodes for a functional HA-tagged Snf1. Protein extracts were immunoprecipitated using an anti-HA antibody, and the level of Mig2-

GFP in the different samples was determined by western-blot using a GFP antibody.

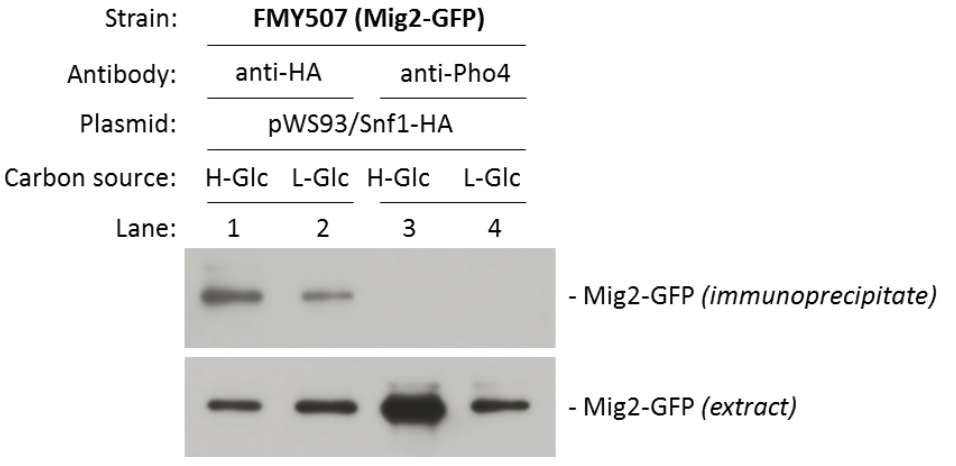


Figure 16. . ***Interaction of Snf1 with Mig2.*** In vivo co-immunoprecipitation of Mig2-GFP with Snf1-HA. The FMY507 strain, expressing a Mig2 GFP-tagged fusion protein, was transformed with plasmids pWS93/Snf1 which encode an HA-tagged Snf1. Cell extracts were immunoprecipitated with a monoclonal anti-HA (lanes 1 and 2), and a polyclonal antibody to Pho4 (lanes 3 and 4). Immunoprecipitates were separated by 12% SDS-PAGE, and the level of immunoprecipitated Mig2-GFP in the blotted samples was determined by using anti-GFP antibody. The level of Mig2-GFP present in the different extracts was determined by western blot using anti-GFP antibody.

In (Figure 16), it is shown that Mig2 also interacts with Snf1 in high and low glucose growth conditions.

Taken all together, we can conclude that Mig2 associates with Mig1, Hxk2 and Snf1 *in vivo* and that the association is independent of the glucose levels in the culture, from both glucose-starved cells and glucose-rich medium grown cells.

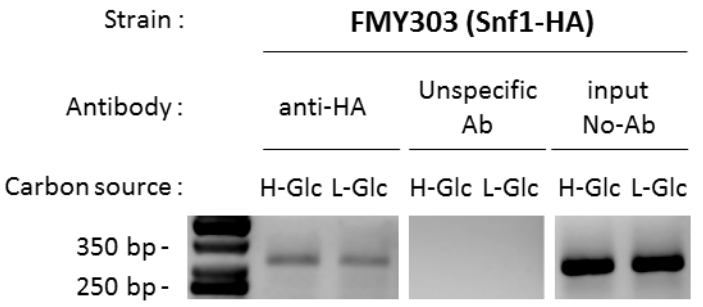
1.4. SNF1 in the repressor complex.

It was previously described, SNF1 complex plays an important role in glucose repression. As shown in Figure 5, in a *Δsnf1* and a *Δsnf4* mutant strains, *SUC2* gene is repressed under high and low glucose growing conditions. Moreover, Snf1 is constitutively associated with Hxk2, and Hxk2 accumulates in the nucleus upon *SNF1* gene disruption (Fernandez-Garcia et al., 2012). All These facts, led us to consider a model in which the main regulatory proteins of the phosphorylation state of Mig1 and Hxk2, the kinase Snf1 and the phosphatase Glc7-Reg1, act by stable association with SUC2-URS on the *SUC2* promoter. To test this model ChIP analysis were perform with Snf1, Snf4, Gal83 and Reg1 proteins.

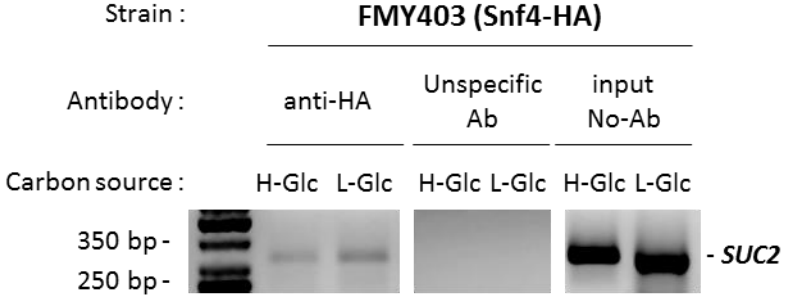
Firstly, we investigated if the SNF1 complex proteins interact with SUC2-URS of the *SUC2* promoter. To study its union with the *SUC2* promoter, three wild-type cells expressing a functional HA-tagged form of the proteins Snf1, Snf4 and Gal83, were generated. These strains were used to perform ChIP experiments, with samples of glucose-starved cells and glucose-rich growing cells, that were immunoprecipitated with an anti-HA antibody.

FMY303	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 SNF1-HA*
FMY403	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 SNF4-HA*
FMY833	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 GAL83-HA*

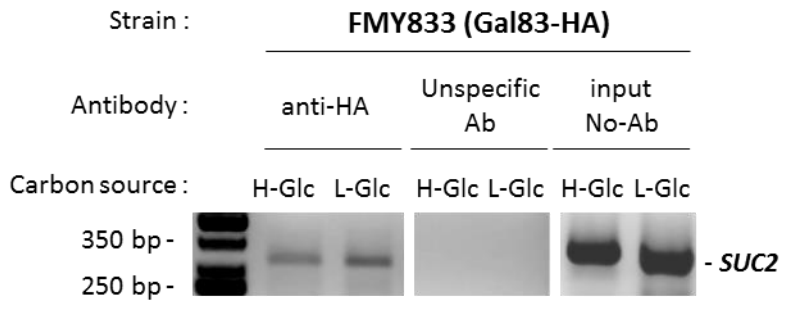
A


Strain :
FMY303 (Snf1-HA)
Antibody :
anti-HA
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -

B


Strain :
FMY403 (Snf4-HA)
Antibody :
anti-HA
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2

C


Strain :
FMY833 (Gal83-HA)
Antibody :
anti-HA
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2

D

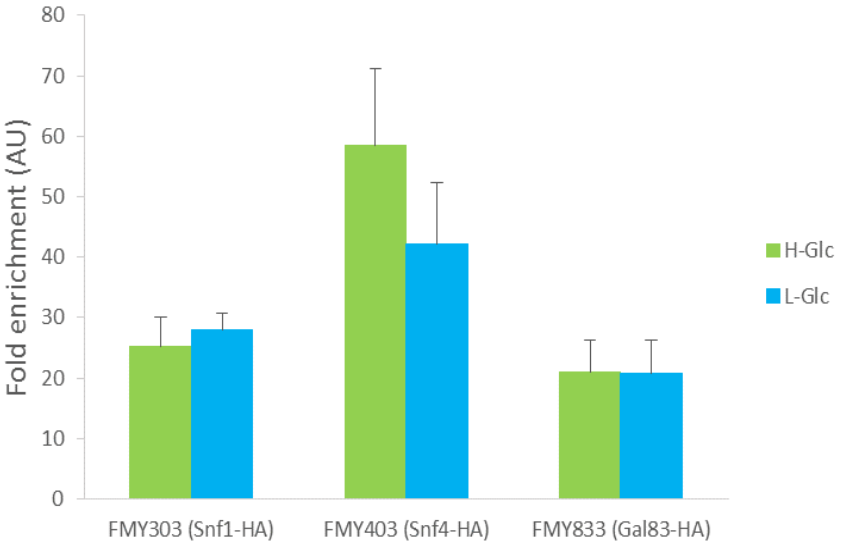


Figure 17. ***Association of Snf1-HA, Snf4-HA and Gal83-HA with the SUC2 promoter.*** Association of Snf1, Snf4 and Gal83 with the *SUC2* promoter as measured by ChIP. The strains FMY303 (**A**), FMY403 (**B**) and FMY833 (**C**) express a functional HA-tagged Snf1, Snf4 and Gal83 protein, respectively. **A**, **B** and **C** represent the PCR analysis, in each image the first two lanes are the ChIP results, the immunoprecipitation with an anti-GFP antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **D** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

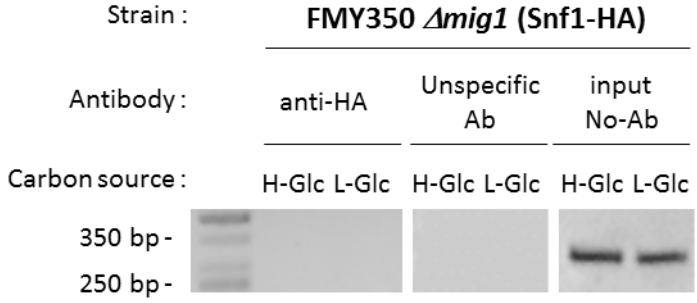
As shown in (Figure 17), the three subunits of SNF1 complex, Snf1, Snf4 and Gal83, bind specifically to the SUC2 elements of the *SUC2* promoter, in both high and low glucose conditions. Comparing the recruitment of the different subunits at high glucose conditions, we can observed that Snf4 recruitment (100%) was more pronounced than the recruitment observed in Snf1 (43%) and Gal83 (36%) (Figure 17D). In low glucose conditions, 72% of Snf4 remains at the *SUC2* promoter, while

Snf1 (48%) and Gal83 (35%) proteins are present in a similar amount to that found at high glucose conditions (Figure 17D).

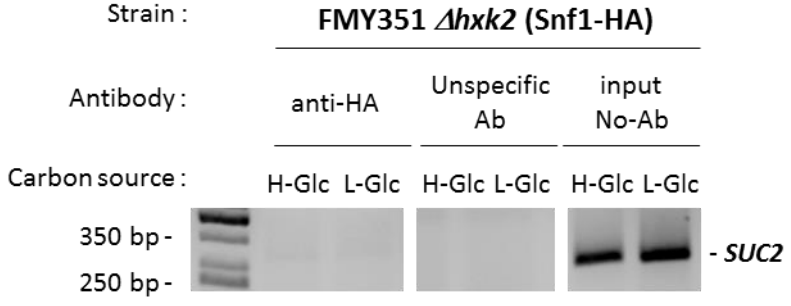
The following step was to determine if the association of Snf1 subunit with the *SUC2* promoter was Mig1 or Hxk2 dependent. For this aim, *SNF1* gene was HA-tagged at its chromosomal locus in a *Δmig1* and *Δhxk2* mutant cells, and these two strains were used to perform ChIP experiments under the same conditions.

FMY350	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig1::kanMX4 SNF1-HA*
FMY351	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 SNF1-HA*

A



B



C

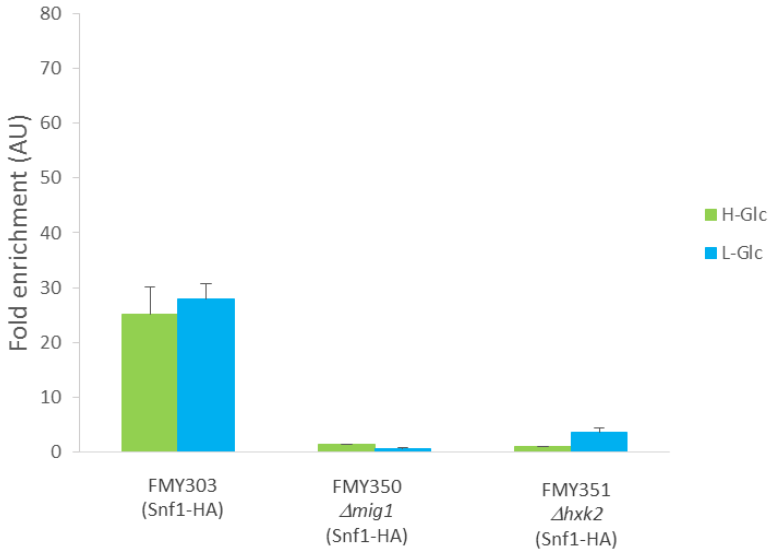


Figure 18. ***Association of Snf1-HA with the SUC2 promoter in Mig1 and Hxk2 absence.*** Association of Snf1 with the *SUC2* promoter in the *Δmig1* and *Δhxk2* mutant strains as measured by ChIP. The strains FMY350 (*Δmig1*)(**A**) and FMY351 (*Δhxk2*)(**B**) express a functional HA-tagged Snf1. **A** and **B** represent the PCR analysis of the strains FMY350 and FMY351, respectively, in each image the first two lanes are the ChIP results, the immunoprecipitation with an anti-GFP antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **C** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). qPCR data of Snf1 binding to the SUC2 promoter in the *Δmig1* and *Δhxk2* mutant strains is compared with Snf1 union in a wild-type. Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

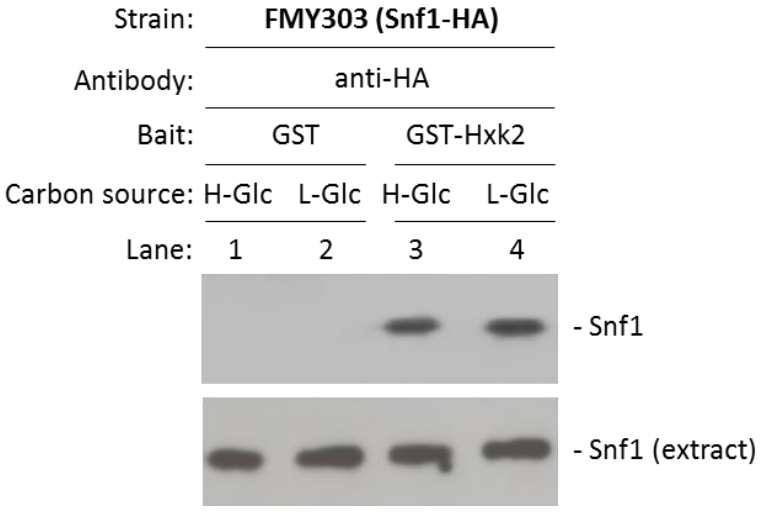
As shown in (Figure 18), in the absence of the proteins Mig1 or Hxk2, Snf1 is not detected in association with the *SUC2* promoter. In this two mutant strains, in either low or high glucose conditions, the amount of Snf1 bound to SUC2 elements of the *SUC2* promoter, was less than 5% in comparison with Snf1 union in a wild-type strain (Figure 18C).

As seen before, in (Figure 12), Hxk2 does not interact directly with *SUC2* gene, its association with the *SUC2* promoter is mediated by Mig1. Snf1 recruitment to the *SUC2* promoter is affected by the absence of

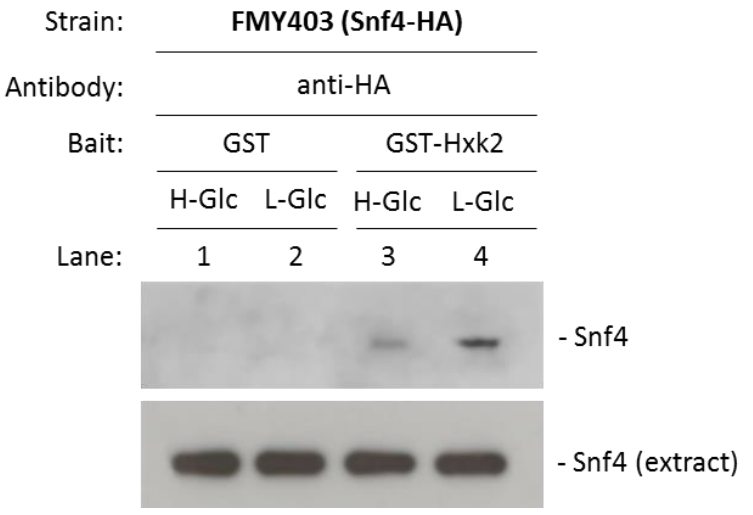
both Mig1 and Hxk2 proteins, so these results suggest that Snf1 association with the *SUC2* promoter is mediated by Hxk2.

To analyse the specific requirements for incorporation of Snf1, Snf4 and Gal83 to the repressor complex at the *SUC2* promoter, we conducted GST pull-down experiments to characterize physical interactions of these proteins with Hxk2. Protein extracts from the three wild-type strains expressing Snf1-HA, Snf4-HA and Gal83-HA, respectively, were used to perform these experiments. GST-Hxk2 expressed in bacteria was used as a bait.

A



B



C

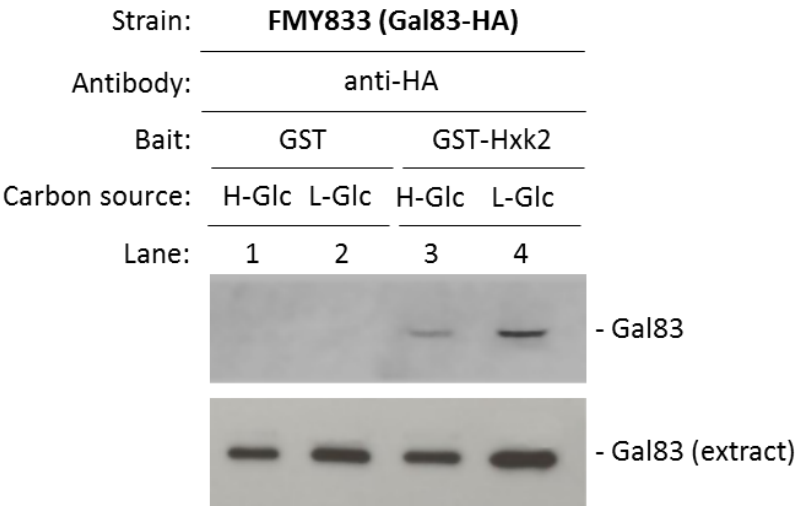


Figure 19. ***GST pull-down assays of the interaction of Snf1, Snf4 and Gal83 with Hxk2*****.** A GST-Hxk2 fusion protein was purified on glutathione-sepharose columns. Equal amounts of GST-Hxk2 were incubated with cell extracts from FMY303, FMY403 and FMY833 strains expressing Snf1-HA (**A**), Snf4-HA (**B**) and Gal83-HA (**C**) fusion proteins, respectively. Lanes 3 and 4 represent the interaction of Snf1 (**A**), Snf4 (**B**) and Gal83 (**C**) with Hxk2. For the control samples, GST protein was also incubated with the H-Glc and L-Glc cell extracts but no signals were detected (lanes 1 and 2). The level of Snf1, Snf4 and Gal83 present in the different extracts used were determined by western blot using a monoclonal anti-HA antibody. The western blots shown are representative of results obtained from three independent experiments.

The results show that there is a strong interaction between Snf1 and Hxk2 in high and low glucose grown cells. Snf4 and Gal83 also interact with Hxk2, however, the interaction is reduced if it is compared with the Snf1 retained by GST-Hxk2. In every sample the amount of each protein: Snf1, Snf4 and Gal83 was checked by western-blot analysis, confirming that the amount of each protein was the same in every sample. Considering that the amount of protein is constant in every sample, and that the interaction of Snf4 and Gal83 is greatly reduced in comparison with Snf1, we believe that these results suggest that the interaction is taking place at the level of Snf1 subunit, so Snf4 and Gal83 interaction with Hxk2 could be mediated by Snf1 subunit. In order to test this hypothesis, we carry out a GST pull-down assay in a *Δsnf1* mutant strain expressing a functional HA-tagged form of the Snf4 protein at its chromosomal locus.

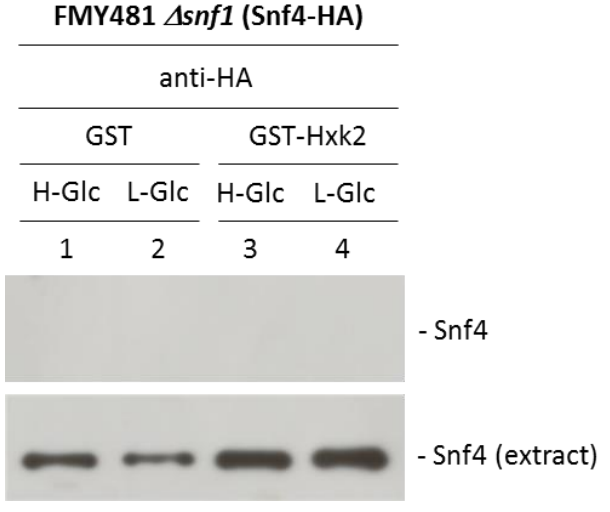


Figure 20. ***GST pull-down assays of the interaction of Snf4 with Hxk2 in Snf1 absence*****.** A GST-Hxk2 fusion protein was purified on glutathione-sepharose columns. Equal amounts of GST-Hxk2 were incubated with cell extracts from FMY481 strain, which

express Snf4-HA in the absence of Snf1. Lanes 3 and 4 represent the interaction between Snf4 and Hxk2. For the control samples, GST protein was also incubated with the H-Glc and L-Glc cell extracts but no signals were detected (lanes 1 and 2). The level of Snf4 present in the different extracts used were determined by western blot using a monoclonal anti-HA antibody. The western blots shown are representative of results obtained from three independent experiments.

In Figure 20 we can observe that Snf4 was not detected in complex with Hxk2 in either high or low glucose-grown cells when Snf1 is absent. From this result, we can conclude that Snf4 association with Hxk2 is Snf1-dependent.

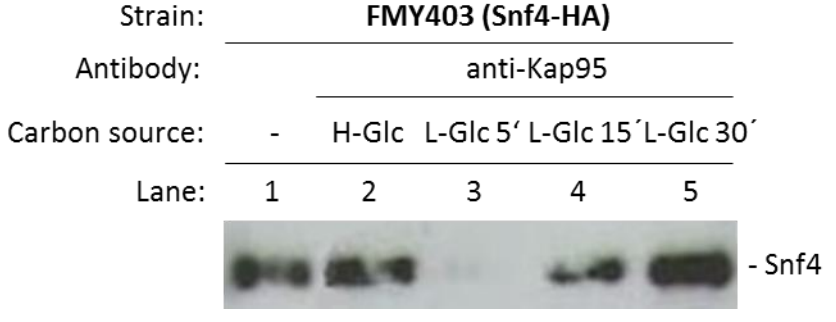
1.4.1. Nucleocytoplasmic transport of the SNF1 complex subunits.

One of the mechanism of regulation of the repression pathway is the subcellular localization of the different elements. It has been described that in high glucose conditions Snf1 and the three beta subunits are cytoplasmic. When glucose becomes limiting, Gal83 relocalizes to the nucleus, and it also directs Snf1 localization. Snf4 is present in excess in both compartments, nucleus and cytoplasm, regardless the nutritional status. Nevertheless, since our results demonstrate that Snf1, Snf4 and Gal83 are part of the repressor complex formed in the *SUC2* promoter under high and low glucose conditions, we decided to study the interaction of the SNF1 complex subunits with the nuclear import and export systems implied in the Hxk2 nucleocytoplasmic shuttling.

Identification of the import system used by Snf4

First, we try to identify the proteins involved in the nuclear transport of Snf4, so we study if there is an interaction with the karyopherins of the classic import system: Kap95 and Kap60. We performed a co-immunoprecipitation of proteins using the wild type strain FMY403 expressing a functional HA-tagged Snf4. Cells were grown overnight in a high glucose medium, and after they reach 0.8 OD_{600}, half of the culture was washed and shifted to a low glucose medium. We realized a time course assay taking a sample from the high glucose growing cells and three samples of the low glucose medium shift at 5, 15 and 30 minutes. Protein extracts of these samples were immnunoprecipitated using an anti-Kap95 and anti-Kap60 antibodies. After incubation with protein-A-sepharose, a western-blot was done and Snf4-HA retained was detected with a specific HA antibody.

A



B

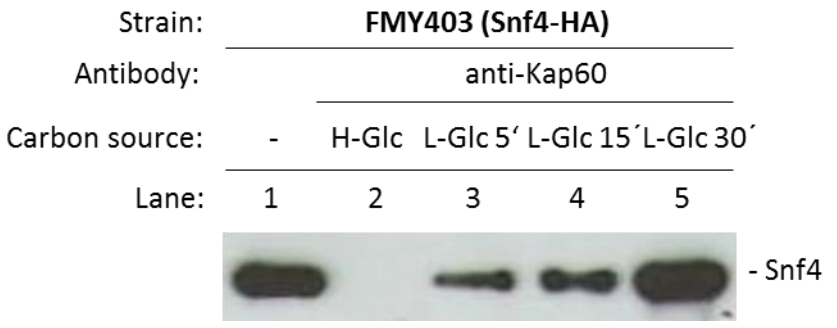


Figure 21. ***Co-immunoprecipitation assay of the interaction of Snf4 with the importins Kap95 and Kap60*****.** Protein extracts of the FMY403 strain which express a functional Snf4-HA, were immnunoprecipitated using an anti-Kap95 (**A**) and an anti-Kap60 (**B**) specific antibodies. Lanes 2-5 represent the interaction between Snf4 and Kap95 (**A**) and Kap60 (**B**) in a time course of cells growing in glucose (lane 2) and in a shift to low glucose media with samples taken at 5 (lane 3), 15 (lane 4) and 30 (lane 5)minutes. The level of Snf4 present in the different extracts used were determined by western blot using a monoclonal anti-HA antibody. The western blots shown are representative of results obtained from three independent experiments.

As observed in (Figure 21), there is a physical association between Snf4 and the two karyopherins, Kap95 and Kap60. We can observed that during the growth in high glucose conditions, Snf4 interacts with Kap95. This interaction disappear at 5 minutes after the shift to low glucose media and then is increasing after 15 and 30 minutes in low glucose conditions (Figure 21A). With regard to Kap60, we can observe that there is an interaction with Snf4 under high and low glucose conditions, but this interaction becomes stronger after 15 and 30 minutes in this media (Figure 21B). These results suggest that Snf4 uses the Kap95/Kap60 import pathway to entry in the nucleus, and according to what is described it is transported into the nucleus in high and low glucose conditions.

As described in the introduction, Kap60 is the mediator protein in charge of cargo recognition, and the dimer formed by Kap60 and the cargo, is able to associate with Kap95 forming an import complex which crosses the nuclear envelope. What we did next was to study if the absence of one these karyopherins, Kap95 or Kap60, affect the interaction of Snf4 with Kap60 or Kap95, respectively. The mutation of these genes is lethal for the yeast cells, so we used temperature-sensitive mutant strains (ts), JCY1410 (*kap60^{ts})* and JCY1407 (*Kap95^{ts}*). These strains are able to synthetize a functional importin during its growth at 25^{o}C (permissive temperature), but non-functional at 37^{o}C (non-permissive temperature).

JCY1407	*MATa; ade2-1; ura3-1; his3-11,15;trp1-1; leu2-3,112; can1-100; kap95::HIS3.*
JCY1410	*MATa; ade2-1; ura3-1; his3-11,15; trp^63; leu2-3,112; can1-100; srp1-31.*

We transformed the JCY1410 (*kap60^{ts})* and JCY1407 (*Kap95^{ts}*) strains with the plasmid pJJH1599/Snf4-GFP, which express a functional GFP-tagged Snf4. Cells were grown in a high glucose and low glucose medium at the permissive temperature, and part of the culture of both conditions were incubated during 90 minutes at 37^{o}C. Protein extracts were immunoprecipitated with anti-Kap95 and anti-Kap60 antibodies, and retained Snf4 was detected using an anti-GFP antibody.

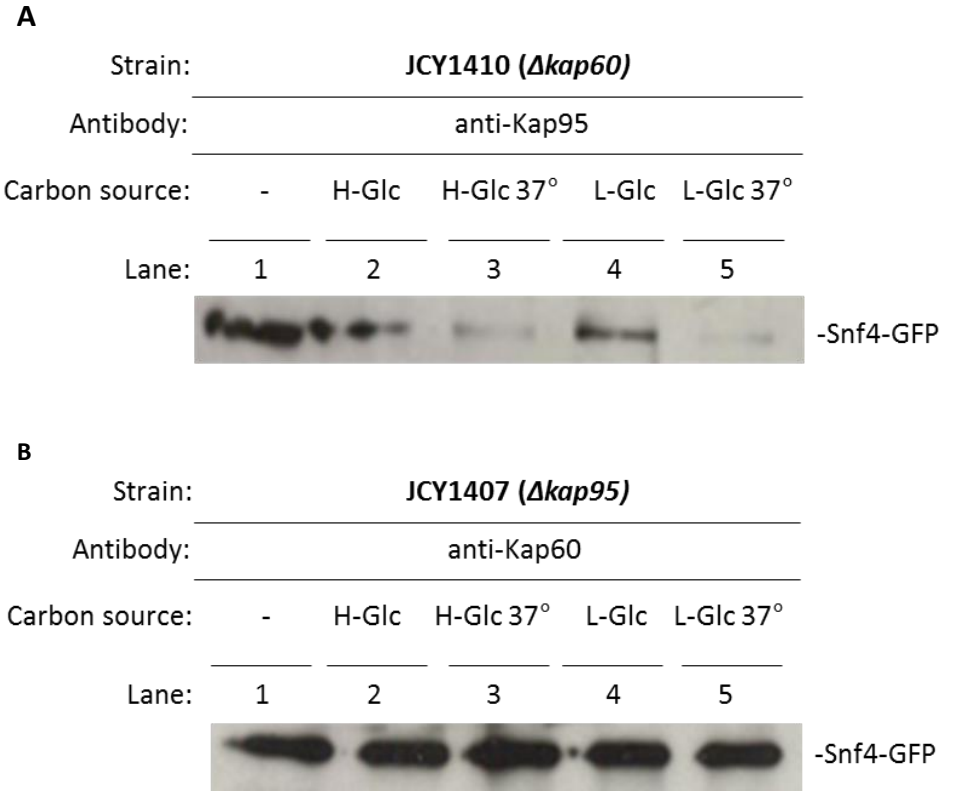


Figure 22. ***Co-immunoprecipitation assay of Kap95 and Kap60 interaction with Snf4 in the absence of Kap60 and Kap95, respectively.*** JCY1410 (kap60^{ts}) and JCY1407 (Kap95^{ts}) mutant strains were transformed with the plasmid pJJH1599/Snf4-GFP. **A** Interaction of Snf4 with Kap95 in high and low glucose conditions at 25°C (permissive temperature) (lanes 2 and 4), and at non-permissive temperature, 37°C (Kap60 is non-functional at 37°C) (lanes 3 and 5). **B** Interaction of Snf4 with Kap60 in high and low glucose conditions at 25°C (permissive temperature) (lanes 2 and 4), and at non-permissive temperature, 37°C (Kap95 is non-functional) (lanes 3 and 5).

In Figure 22A, we can see that during non-permissive conditions, in the lack of a functional Kap60, the interaction of Snf4 and Kap95 is almost abolished in both high and low glucose medium. However, the absence of Kap95 does not seem to affect Snf4-Kap60 interaction (Figure 22B). These results suggest that Kap60 is important to recognize Snf4, and when this mediator protein is not present, Kap95 is not able to interact

with Snf4, impeding the formation of the import complex and therefore Snf4 is not transported into the nucleus.

In vitro reconstruction of the Snf4 nuclear import complex

As explained in the introduction, the classic import pathway involves the formation of a trimeric complex: cargo, the adaptor protein Kap60 and Kap95, which is transported from the cytoplasm into the nucleus. The directionality of the nucleocytoplasmic transport is determined by the asymmetrically distributed Gsp1-GDP and Gsp1-GTP. In the cytoplasm the majority of Gsp1 is bound to GDP, and in the nucleus is bound to GTP. Gsp1GDP and Gsp1GTP levels are controlled by the presence of a RanGAP in the nucleus, and a RanGEF in the nucleus. Gsp1-GDP favors the assembly of the import complex, and Gsp1-GTP triggers the import complex dissociation, releasing the cargo in the nucleus.

To confirm that the transport directionality is regulate by the levels of Gsp1GDP/Gsp1GTP, we tried to reconstruct Snf4 import complex *in vitro* to study if the affinity of Snf4 and the two karyopherins was affected by the levels of Gsp1GDP/Gsp1GTP

To perform these experiments we purified the proteins: Kap95, Kap60 and Gsp1 in bacteria. Gsp1 was charged *in vitro* with GDP and GTP. As a bait GST-Snf4 was used and it was incubated with different combinations of the other factors.

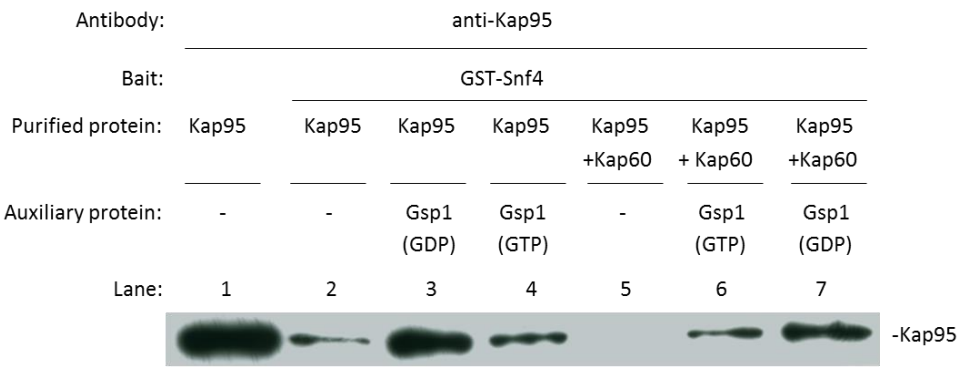


Figure 23. ***In vitro reconstruction of the import system.*** Interaction of Snf4 with purified Kap95 (lane 2), in the presence of Gsp1GDP (lane 3) and Gsp1GTP (lane 4). There is also shown, the interaction between Snf4 and Kap95 in the presence of purified Kap60 (lane 5), and when Gsp1GTP (lane 6) or Gsp1GDP (lane7) are present.

As is it shown in Figure 23, the affinity between Snf4 and Kap95 is increased in the presence of Gsp1GDP, and decreased in the presence of Gsp1GTP. This result suggest that the interaction of Snf4 with Kap95 is regulated by Gsp1GDP/Gsp1GTP. Thus, when Gsp1 is in Gsp1GDP form, the affinity of Snf4 with Kap95 is higher, so its interaction is favoured, as happens in the cytoplasm. However, in the presence of Gsp1GTP the affinity is reduced and the complex dissociation arises, as it happens in the nucleus.

Identification of the export system used by Snf4

We also study if Snf4 uses the same export pathway as Hxk2, the export pathway mediated by the exportin Xpo1.

A GST pull-down was performed using as a bait GST-Xpo1 produced in bacteria. Cells were grown in high glucose overnight and when the cells reached 0.8 OD_{600}, part of the culture was shifted to a low glucose medium. We made a time course assay taking a sample from the high glucose grown cells and three samples from the low glucose containing-medium shift at 5, 15 and 30 minutes. Protein extracts of these samples were incubated with GST-Xpo1, and after western-blot, Snf4 was detected using an anti-HA monoclonal antibody.

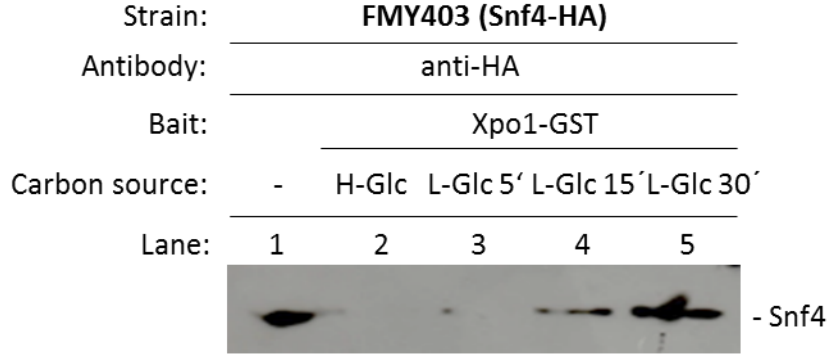


Figure 24. ***GST pull-down assay of the interaction of Snf4 with Xpo1*****.** A GST-Xpo1 fusion protein was purified on glutathione-sepharose columns. Equal amounts of GST-Xpo1 were incubated with cell extracts from FMY403 strain, which express a functional Snf4-HA. Lane 2 represents the interaction at high glucose conditions and from lane 3 to 5, interaction in low glucose-containing medium at the times of 5, 15 and 30 minutes, respectively.

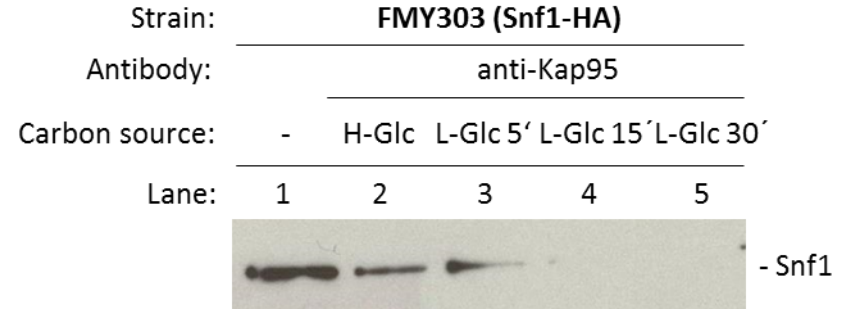
Physical association of Snf4 with the exportin Xpo1 was detected after 15 minutes in a low glucose media, suggesting that Snf4 uses the Xpo1 pathway to exit the nucleus, but this only occurs in low glucose conditions.

Identification of the import system used by Snf1

Although Snf1 subunit was described to have only a nuclear localization under low glucose conditions, our results indicate that it is also present in the nucleus during the growth in high glucose media. This led us to study if Snf1 is imported into the nucleus by the same import system as Hxk2.

We perform a co-immunoprecipitation using protein extracts from the FMY303 strain, which express a functional Snf1 tagged with HA at its C-terminal region. A time course was done, taking samples of high glucose-growing cells, and at 5, 15 and 30 minutes after a shift to low glucose conditions. These samples were immunoprecipitated with an anti-Kap95 and an anti-Kap60 antibodies, and the amount of Snf1 retained was detected using a monoclonal HA antibody.

A



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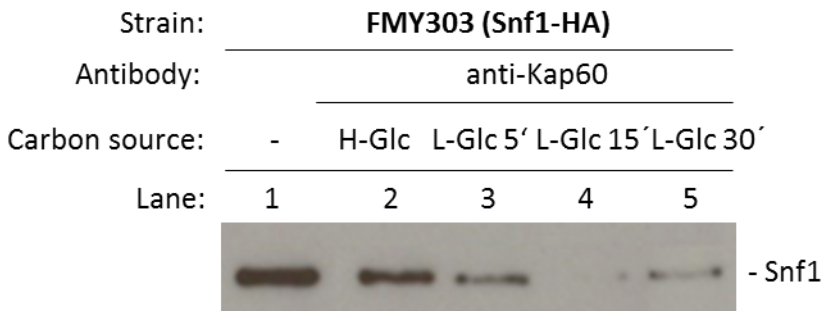


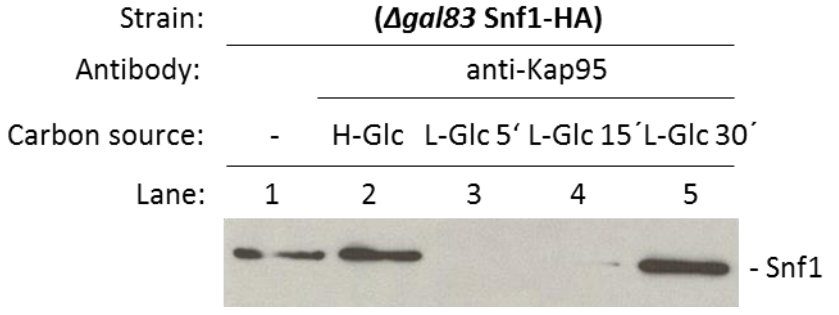
Figure 25. ***Co-immunoprecipitation assay of the interaction of Snf1 with the importins Kap95 and Kap60.*** Protein extracts of the FMY303 strain which express a functional Snf1-HA, were immnunoprecipitated using an anti-Kap95 (**A**) and an anti-Kap60 (**B**) specific antibodies. Lanes 2-5 represent the interaction between Snf1 and Kap95 (**A**) and Kap60 (**B**) in a time course of cells growing in glucose (lane 2) and in a shift to low glucose media with samples taken at 5 (lane 3), 15 (lane 4) and 30 (lane 5)minutes. The level of Snf1 present in the different extracts used were determined by western blot using a monoclonal anti-HA antibody. The western blots shown are representative of results obtained from three independent experiments.

In Figure 25, we can observe that Snf1 interacts with the two karyopherins of the classic import system. Kap95 and Kap60 association with Snf1 is mainly detected during the growth in high glucose conditions, and the first minutes in low glucose medium. In agreement with the ChIP results, where Snf1 was detected in association with the *SUC2* promoter under high and low glucose conditions, these results show how Snf1 interacts with the import machinery in both glucose conditions, indicating that Snf1 is transported into the nucleus not only in low glucose conditions but also in high glucose conditions.

It was also described that Snf1 subcellular localization is mediated by Gal83 subunit. Thus, when glucose becomes limiting, Gal83 directs Snf1 to the nucleus (Vincent et al., 2001). Since we observe that Snf1 is able to interact with the import machinery in high glucose conditions, we

wonder if this association was Gal83-dependent. To test it, we generate a *Δgal83* mutant strain, which express a functional HA-tagged Snf1. Using this strain, we performed a co-immunoprecipitation assay, in the same conditions as done before in the wild-type strain.

A



B

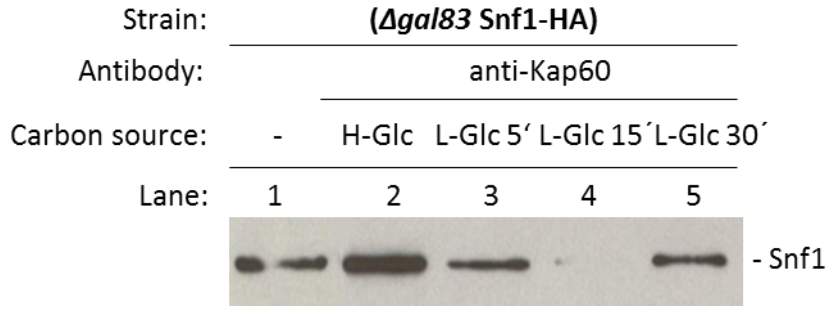


Figure 26. ***Co-immunoprecipitation assay of the interaction of Snf1 with the importins Kap95 and Kap60 in the absence of Gal83.*** Protein extracts of the FMY353 strain which express a functional Snf1-HA, were immnunoprecipitated using an anti-Kap95 (**A**) and an anti-Kap60 (**B**) specific antibodies. Lanes 2-5 represent the interaction between Snf1 and Kap95 (**A**) and Kap60 (**B**) in a time course of cells growing in glucose (lane 2) and in a shift to low glucose media with samples taken at 5 (lane 3), 15 (lane 4) and 30 (lane 5)minutes. The level of Snf1 present in the different extracts used were determined by western blot using a monoclonal anti-HA antibody. The western blots shown are representative of results obtained from three independent experiments.

This results demonstrate that Snf1 interaction with the import machinery is not Gal83-dependent (Figure 26), suggesting that Snf1 can be transported alone into the nucleus.

In vitro reconstruction of the Snf1 nuclear import complex

In order to study if Snf1 nuclear import directionality is regulated by the levels of Gsp1GDP/Gsp1GTP, we analyse the *in vitro* interaction of Snf1 with the importins Kap95 and Kap60. Importins Kap95, Kap60, Gsp1 and the bait GST-Snf1 were produced and purified in bacteria. After purification, Gsp1 was charged with GDP and GTP.

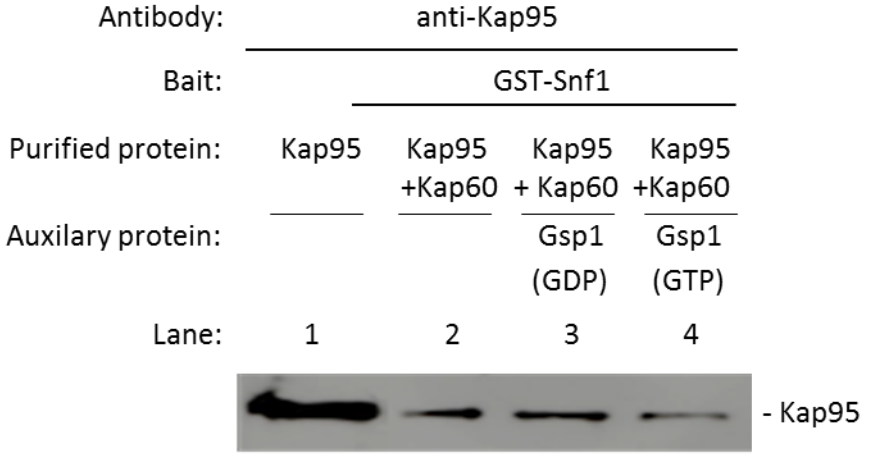


Figure 27. ***In vitro reconstruction of the import system.*** Interaction of Snf1 with purified Kap95 in the presence of purified Kap60 (lane 2), and when Gsp1GTP (lane 6) or Gsp1GDP (lane7) are present.

As observed in Figure 27, Snf1 interaction with Kap95 is regulated Gsp1GDP/Gsp1GTP. In the presence of Gsp1GDP the ternary complex Snf1-Kap60-Kap95 is stable, as it happens in the cytoplasm. However in the presence of Gsp1GTP, the interaction is decreased, as it happens in the nucleus, suggesting that the presence of Gsp1GTP produces the nuclear import complex dissociation.

Identification of the export system used by Snf1

The interaction of Snf1 with the export system mediated by Xpo1 was also studied. For this aim, we performed a GST pull-down assay using as a bait Xpo1-GST. Cells of the FMY303 (Snf1-HA) strain, were growth under high glucose conditions, and after the cells reached the 0.8 OD_{600}, part of the culture was shifted to a low glucose-containing media. Protein extracts correspond to samples of glucose media and three samples at different times after the shift to the low glucose media (5, 15 and 30 minutes). Samples were incubated with GST-Xpo1, and after western-blot the retained Snf1 was detected using a specific anti-HA antibody.

As shown in Figure 28, Snf1 also interacts with the exportin Xpo1, but this interaction is only detected under high glucose conditions. This result suggest that Snf1 uses the Xpo1 export pathway to exit the nucleus, and its export only occurs under low glucose conditions.

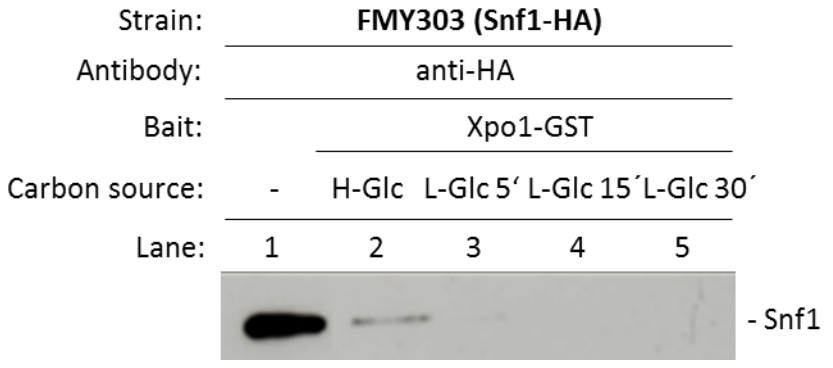


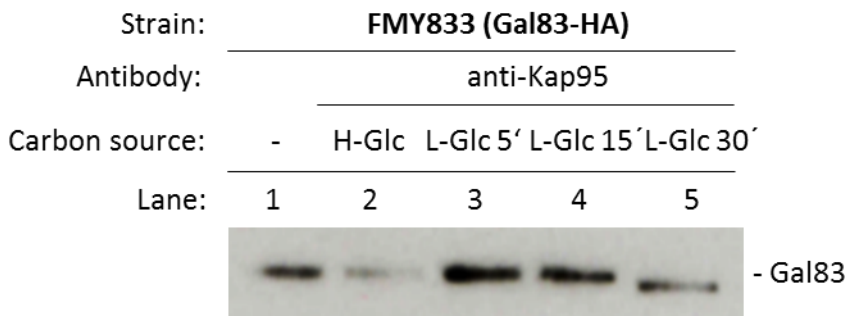
Figure 28*. GST pull-down assays of the interaction Snf1 with Xpo1*. A GST-Xpo1 fusion protein was purified on glutathione-sepharose columns. Equal amounts of GST-Xpo1 were incubated with cell extracts from FMY303 strain, which express a functional Snf1-HA. Lane 2 represents the interaction at high glucose conditions and from lane 3 to 5, interaction in low glucose-containing medium at the times of 5, 15 and 30 minutes, respectively.

Identification of the import system used by Gal83

Since it is described that Gal83 has a cytoplasmic distribution during the growth in glucose rich medium, and when glucose becomes limiting is relocated in the nucleus, we decided to study its interaction with the Kap95/Kap60 import pathway.

We used the strain FMY833 which express a functional HA-tagged Gal83 at its chromosomal locus, to perform a co-immunoprecipitation of proteins. Protein extracts from samples of high glucose and low glucose (at 5, 15 and 30 minutes) growing cells were immunoprecipitated with anti-Kap95 and anti-Kap60. Retained Gal83, was detected using a specific anti-HA antibody.

A



B

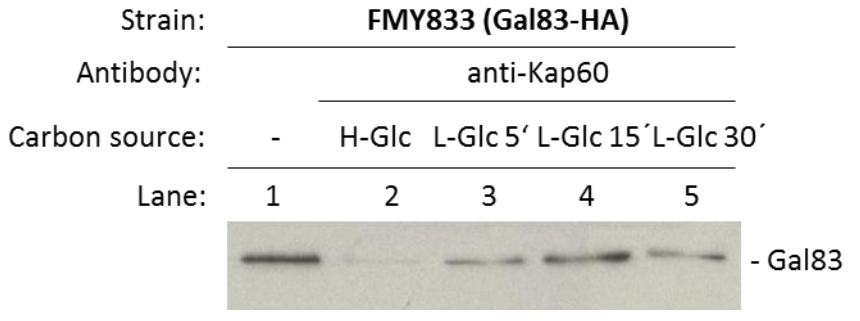


Figure 29. ***Co-immunoprecipitation assay of the interaction of Gal83 with the importins Kap95 and Kap60.*** Protein extracts of the FMY833 strain which express a functional Gal83-HA, were immnunoprecipitated using an anti-Kap95 (**A**) and an anti-Kap60 (**B**) specific antibodies. Lanes 2-5 represent the interaction between Gal83 and Kap95 (**A**) and Kap60 (**B**) in a time course of cells growing in glucose (lane 2) and in a shift to low glucose media with samples taken at 5 (lane 3), 15 (lane 4) and 30 (lane 5)minutes. The level of Gal83 present in the different extracts used were determined by western blot using a monoclonal anti-HA antibody. The western blots shown are representative of results obtained from three independent experiments.

Gal83 also interacts with the importins Kap95/Kap60 (Figure 29). In accordance with its nuclear localization, its interaction with the import system is higher in low glucose conditions. However, Gal83 also interacts with Kap95 and Kap60 in high glucose conditions, which supports the results obtained by ChIP in which we demonstrate that Gal83 associates with *SUC2* promoter constitutively.

Identification of the export system used by Gal83

It has been described that Gal83 contains a leucine-rich export signal (LR-NES) in its N-terminal domain (Hedbacker and Carlson, 2006). The karyopherin which is normally in charge of LR-NES- containing proteins transport, is the exportin Xpo1. To test is there is an interaction of Gal83 with this karyopherin, we perform a GST pull-down experiment in the same conditions as the previous Xpo1-GST pull- down assays.

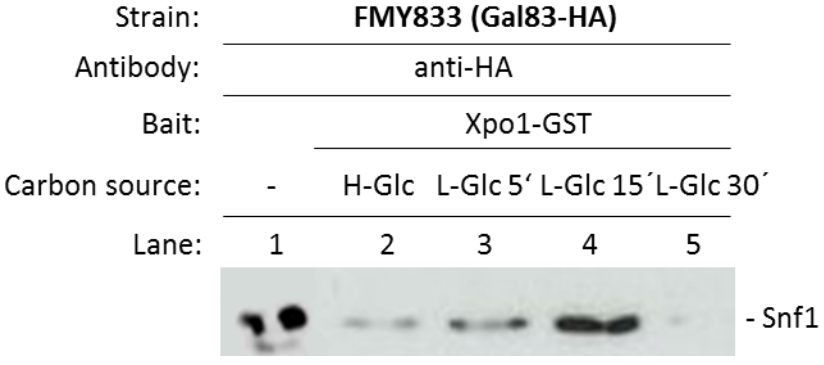


Figure 30*. GST pull-down assays of the interaction Snf1 with Xpo1.* A GST-Xpo1 fusion protein was purified on glutathione-sepharose columns. Equal amounts of GST-Xpo1 were incubated with cell extracts from FMY833 strain, which express a functional Gal83-HA. Lane 2 represents the interaction at high glucose conditions and from lane 3 to 5, interaction in low glucose-containing medium at the times of 5, 15 and 30 minutes, respectively.

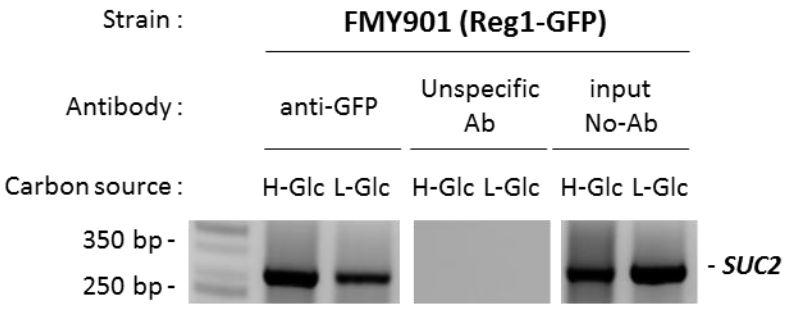
This result (Figure 30), demonstrate that Gal83 interacts with the exportin Xpo1 in both high and low glucose conditions, suggesting that the Gal83 nuclear export is through the Xpo1 pathway.

1.5. Reg1 in the repressor complex

As demonstrated before, the Snf1 kinase is constitutively associated with Hxk2, in both high and low glucose-growth conditions, and therefore is part of the repressor complex formed in the *SUC2* promoter. This led us to wonder if the other protein in charge of controlling Mig1 and Hxk2 phosphorylation state, the protein phosphatase Reg1-Glc7, was also taking part of the repressor complex. To test this hypothesis, we generate a wild-type strain which express a functional GFP-tagged Reg1 and we used to study the *in vivo* interaction of Reg1 with the SUC2 elements of the *SUC2* promoter.

FMY901	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 REG1-GFP*

A



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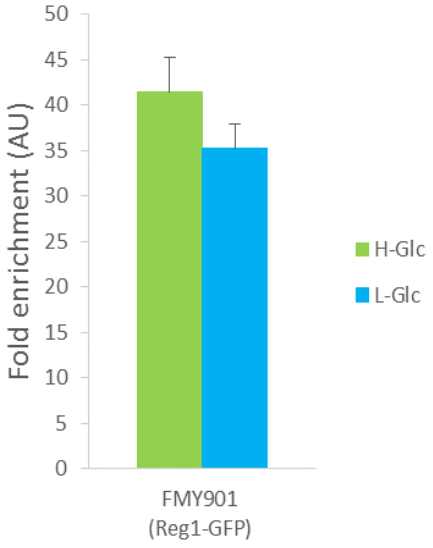


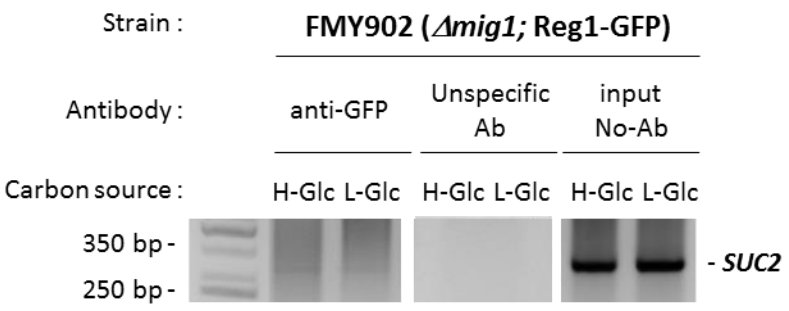
Figure 31. ***Association of Mig1 repressor with the SUC2 promoter*****.** Association of Reg1-GFP with the *SUC2* promoter measured by ChIP, using the strain FMY901 expressing a GFP-tagged Reg1 protein. **A** Represents the PCR analysis, the first two lanes are the ChIP result of the immunoprecipitation with anti-GFP antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **B** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

As observed in Figure 31, there is a strong association of Reg1 with the *SUC2* promoter, in both growth conditions, high and low glucose media.

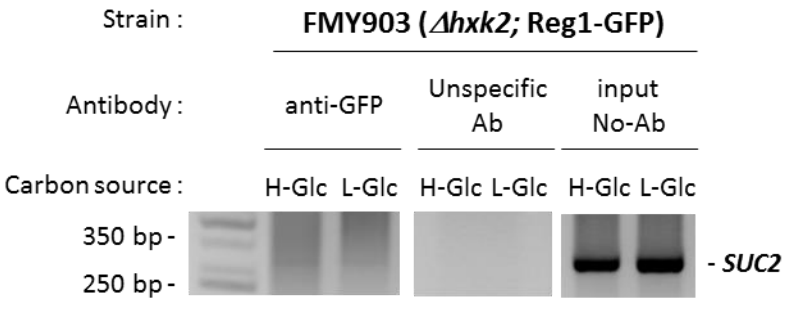
However, when we perform the same experiments in the strains *Δmig1* and *Δhxk2* expressing a functional Reg1-GFP, we observed that Reg1 union with the *SUC2* promoter is highly impaired (Figure 32). In the absence of Mig1 and Hxk2 less than 5% of Reg1-GFP remains associated with SUC2-URS (Figure 32C).

FMY902	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig1::kanMX4 REG1-GFP*
FMY903	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 REG1-GFP*

A



B



C

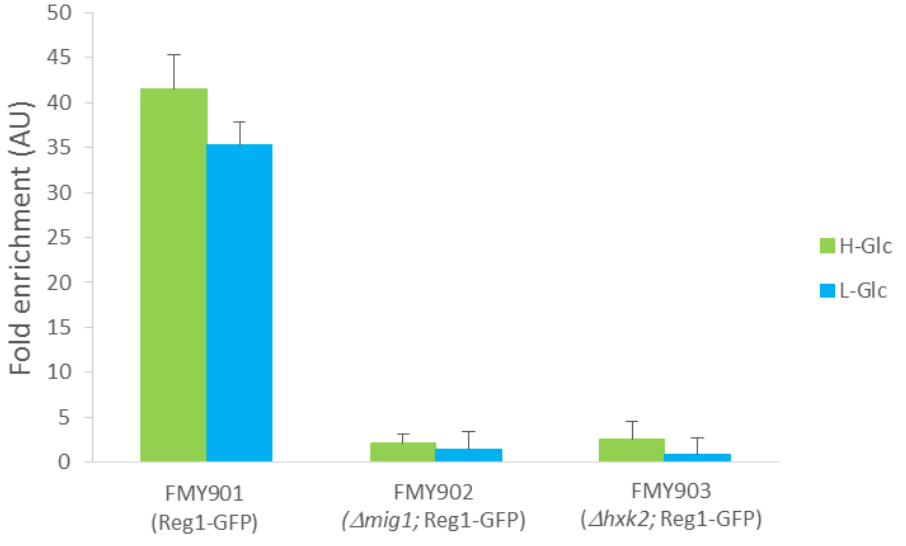


Figure 32. ***Association of Reg1-GFP with the SUC2 promoter in Mig1 and Hxk2 absence.*** Association of Reg1 with the *SUC2* promoter in the *Δmig1* and *Δhxk2* mutant strains as measured by ChIP. The strains FMY901 (*Δmig1*)(**A**) and FMY903 (*Δhxk2*)(**B**) express a functional HA-tagged Reg1. **A** and **B** represent the PCR analysis of the strains FMY902 and FMY903, respectively, in each image the first two lanes are the ChIP results, the immunoprecipitation with an anti-GFP antibody. The following two are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **D** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). qPCR data of Snf1 binding to the *SUC2* promoter in the *Δmig1* and *Δhxk2* mutant strains is compared with Reg1 union in a wild-type. Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

To confirm the association of Reg1 with the repressor complex, we also determined the ability of Reg1 to interact with Hxk2 *in vivo*. For this aim, a co-immunoprecipitation of proteins was done, using the wild-type expressing a functional GFP-tagged Reg1. Protein extracts from high and low glucose conditions were immunoprecipitated with an anti-GFP antibody, and the samples were subjected to wester-blot analysis.

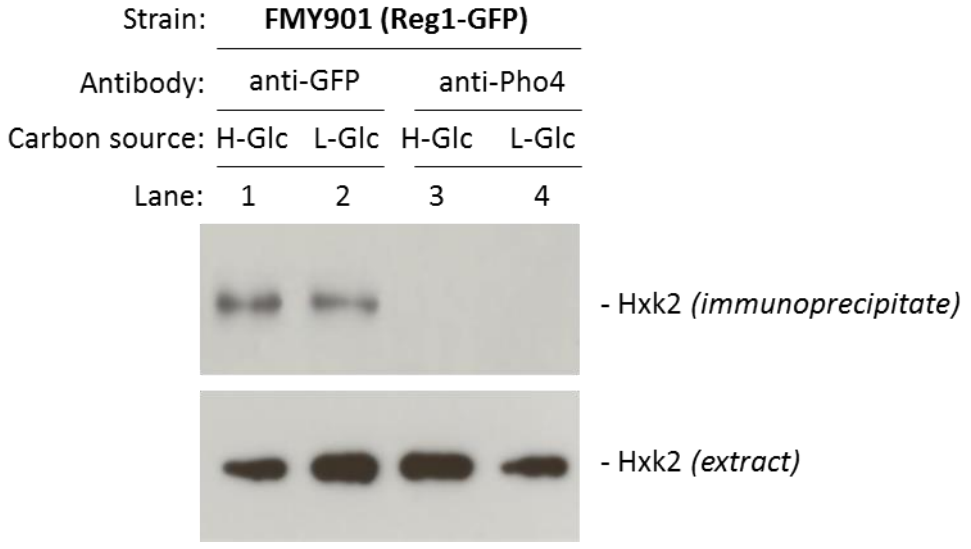


Figure 33. ***Co-immunoprecipitation assay of the interaction of Reg1 with Hxk2.*** Protein extracts of the FMY901 strain which express a functional Reg1-GFP, were immnunoprecipitated using an anti-GFP antibody (lanes 1 and 2), and a polyclonal

antibody to Pho4, as negative control (lanes 3 and 4). Immunoprecipitates were separated by 12% SDS-PAGE, and the level of immunoprecipitated Reg1-GFP in the blotted samples was determined by using anti-GFP antibody. The level of Reg1-GFP present in the different extracts was determined by western blot using anti-GFP antibody.

This experiment confirm (Figure 33) that there is an interaction between Reg1 and Hxk2 and this interaction is independent of the glucose levels in the medium.

Although Reg1 has been described to be predominantly cytoplasmic (Dombek et al., 1999), our results indicate that Reg1 has also a nuclear localization. This led us to examine its interaction with the nuclear import machinery (Kap95-Kap60) and export systems (Xpo1) used also by Hxk2 to enter and exit the nucleus.

To study Reg1 interaction with the karyopherins of the classic import system, Kap95 and Kap60, a co-immunoprecipitation assay was done. The wild-type strain FMY901 expressing Reg1-GFP was used, and protein extracts of high glucose and low glucose grown-cells were used and immunoprecipitated with antibodies anti-Kap95 and anti-Kap60. The resulting immnoprecipitates were assayed for the presence of Reg1-GFP by immunoblot analysis with anti-GFP antibodies.

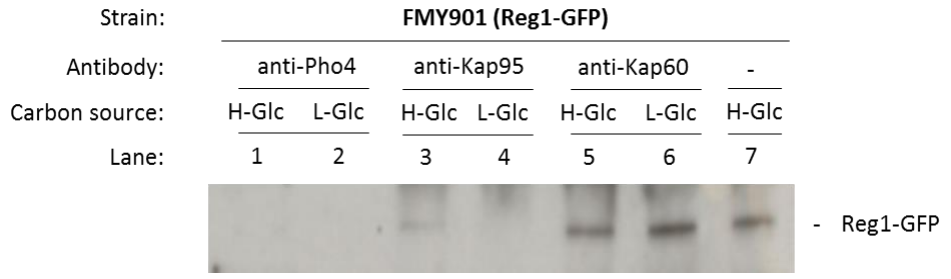


Figure 34. ***In vivo interaction of Reg1 with Kap60 and Kap95.*** Protein extracts of the FMY901 strain, expressing a Reg1-GFP fusion protein, were immunoprecipitated with polyclonal anti-Kap95 and anti-Kap60 antibodies (lanes 3 to 6), or a polyclonal antibody to Pho4, negative controls (lanes 1 and 2). Imunoprecipitates were separated by 12% SDS-PAGE, and co-precipitated Reg1-GFP was visualized on a Western blot with a polyclonal anti-GFP antibody. The level of Reg1 present in the different extracts was determined by western blot using an anti-GFP antibody.

As shown in Figure 34, a strong and specific Reg1-GFP signal was observed with samples immunoprecipitated with an anti-Kap60 antibody in cells grown in high and low glucose conditions. However, no or very weak interaction was observed when the experiment was done using samples immunoprecipitated with anti-Kap95 antibody, although similar amounts of Kap60 and Kap95 proteins were detected in the immunoprecipitates. Similarly, the same amount of the Reg1-GFP protein was detected by immunoblot analysis with anti-GFP antibody from the protein extract used for immunoprecipitation (Figure 34, lane 7). These results suggest that Reg1 enters the nucleus via the canonical route by directly binding to the α-importin Kap60 and the β-importin Kap95 is recruited to the ternary complex mainly during high-glucose growth conditions. Then, the tripartite protein complex could be recognized by the nuclear pore for transport into the nucleus.

To investigate Reg1 interaction with the exportin Xpo1, a GST pull-down was performed. The fusion protein Xpo1-GST was produced and purified in bacteria, and incubated with raw extracts from the wild-type strain FMY901 expressing Reg1-GFP.

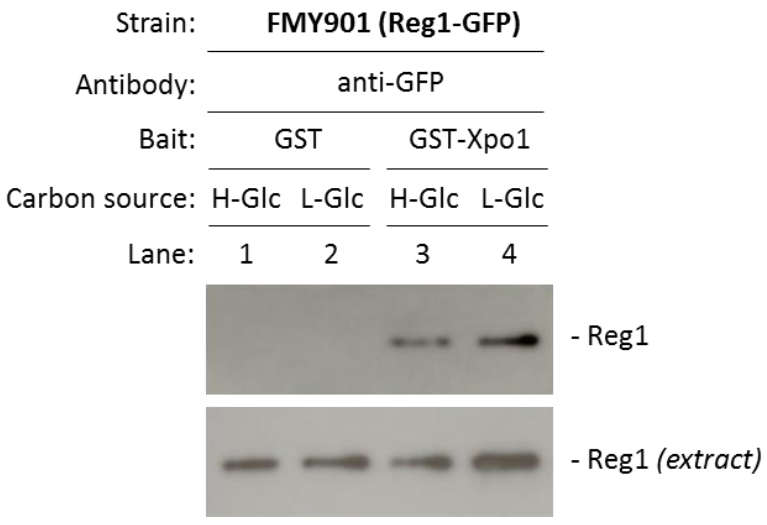


Figure 35. ***GST pull-down assays of the interaction Reg1 with Xpo1.*** A GST-Xpo1 fusion protein was purified on glutathione-sepharose columns. Equal amounts of GST-Xpo1 were incubated with cell extracts from FMY901 strain, which express a functional Snf1-GFP. Lanes 2 and 3 represent the interaction at high and low glucose conditions, respectively, and lanes 1 and 2 are the negative controls, protein extracts incubated with only GST.

As shown in Figure 35, a clear retention of Reg1 protein was observed in the sample containing GST-Xpo1 and raw extract from the FMY901 strain grown in high and low-glucose conditions. However, the interaction between Reg1-GFP and Xpo1 was systematically stronger with samples from low glucose-grown cultures than from high glucose cultures, suggesting that the low glucose conditions increases the affinity

of Reg1 for the export receptor. When a control with GST protein alone in the reaction mixture was used, no signal was observed. These results suggest that Reg1 is a cargo of Xpo1 to exit the nucleus in *S. cerevisiae*. Since results from numerous studies support a direct substrate interaction mechanism to explain the ability of Reg1 to direct Glc7 to its substrates of interest, it is tempting to speculate that the Glc7 phosphatase could be recruited by Reg1 to the repressor complex of the *SUC2* promoter to act over its substrates Snf1, Mig1 and Hxk2.

Considering all the results, what we can conclude that the repressor complex of the *SUC2* promoter is an assembly of proteins that bind to DNA, Mig1 and Mig2, as well as the machinery regulating the Mig1 repressing activity, Hxk2, SNF1 complex and Reg1.

2. Subnuclear localization of the *SUC2* repressor complex.

2.1. *SUC2* recruitment to the nuclear pore complexes.

During the last chapter, we have described the composition of the repressor complex of the *SUC2* gene, and how the assemble and disassemble of this repressor complex occurs and controls the expression of the *SUC2* gene. However, there are other mechanisms which can regulate the expression of the *SUC2* gene.

Since recently it has been described that the nuclear pore complexes apart from its role in nucleocytoplasmic transport, are also involved in gene expression regulation and transcriptional memory, and the *SUC2* gene has been demonstrated to be associated with the NPC, we decided to investigate the role of *SUC2* association with the NPC.

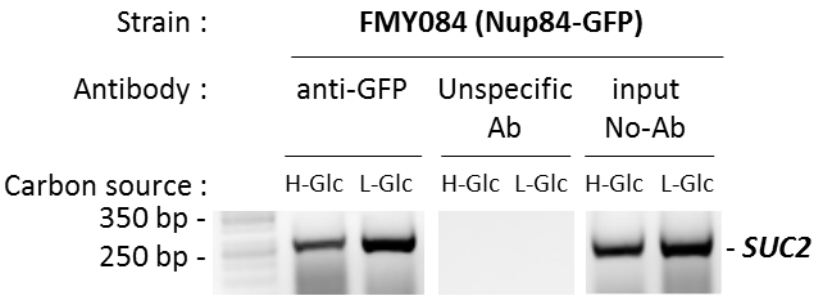
It was observed that exists a physical association of the *SUC2* gene with the nuclear pore complexes in either repressing and derepressing conditions, and that the absence of nuclear pore components causes a glucose repression defect. Two of the nucleoporins which have been seen to affect *SUC2* gene expression are Nup84 and Nup120. They are two scaffold nucleoporins located in the outer ring of the nuclear pore complexes. Deletion of NUP84 or NUP120 causes an approximately 40% decrease in invertase production when cells are growing under derepressing conditions. However, the elimination of Nup120 also results in a severe defect in repression of the *SUC2* gene under repressing conditions (high glucose media) (Sarma et al., 2011).

This observations, led us to study the association of Nup84 and Nup120 nucleoporins with the *SUC2* promoter. For this aim we generate two wild-type strains which express a functional GFP-tagged form of Nup84 and Nup120, respectively, at its chromosomal locus. The two strains were used to perform ChIP experiments. Cells were grown in a high glucose media and part of the culture was shifted to low glucose-containing media. Samples were immunoprecipitated using a specific anti-GFP antibody.

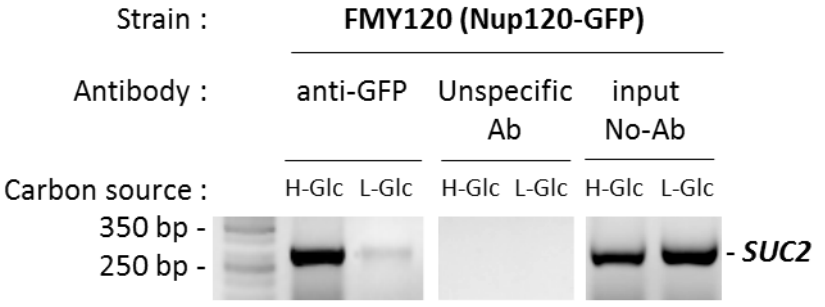
The results of these experiments were compared with the association of Mig1 with the *SUC2* promoter in a wild-type strain.

FMY084	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 Nup84-GFP*
FMY120	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 Nup120-GFP*

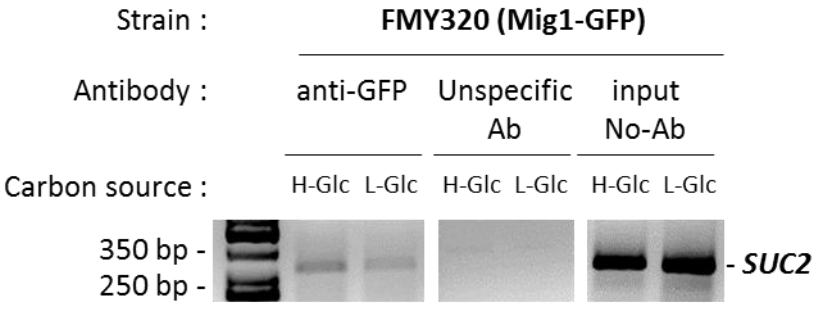
A



B



C



D

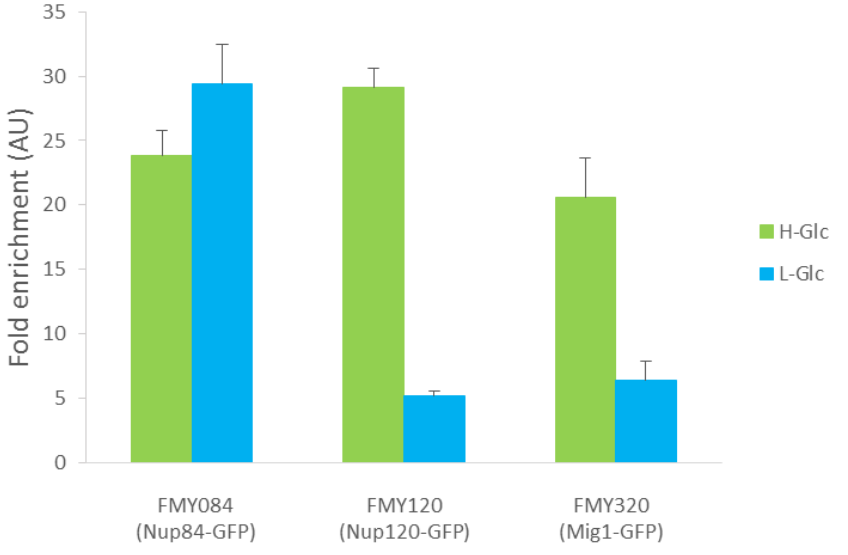


Figure 36. ***Association of Nup84, Nup120 and Mig1 with the SUC2 promoter.*** Association of Nup84, Nup120 and Mig1 with the *SUC2* promoter in a wild-type strain as measured by ChIP. **A**, **B** and **C**, represent the PCR analysis of the strains FMY084 (Nup84-GFP), FMY120 (Nup120-GFP) and FMY320 (Mig1-GFP), respectively, in each image the first two lanes are the ChIP results, the immunoprecipitation with an anti-GFP antibody. The two following are the immunoprecipitation with an unspecific antibody (negative controls), and the last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **D** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). qPCR data of Nup84, Nup120 and Mig1 binding to the *SUC2* promoter. Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

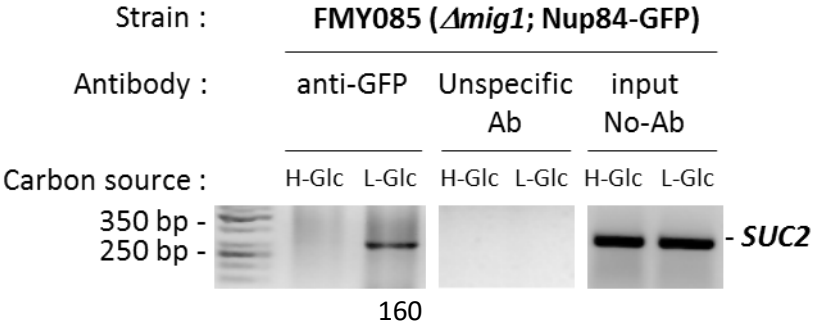
As it is observed in Figure 36, an association between Nup84 and the *SUC2* promoter is detected in both high and low glucose conditions, being slightly higher in glucose-starved cells. Nup120 also was also detected in association with the *SUC2* promoter, but in this case its union mainly occurs under high glucose conditions, because in low glucose conditions less than 20% of Nup120 remains at the *SUC2* promoter.

Nup120 recruitment to the *SUC2* promoter occurs in the same glucose-sensitive manner as Mig1, suggesting that nucleoporins could be also structurally important for the assembly and disassembly of the repressor complex. To understand better its recruitment with the *SUC2* promoter, we decided to investigate whether this association was influenced by Mig1 or Hxk2 absence.

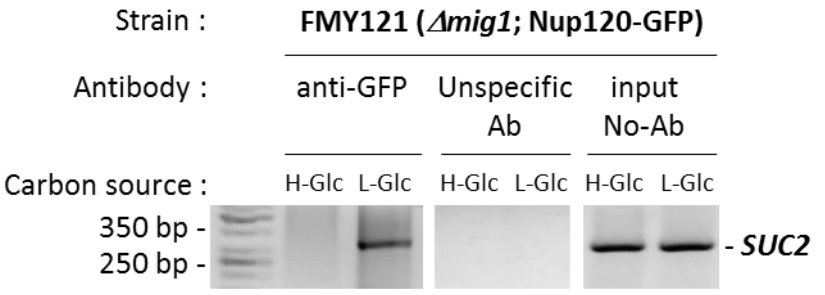
To study Nup84 and Nup120 association with the *SUC2* promoter in Mig1 absence, we generated two *Δmig1* mutant strains which contain a functional GFP-tagged form of the proteins Nup84 and Nup120, respectively. These two strains were used to perform ChIP assays in the same high and low glucose conditions as done for the wild type strain.

FMY085	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6J::LEU2 Nup84-GFP*
FMY121	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6J::LEU2 Nup120-GFP*

A



B



C

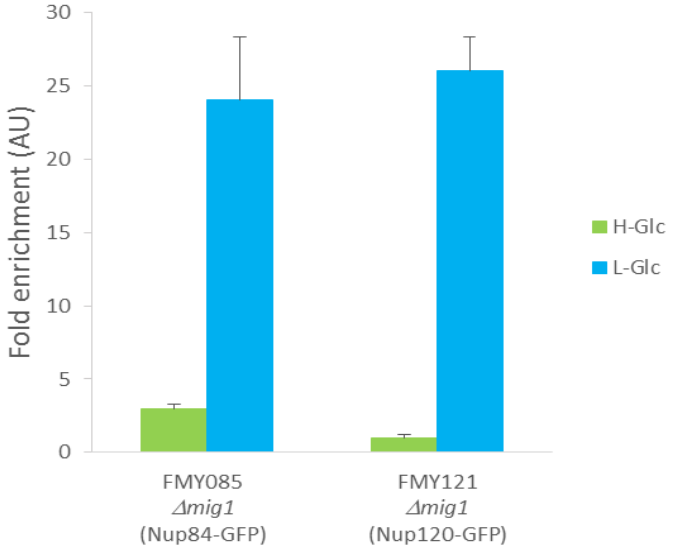


Figure 37. ***Association of Nup84, Nup120 with the SUC2 promoter in Mig1 absence.*** Association of Nup84 and Nup120 with the *SUC2* promoter *Δmig1* mutant strain as measured by ChIP. The strains FMY085 and FMY121 express a functional GFP-tagged form of Nup84 and Nup120, respectively. **A** and **B** represent the PCR analysis of the strains FMY085 and FMY121, respectively, in each image the first two lanes are the ChIP results, the immunoprecipitation with an anti-GFP antibody. The two following are the immunoprecipitation with an unspecific antibody (negative controls), and the last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **C** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). qPCR data of Nup84 and Nup120 binding to the *SUC2* promoter in Mig1 absence. Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

In Figure 37, we can observe how Mig1 absence highly impaired the union of both Nup84 and Nup120 with the *SUC2* promoter. In both

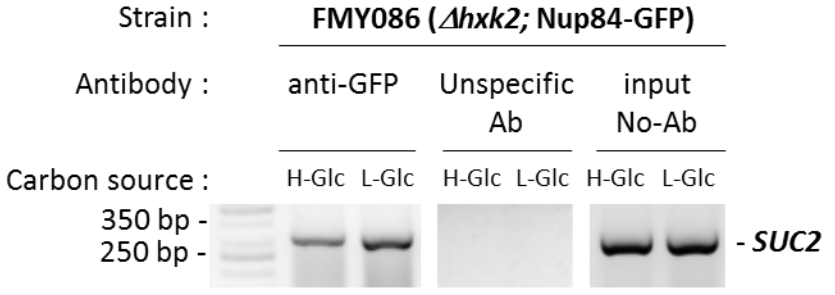
mutant strains the association of both nucleoporins with *SUC2* gene under high is totally lost in comparison with the wild-type strain.

During the growth in low glucose conditions, Nup84 association with *SUC2* promoter does not seem to be affected, and Nup120 have an interaction with SUC2 promoter that was not detected in the wild-type strain.

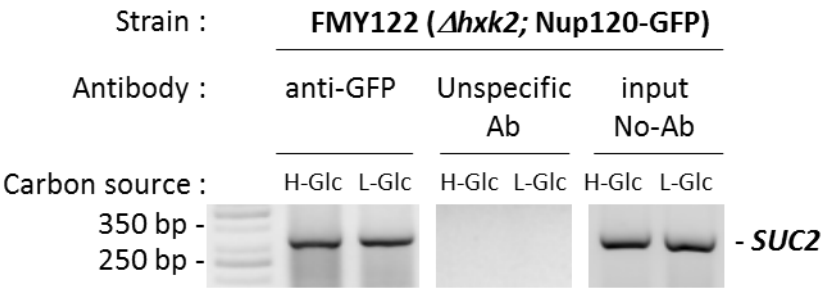
When we perform the same experiments in *Δhxk2* mutant strains, we observe that Nup84 union with the *SUC2* promoter is not affected by Hxk2 absence, as it follows the same pattern as in a wild-type strain. Nevertheless, Nup120 association is also affected by Hxk2 absence. In cells growing in rich glucose medium, Nup120 is detected in association with *SUC2* gene, as is happens in the wild-type, but during the growth in low glucose conditions this association was also detected, which differs from the result in the wild-type strain. Thus, Hxk2 absence strongly affect the regulation of Nup120 interaction with SUC2-URS element of the *SUC2* promoter.

FMY086	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 Nup84-GFP*
FMY122	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 Nup120-GFP*

A



B



C

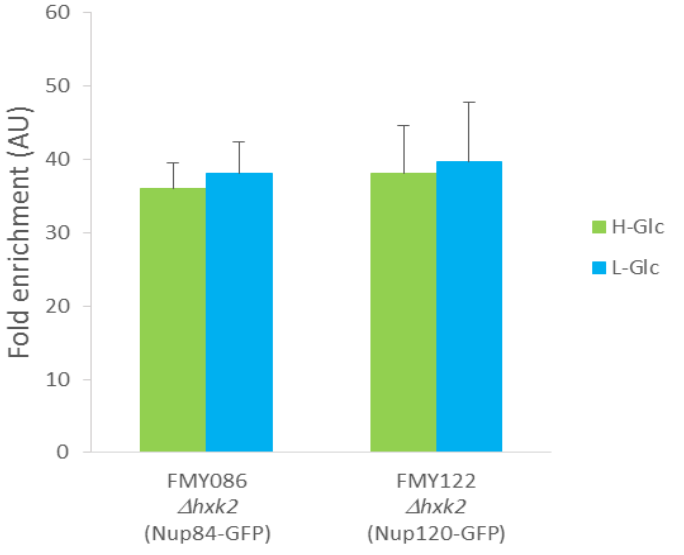
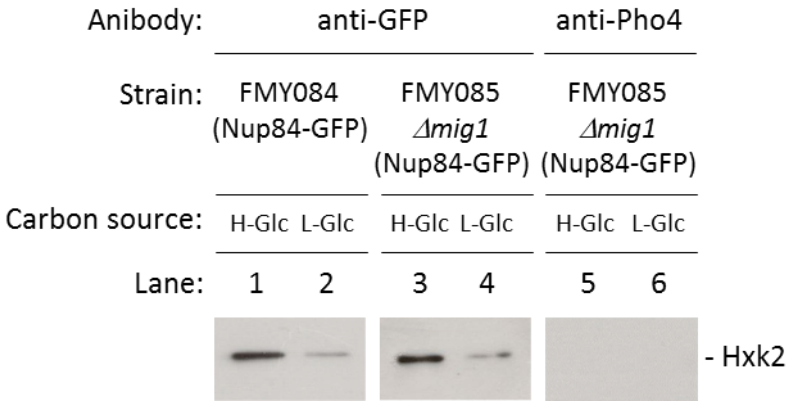


Figure 38. ***Association of Nup84, Nup120 with the SUC2 promoter in Hxk2 absence.*** Association of Nup84 and Nup120 with the *SUC2* promoter *Δhxk2* mutant strain as measured by ChIP. The strains FMY086 and FMY122 express a functional GFP-tagged form of Nup84 and Nup120, respectively. **A** and **B** represent the PCR analysis of the strains FMY086 and FMY122, respectively, in each image the first two lanes are the ChIP results, the immunoprecipitation with an anti-GFP antibody. The two following are the immunoprecipitation with an unspecific antibody (negative controls), and last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **C** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). qPCR data of Nup84 and Nup120 binding to the *SUC2* promoter in Hxk2 absence. Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

Since Hxk2 is essential to maintain the structure and function of the repressor complex of the *SUC2* promoter, and its absence also affects the

recruitment of Nup120, we wonder if Hxk2 was also indispensable for *SUC2* recruitment to the NPC. To test this idea we study its interaction with Nup84 and Nup120 in a wild-type strain, and also if this interaction was Mig1-dependent. For this aim, we perform co-immunoprecipitation assays using wild-type strain and a *Δmig1* mutant strain, expressing Nup84-GFP and Nup120-GFP. Protein extracts of high glucose growing cells, and glucose-starved cells were immunoprecipitated with an anti-GFP antibody and the retained Hxk2 was detected using a specific anti-Hxk2 antibody.

A



B

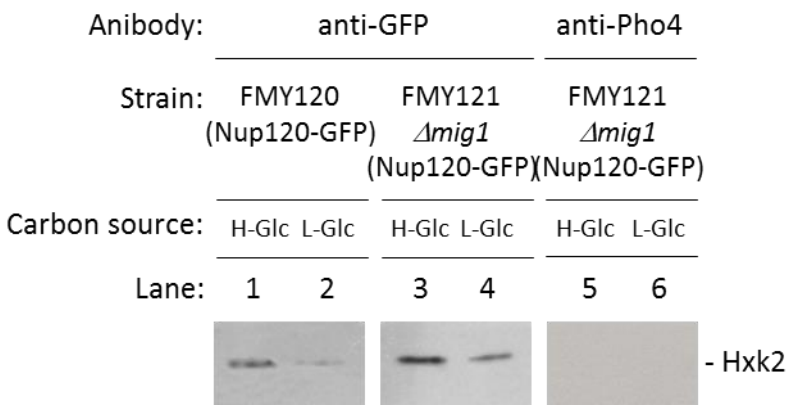


Figure 39. ***Co-immunoprecipitation assay of the interaction of Nup84 and Nup120 with Hxk2 in a wild-type strain and in a Δmig1 mutant strain.*** Protein extracts of the FMY084 and FMY085 (*Δmig1*) strains which express a functional Nup84-GFP (**A**), and proteins extracts of the FMY120 and FMY121 (*Δmig1*) which express a functional

Nup120-GFP (**B**), were immnunoprecipitated using an anti-GFP antibody (lanes 1 -4), and a polyclonal antibody to Pho4, as negative control (lanes 5 and 6). Immunoprecipitates were separated by 12% SDS-PAGE, and the level of immunoprecipitated Hxk2 in the blotted samples was determined by using anti-Hxk2 antibody. The level of Hxk2 present in the different extracts was determined by western blot using anti-Hxk2 antibody.

As the Figure 39 indicate, there is an interaction of Nup84 and Nup120 with the protein Hxk2 which occurs in a glucose-dependent manner. Moreover, this interaction is independent of Mig1, because as seen in lanes 3 and 4 of both Figure 39 A and B, Hxk2 association with Nup84 and Nup120 is not abolished in Mig1 absence.

Taken all together, these results suggest that Hxk2 is also essencial for the recruitment of the *SUC2* gene to the NPC.

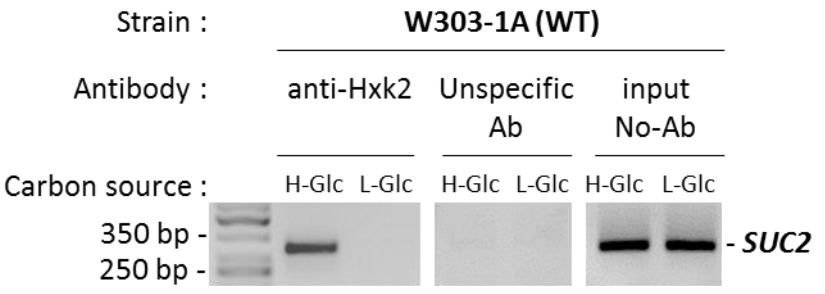
2.2. Role of *SUC2* gene recruitment to the NPC.

Until know we have studied how the recruitment *SUC2* gene to the NPC is done and which factors are involved in its recruitment. However, we did not answer the question of which is the role of *SUC2* gene association with the NPC.

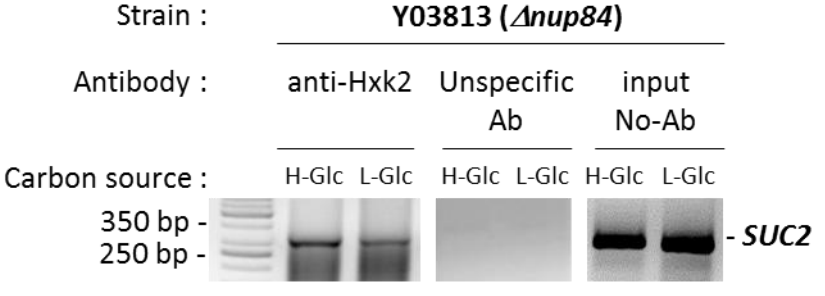
One of the theories regarding the subnuclear localization of genes and its association with the NPC, support that the nucleoporins may facilitate the binding of proteins to consensus sites within the genes. Since Hxk2 seems to be the critical factor involved in the interaction with the nucleoporins, we thought that Nup84 and Nup120 might be involved in possitioning Hxk2 at the *SUC2* promoter. In order to test this idea, we study the Hxk2 association with the *SUC2* promoter in *Δnup84* and *Δnup120* mutant strains. ChIP experiments were done with samples from high and low glucose conditions that were immunoprecipitated with a

specific anti-Hxk2 antibody. The results of these two mutant strains were compared with Hxk2 union in a wild-type strain.

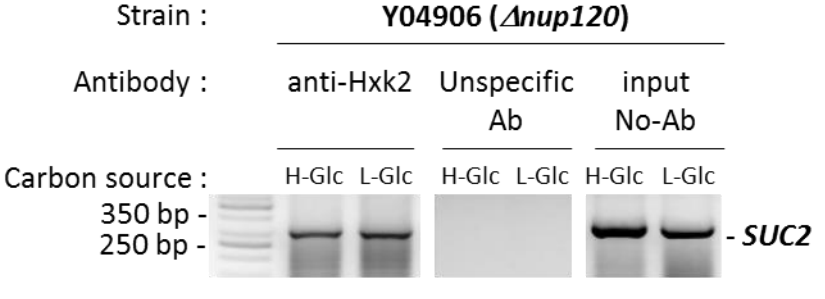
A



B



C



D

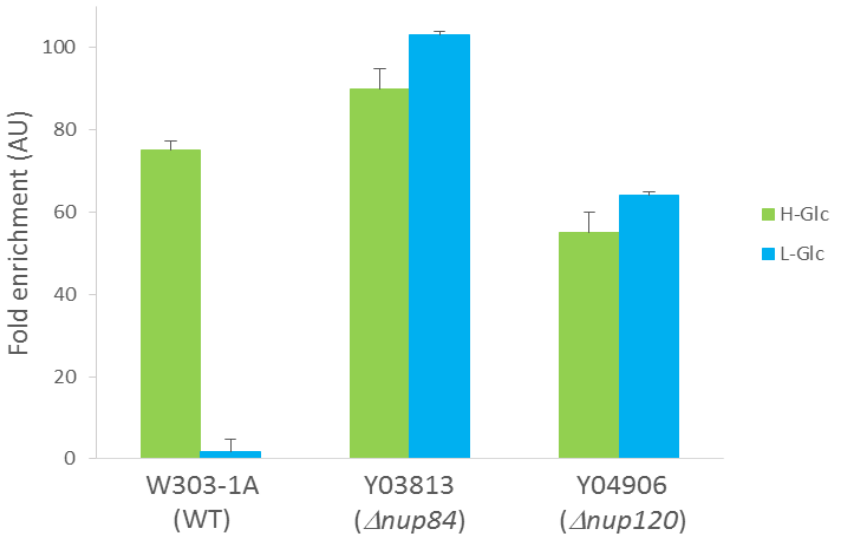


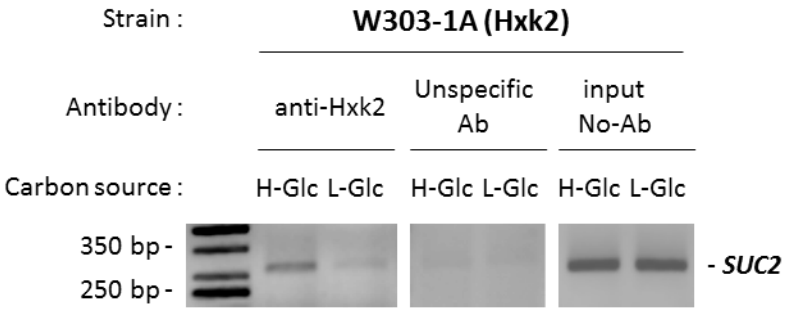
Figure 40. ***Association of Hxk2 with the SUC2 promoter in a WT, and in Nup84 and Nup120 absence.*** Association of Hxk2 with the *SUC2* promoter in a WT strain, a *Δnup84* and a *Δnup120* mutant strains as measured by ChIP. **A**, **B** and **C** represent the PCR analysis of the strains W303-1A (WT), Y03813 (*Δnup84*) and Y04906 (*Δnup120*), respectively, in each image the first two lanes are the ChIP results, the immunoprecipitation with an anti-Hxk2 antibody. The two following are the immunoprecipitation with an unspecific antibody (negative controls), and the last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **D** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). qPCR data of Hxk2 binding to the *SUC2* promoter a WT strain, and in Nup84 and Nup120 absence. Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

In a wild-type strain, Hxk2 is binding to the *SUC2* promoter elements in a glucose-dependent manner. However, when Nup84 or Nup120 are absent, we found that Hxk2 is constitutively associated with the *SUC2* promoter, suggesting that the presence of either of this nucleoporins is essential to maintain the structure and regulate the formation and dissociation of the repressor complex.

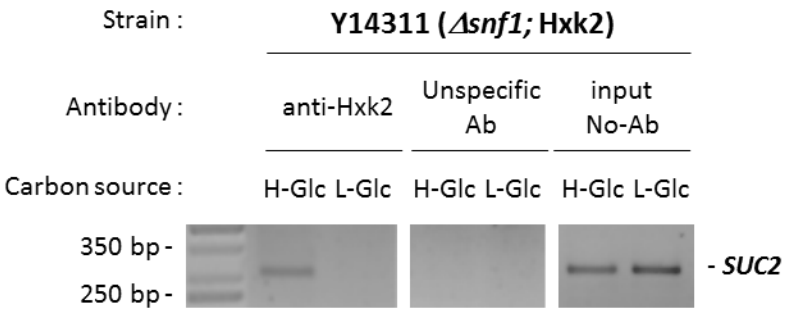
3. Regulation of the Hxk2 incorporation to the repressor complex of the *SUC2* promoter.

Hxk2 protein shuttles in and out the repressor complex of the *SUC2* promoter in a glucose-dependent manner. Since the shuttling back and forth between the nucleus and the cytoplasm of Hxk2 is also controlled by glucose availability and glucose determines the phosphorylation state of Hxk2 by controlling Snf1 kinase and Reg1-Glc7 phosphatase activities (Fernandez-Garcia et al., 2012), an interesting hypothesis could be that the phosphorylation state of Hxk2 also regulates its incorporation and dissociation from the repressor complex of the *SUC2* promoter. To address this hypothesis, we analyse the association of Hxk2 with the *SUC2* promoter in the absence of Snf1. In *Δsnf1* mutant strain Hxk2 is not phosphorylated under low glucose conditions, so if the phosphorylation state of Hxk2 controls its incorporation and dissociation of the repressor complex, in Snf1 absence we would expect that it remains associated with *SUC2* gene under low glucose conditions. Nevertheless, when we perform a ChIP experiment in *Δsnf1* mutant strain FIGURAX, what we see is that Hxk2 has the same behaviour that in a wild-type strain (Figure 41). In the absence of Snf1, Hxk2 is able to interact with the *SUC2* promoter in high glucose (95%) but less than 8% of Hxk2 was bound to the SUC2 elements in low glucose conditions, which is a similar result to that obtained using a wild-type strain. Thus, this result supports that phosphorylation of Hxk2 by the Snf1 kinase is not essential to regulate Hxk2-*SUC2* promoter binding.

A



B



C

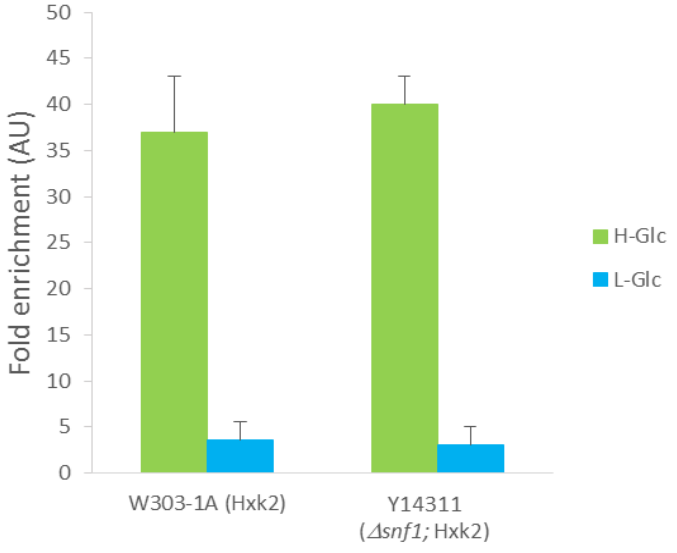


Figure 41. ***Association of Hxk2 with the SUC2 promoter in a WT and in Snf1 absence.*** Association of Hxk2 with the *SUC2* promoter in a WT strain and in a *Δsnf1* mutant strain as measured by ChIP. **A** and **B** represent the PCR analysis of the strains W303-1A (WT) and Y14311 (*Δsnf1*), respectively, in each image the first two lanes are the ChIP results, the immunoprecipitation with an anti-Hxk2 antibody. The two following are the immunoprecipitation with an unspecific antibody (negative controls), and the last two

lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **D** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). qPCR data of Hxk2 binding to the *SUC2* promoter a WT strain, and in Snf1 absence. Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

Seen that phosphorylation state of Hxk2 does not affect its recruitment to the repressor complex, we decided to test if conformational changes in Hxk2 could affect its binding to both, Mig1 and the *SUC2* promoter.

D-xylose is a glucose non phosphorylable analogue that binds in the presence of ATP to Hxk2 and it promotes a conformation change in Hxk2 and its inactivation by autophosphorylation of the serine-157 (DelaFuente, 1970; Fernandez et al., 1986; Fernandez et al., 1985; Heidrich et al., 1997). Additionally, glucose protects against xylose-induced inactivation with efficiencies closely related to their respective glucose-*Km* or xylose-*Ki* values (DelaFuente, 1970). Thus, it seems likely that xylose could induce an open conformation of Hxk2 that mimics the conformation of low glucose conditions.

To address this possibility, we first investigated how the presence of xylose in the media affects the interaction between Hxk2 and Mig1 proteins. For this aim we perform GST pull-down assays, purifying from bacteria GST and Mig1-GST with the help of sepharose beads. Hxk2 was also produced and purified in bacteria, and equal amounts of Hxk2 were incubated with the beads in the presence of different metabolites. After incubation, the samples were washed and the Hxk2 bound was detected by western blot using a specific antibody anti-Hxk2.

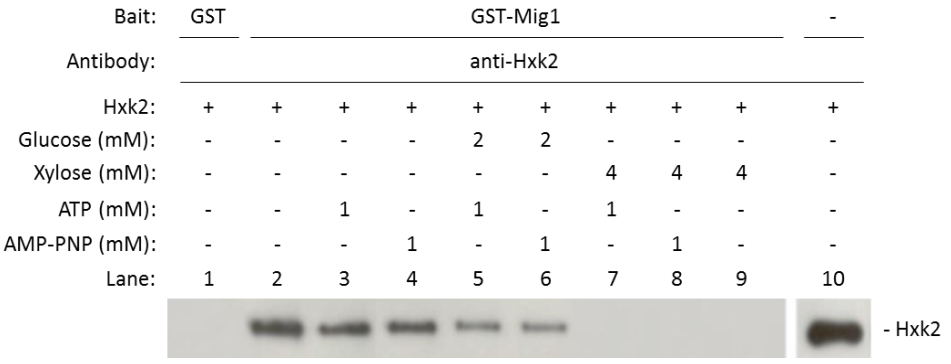


Figure 42. ***In vitro interaction of Hxk2 and Mig1.*** Identical amounts of Hxk2 affinity purified protein from bacteria were incubated in the presence of 2mM glucose, 4mM D-xylose, 1mM $MgATP^{2-}$ and 1mM MgAMP-PNP for 30 min at 20ºC in PBS buffer.

In Figure 42, we can observed that GST-Mig1, but not GST (lane1), was able to precipitate the purified Hxk2 (lane 2). Either in the presence of glucose, ATP or the ATP analogue, AMP-PNP, an interaction between GST-Mig1 and Hxk2 was observed (lane 3-6). However, no signal was observed when the assay is done in the presence of 4mM xylose (lanes 7-9).

This experiment supports the idea that Mig1-Hxk2 interaction is abrogated by xylose, and this interaction does not require neither ATP nor AMP-PNP. This result also suggest that the efficiency of xylose as inactivating agent of Hxk2-Mig1 interaction could be related to its ability to bind to Hxk2 and not to inhibit competitively the hexokinase reaction for which a metal-nucleotide complex is also required (DelaFuente, 1970).

To confirm that the xylose-Hxk2 binding is critical for suppression of Hxk2-Mig1 interaction, we measure by GTS pull-down the interaction of

Mig1 and Hxk2 under fixed glucose concentration (0.5mM) and increasing xylose concentrations from 0.5mM to 4mM.

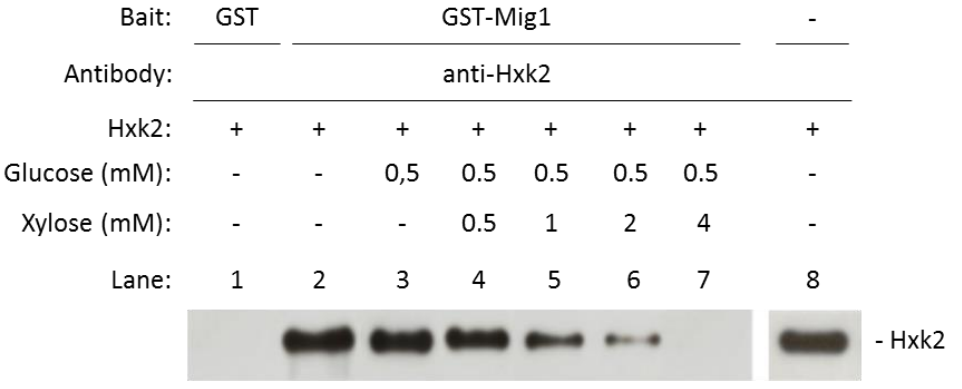


Figure 43. ***In vitro interaction of Hxk2 and Mig1.*** Time course of D-xylose inhibition of Hxk2-Mig1 interaction. Identical amounts of Hxk2 affinity purified protein from bacteria were incubated in the presence of 0.5mM glucose and increasing amounts of D-xylose, from 0.5 to 4mM (lanes 4-7) for 30 min at 20ºC in PBS buffer.

As shown in Figure 43, there is an interaction of Mig1 and Hxk2 in the presence or glucose absence (line 2 and 3, Figure 43), however, when we add increasing concentrations of xylose, the interaction is decreasing until the interaction is completely abrogated at 4mM xylose (lane 4-7, Figure 43).

A similar GST pull-down assay was performed, but this time fixing the concentration of xylose (2mM) and with increasing concentrations of glucose, from 0.5mM to 8mM.

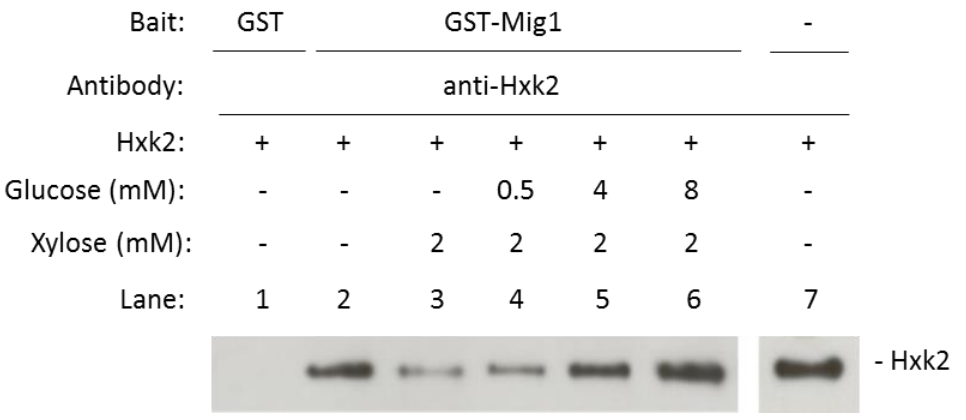


Figure 44. ***In vitro interaction of Hxk2 and Mig1.*** Time course of glucose activation of Hxk2-Mig1 interaction. Identical amounts of Hxk2 affinity purified protein from bacteria were incubated in the presence of 2mM D-xylose and increasing amounts of glucose, from 0.5 to 8mM (lanes 4-6) for 30 min at 20ºC in PBS buffer. Then, the GST-Mig1 fusion protein coupled to glutathione-Sepharose was incubated with the assay mixtures for 90 min at 4ºC in PBS buffer.

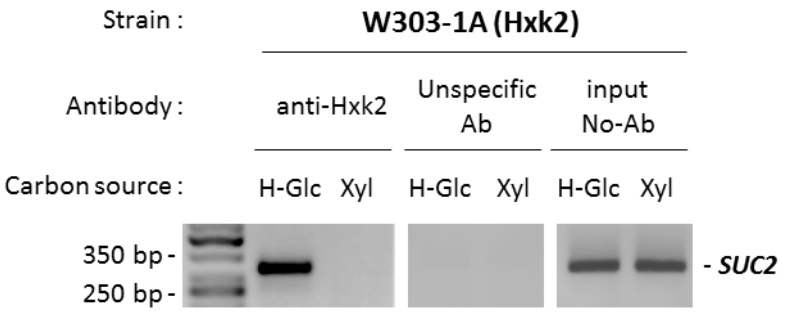
Figure 44 shows the same result as the previous experiment (Figure 43) in this case we can see how increasing glucose concentrations increase the interaction between Mig1 and Hxk2 (lane 4-6, Figure 44), suggesting that glucose acts as a protecting agent with high efficiency to revert the changes induced by xylose.

Taken together, these results show that the regulation of Hxk2 interaction with Mig1 involves a conformational change of the protein induced by the sugar substrate glucose and its analogue xylose. Thus, it could be assumed that the same flexibility of the Hxk2 active site which allows a “induce fit” (Koshland, 1958) can promote an inactive conformation.

In order to confirm these results obtained *in vitro* with purified proteins, we performed ChIP assays to analyse the union of Hxk2 with the *SUC2* promoter in the presence of xylose. The first experiment carry

out was in a wild type strain, using a specific anti-Hxk2 to precipitate Hxk2 in samples of glucose and xylose growing cells.

A



B

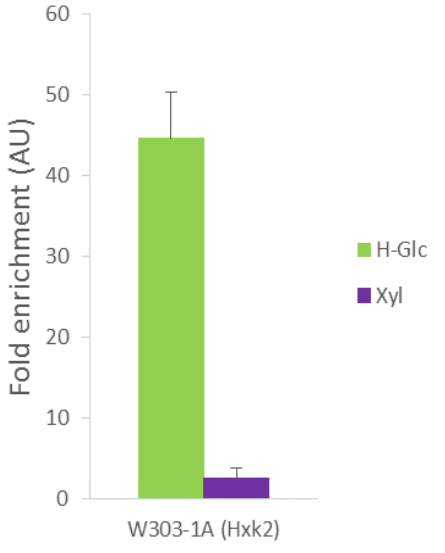
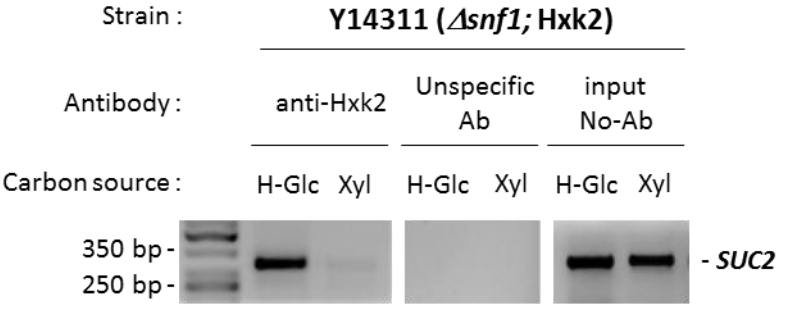


Figure 45. ***Association of Hxk2 with the SUC2 promoter in a WT in glucose and xylose conditions.*** Association of Hxk2 with the *SUC2* promoter in a WT strain as measured by ChIP. **A** Represent the PCR analysis of the strains W303-1A (WT), the first two lanes are the ChIP results, the immunoprecipitation with an anti-Hxk2 antibody. The two following are the immunoprecipitation with an unspecific antibody (negative controls), and the last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **C** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). qPCR data of Hxk2 binding to the *SUC2* promoter a WT strain. Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

In the presence of xylose, Hxk2 binding to the *SUC2* promoter is highly impared, during this growth conditions less than 6% of Hxk2 was bound to the SUC2-URS of the *SUC2* promoter (Figure 45).

The same experiment was performend in a *Δsnf1* mutant strain, to study the Hxk2 association with the *SUC2* promoter elements in the absence of Snf1 kinase.

A



B

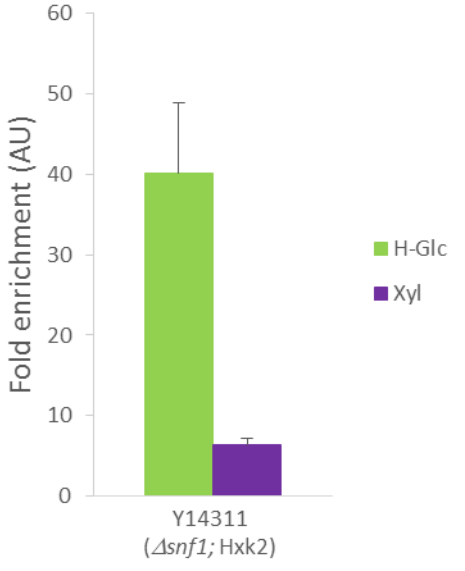


Figure 46. ***Association of Hxk2 with the SUC2 promoter in a Δsnf1 mutant strain, in glucose and xylose conditions.*** Association of Hxk2 with the *SUC2* promoter in a *Δsnf1* mutant strain as measured by ChIP. **A** Represent the PCR analysis of the strains Y14311,

the first two lanes are the ChIP results, the immunoprecipitation with an anti-Hxk2 antibody. The two following are the immunoprecipitation with an unspecific antibody (negative controls), and the last two lanes are the extracts prior to immunoprecipitation (input, whole-cell extract). **B** ChIP results were also analysed by quantitative real-time PCR (RT-PCR). qPCR data of Hxk2 binding to the *SUC2* promoter a *Δsnf1* mutant strain. Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

As seen in Figure 46, the occupation rate of the SUC2 elements of the *SUC2* promoter by Hxk2 in the absence of Snf1 protein is also affected by the presence of xylose. A comparison between high glucose and xylose conditions revealed that nuclear Hxk2 was well engaged with *SUC2* promoter in high glucose, but only 14% of Hxk2 was bound to the SUC2-URS in xylose conditions. In a *Δsnf1* mutant strain, Mig1 and Hxk2 are dephosphorylated and cells are repressed at high and low glucose conditions (Figure 47). Although in this mutant strain an inactivating Hxk2 conformational change induced by xylose was observed, *SUC2* gene expression is not promoted. A possible explanation is that although Hxk2 has adopted an inactive structure, since only 14% of the protein remains associated with the *SUC2* promoter, Mig1 is not phosphorylated and stays associated with the SUC2-URS element repressing gene transcription.

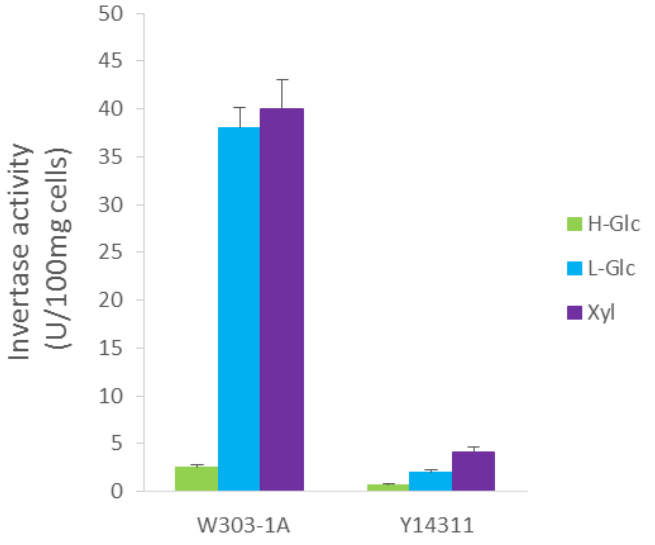


Figure 47. ***Quantitative invertase assays were performed in a WT strain and a Δsnf1 mutant strain.*** Whole cells from the wild-type strain, W303-1A, and the Y14311 (*Δsnf1)* mutant strain, were used for invertase activity determination. Invertase was assayed using cells grown in high-glucose medium (H-Glc,green bars) until an A_{600nm} of 0.8 was reached and then part of the culture was transferred to low-glucose medium for 60 min (L-Glc, blue bars), and to xylose medium (Xyl, purple bars). Error bars represent the standard error of the mean for three independent determinations using three colonies of each strain.

Taken together, these results suggest that Hxk2 would act as an intracellular glucose sensor that communicates the glucose level information from the environment to the Mig1-regulated genes. The signalling activity of Hxk2 is linked to its conformational changes induced by glucose levels. Under high glucose conditions Hxk2 would adopt a close conformation which promote the assembly of the repressor complex, and during the growth under low glucose conditions Hxk2 would have an open conformation that promote the dissociation of the repressor complex.

DISCUSSION

DISCUSSION

Controlling gene expression allows yeast cells to rapid respond and to adapt to environmental fluctuations, such as nutritional stress. A remarkable example of it is the response of *Saccharomyces cerevisiae* to glucose availability. This yeast has multiple glucose signalling pathways such as Snf3/Rgt2 and cAMP/PKA that are quite well characterized, and allow cells to detect extracellular glucose levels and generate an adequate response. However, there are others such the termed repression pathway that although it has been extensively studied over the last years, there are different questions that have not been answered in an appropriate way.

During this work we tried to identify the composition of the repressor complex formed at the *SUC2* promoter, and tried to address two main questions: how the formation and stabilization of a repressor complex at the *SUC2* promoter is promoted, and how glucose level regulates this process.

Mig1 and Mig2 act as transcriptional repressors of the *SUC2* gene, both proteins have similar DNA-binding zinc fingers and recognize identical DNA-binding sequences (Lundin et al., 1994). Mig1 protein is more important than Mig2 in *SUC2* repression, since it is sufficient to achieve complete repression, although the complete derepression of the *SUC2* gene is only reach in *Δmig1Δmig2* double mutant strain.

We first investigate the importance of Hxk2 in the Mig1 binding to the *SUC2* promoter, for this aim we used a ChIP assay and cells from both *Δhxk1Δhxk2* double mutant and *Δhxk2* single mutant strains expressing

a Mig1 GFP-tagged protein. We found that in the absence of both Hxk1 and Hxk2 proteins, Mig1 cannot bind to SUC2-URS region. In either of these mutant cells less than 5% of Mig1 was found in association with the *SUC2* promoter at high and low glucose conditions. The most straightforward interpretation of these results could be that, in the absence of both Hxk1 and Hxk2 proteins, the Snf1 kinase is activated (Mayordomo et al., 2002; Sanz et al., 2000), Mig1 is phosphorylated and is exported to the cytoplasm by binding to the Msn5 transporter (DeVit and Johnston, 1999), and therefore *SUC2* gene is expressed. However, in the *Δhxk2* mutant strain, Mig1 recruitment to the SUC2-URS in both high and low glucose conditions is similar to the one found in a wild-type strain grew in low glucose conditions.

Since *HXK1* gene is only expressed during low glucose conditions, and its expression is repressed by Hxk2 under high glucose conditions, a possible explanation for these results could be that Hxk1 protein could partially mimic Hxk2 function at the *SUC2* promoter repressor complex. This would explain why in a wild-type strain under low glucose conditions 32% of Mig1 remains associated with the *SUC2* promoter, maintaining a 70% of *SUC2* repression, and why in a *Δhxk2* mutant strain the same percentage of Mig1 is recruited to the SUC2-URS. Moreover, Hxk1 and Hxk2 proteins have more than 77% identity, and the first 24 amino acids of the N-terminal region are identical in both proteins. Since Hxk2-Mig1 interaction is mediated by a 10-amino acid motif in Hxk2 located between lysine-6 and methionine-15 (Ahuatzi et al., 2004; Herrero et al., 1998) (Hxk2 is numbered from residues 1 to 485; residue 1 is a valine because the initiator methionine is cleaved off from the primary translation product) and Hxk1 has in its N-terminal region the same 10-

amino acid motif, we can supposed that Hxk1 could also interact with Mig1 protein in these metabolic conditions to prevent, at least in part, the Snf1 kinase-mediated phosphorylation of Mig1 at serine-311. This behaviour has not been previously described because the amount of Mig1-GFP retained in the nucleus is probably not detectable by conventional fluorescent microscopy, so in these conditions it has been claimed that Mig1 is localized exclusively in the cytoplasm (De Vit et al., 1997). Moreover, these results would also explain why fully derepression of the *SUC2* gene is only achieved in the absence of both Hxk1 and Hxk2 proteins.

In addition, our work also shows that the regulatory factors Snf1 and Reg1 are also associated with the *SUC2* promoter, and demonstrate that this association is Hxk2-dependent. In either Mig1 or Hxk2 absence, the recruitment of both Snf1 and Reg1 proteins to the *SUC2* promoter is abolished. As seen before in an *Δhxk2* mutant strain about 32% of Mig1 transcriptional repressor remains associated with the *SUC2* promoter, so if Snf1 and Reg1 association with SUC2-URS occurs through the Mig1 protein, a similar amount of Snf1 and Hxk2 should be detected. However, what we observed is that less than 5% of these proteins were recruited to the *SUC2* promoter in an *Δhxk2* mutant strain, suggesting that Mig1 is not the critical factor for its association with the *SUC2* promoter, and it occurs in an Hxk2-dependent manner. These results are also sustained by the fact that Snf1 and Reg1 interact with Hxk2 in high and low glucose conditions.

Snf4 and Gal83 proteins, the regulatory units of the SNF1 complex, have also been detected in association with SUC2-URS elements at a similar level both in high and low glucose conditions. On the other hand,

GST pull-down experiments show a weak interaction of Snf4 and Gal83 proteins with Hxk2, and in the absence of Snf1 no interaction of Snf4 with Hxk2 was observed. Thus, SNF1 complex regulatory proteins, Snf4 and Gal83 binding to *SUC2* repressor complex appears to be Snf1-dependent.

Here we show that Hxk2 is recruited to the SUC2-URS sequences of the *SUC2* promoter by its interaction with the DNA-bound Mig1 protein and subsequently it promotes the formation of a functional repressor complex. Snf1 and Reg1, whose interaction with Hxk2 was confirmed *in vitro* and *in vivo*, are also components of it. Mig2 appears to play no role in *SUC2* repression when Mig1 is present, since in these conditions the absence of Mig2 has no effect on *SUC2* expression. However, in wild-type cells grown in high and low glucose conditions approximately 25% of the Mig2 detected in the absence of Mig1 was recruited to the *SUC2* promoter. The level of Mig2 associated with the *SUC2* promoter increases in the absence of either Mig1 or Hxk2 proteins, which contributes to maintain approximately 70% of *SUC2* repression, as observed in *Δmig1* and *Δhxk2* mutant cells (Figura 1). Taking into account that Mig2 interacts with Mig1, Hxk2 and Snf1 proteins both in high and low glucose conditions, our results suggest that Mig2 could be a structural component of *SUC2* repressor complex in high glucose. In the absence of Mig1 or Hxk2 proteins an increased amount of Mig2 is dragged to the *SUC2* promoter, causing 70% of *SUC2* repression. In these conditions, SNF1 complex components would remain associated with the *SUC2* promoter due to their interaction with Mig2. A fully derepression of the *SUC2* gene, in high and low glucose conditions, is only achieved in the absence of either Mig1-Mig2 or Hxk1-Hxk2 protein pairs, because in

these conditions the *SUC2* promoter repressor complex is completely disassembled.

Considering all the results we propose the next model, represented in FIGUREX, to explain the repressor composition and how its assemble and disassemble occurs. During the growth on high glucose conditions, glucose is detected and it triggers different pathways, one of them produces the activation of PKA which activates the protein phosphatase Glc7-Reg1. Glc7-Reg1 maintains dephosphorylated Hxk2 and Mig1, which in this phosphorylation state have a nuclear localization. Glc7-Reg1 also dephosphorylates, and therefore maintains inactivated the kinase Snf1. Thus, under high glucose conditions it is formed a repressor complex in the *SUC2* promoter, which repress the gene expression. The repressor complex consist of different elements: the transcriptional repressors Mig1 and Mig2, the protein Hxk2, the phosphatase Reg1-Glc7 and the three subunits of the SNF1 complex: Snf1, Snf4 and Gal83. While Mig1 is the major transcriptional repressor, Mig2 is more a structural component that helps to maintain the repressor complex under high glucose conditions. The protein Hxk2 is the essential factor of this repressor complex, on one hand Hxk2 is required to stabilize the Mig1 association with the *SUC2* promoter, and on the other hand, Snf1 and Reg1 association with the SUC2-URS is Hxk2-dependent. Snf4 and Gal83 proteins also take part in this repression complex, and its union with the *SUC2* promoter is mediated by Snf1.

When glucose becomes limiting, Sak1 kinase activates Snf1. Snf1 phosphorylates Hxk2 and Mig1, which once they are phosphorylated are exported to the cytoplasm. Thus, producing the disassembly of the repressor complex and the *SUC2* gene is derepressed.

Moreover, not all Mig1 is exported to the cytoplasm. Under low glucose conditions, about 30% of Mig1 remains associated with the *SUC2* promoter due to the presence of Hxk1, which can mimics Hxk2 function sustaining the structure of the repressor complex.

This model explain why the full derepression of the *SUC2* gene is only achived in the double mutant strains *Δmig1Δmig2* or *Δhxk1Δhxk2*.

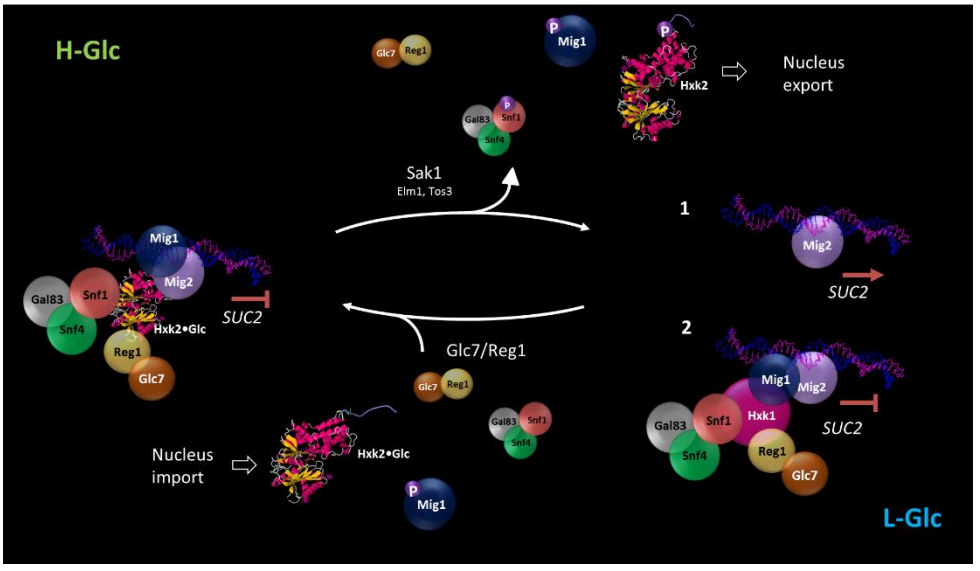


Figure 48. Diagram representing the proposed model for the assembly and disassembly of the repressor complex of the *SUC2* promoter.

The formation and dissociation of the repressor complex implicates a nucleocytoplasmic transport of the different elements through the NPC. It has been described how Mig1 and Hxk2 nucleocytoplasmic distribution is regulated by glucose, being predominantly nuclear under high glucose conditions, and shuttled to the cytoplasm when the glucose becomes limiting. However, there is little known about the nucleocytoplasmic transport of the rest of the repressor complex components.

Snf1 and Gal83, have been described to have a cytoplasmic distribution in cells growing under high glucose conditions, and when glucose becomes limiting they relocalize to the nucleus directed by Gal83. However, our results determined that Snf1 is constitutively associated with the *SUC2* promoter. This led us to try to study if this two proteins use the same import and the same export pathway used by Hxk2 to entry and exit the nucleus. When we study Snf1 interaction with the classic import machinery, our results show that Snf1 is detected in association with the importins Kap95 and Kap60 not only in low glucose conditions but also in high glucose conditions, supporting that Snf1 could be transported in high glucose conditions to the nucleus, in accordance with our ChIP results. In addition, our data also demonstrate that Snf1 import is independent of the Gal83 subunit. We have also reconstructed from bacterially produced proteins the Snf1 import complex *in vitro*, and the GST pull-down assays suggest that the Snf1-Kap60-Kap95 ternary complex is stable in the presence of Gsp1-GDP as happens in the cytoplasm. These experiment, also allows us to exclude the possibility that some unknown protein(s) could mediate Snf1 interaction with the importins Kap95 and Kap60.

Snf1 also present an interaction with the exportin Xpo1, but only in high glucose conditions, suggesting that in glucose-starved cells Snf1 lost its affinity for Xpo1, and Snf1 would be accumulated in the nucleus, where its substrates Mig1 and Hxk2 are also located.

Gal83 protein also interacts with Kap95 and Kap60, in high and low glucose conditions, but the greater interaction occurs in low glucose conditions, suggesting that as demonstrated by ChIP experiments, Gal83 is transported into the nucleus in both glucose conditions but more

actively when glucose becomes limiting. Our results also show that exist a physical association of Gal83 with the exportin Xpo1, as predicted before as it have in its sequence an LR-NES in the N-terminal region.

With regard to Snf4, it has been described that this protein is present in excess in both cytoplasm and nucleus compartments with independence of glucose levels. Our results show that like Hxk2, is transported to the nucleus by the classic import system, as it interacts with both Kap95 and Kap60 in high and low glucose conditions. In addition we have also reconstructed in vitro Snf4 import system, demonstrating that the formation of the import complex Snf4-Kap60-Kap95 is favoured in the presence of Gsp1-GDP which is present in the cytoplasm, and the complex is disrupted in the presence of high levels of Gsp1-GTP, as it happens in the nucleus. Using the thermosensitive strains, which synthetize a non-functional Kap95 and Kap60 proteins during it growth at 37^{o}C, we have also demonstrate that when Kap60 is absent, Snf4 is not able or weakly interacts with Kap95, suggesting that Kap60 is key for the nuclear transport of Snf4, as it is necessary to recognize Snf4 and present it to Kap95. Snf4 also have an association with the exportin Xpo1 but only under low glucose conditions. So Snf4 is transported into the nucleus in both high and low glucose conditions but its export to the cytoplasm only takes place when the glucose becomes limiting.

Reg1 regulatory protein has been described to mainly have a cytoplasmic localization. Nevertheless, our results for the first time support that at least a fraction of the Reg1 is constitutively associated with the *SUC2* promoter, together with its substrates of interest, Hxk2 and Mig1, which exhibit the same nuclear localization. Nuclear

localization of Reg1 is also supported by the fact that Reg1 interacts with de classic import system Kap95/Kap60, and also with the exportin Xpo1.

Considering all these results we propose a model in which Snf1, Snf4, Gal83 and Reg1 would be transported into the nucleus in both high and low glucose conditions by the classic import system, Kap95 and Kap60, and exported through the exportin Xpo1 pathway. Moreover each of these proteins would be transported independently, thus, Snf1 would be transported with independence of Gal83. According to literature, Snf1 nuclear concentration is greater under low glucose conditions because under this circumstances Snf1 does not have affinity for the export machinery Xpo1, and therefore is retained in the nucleus. Gal83 also accumulates in the nucleus under low glucose conditions because its import is increased.

Recently it has been described that the *SUC2* gene is also associated with the nuclear pore complexes. Since NPC have been described to be involved not only in cargoes transport, but also in regulation of the gene expression and transcriptional memory, we decided to analyse the role of *SUC2* gene association with the NPC.

We have demonstrated that *SUC2* expression is affected by the absence of the scaffold nucleoporin Nup120. It was observed that in the absence of this nucleoporin *SUC2* gene derepression under low glucose conditions is twice the derepression observed in a wild-type strain. This, suggest that Nup120 is involve in repression of the *SUC2* gene. These idea is also supported by the fact together with Nup84, Nup120 associates with the SUC2-URS of the *SUC2* promoter, taking part of the repressor complex. Nup120 union to the *SUC2* promoter occurs in a glucose

dependent manner and its association is deregulated in the absence of Hxk2 and Mig1. In contrast, Nup84 union with the *SUC2* promoter is constitutive, and is only affected by the Mig1 absence.

In addition, our results suggest that Nup84 and Nup120 are also important to regulate Hxk2 recruitment to the *SUC2* promoter, as it was observed that in the absence of these nucleoporins Hxk2 union is constitutive and not regulated in a glucose-dependent manner. One possible explanation could be that nucleoporins help to position Hxk2 at the *SUC2* promoter.

Until now we have shown how our ChIP experiments support that the *SUC2* repressor complex dynamically disassembles and reassembles in a glucose-dependent manner, and how its association with the NPC is also important to maintain its structure and regulate its expression. However, the problem of how the glucose signal is transmitted from the environment to the nucleus to regulate *SUC2* expression is not solved. The results reported here provide for the first time a much clearer picture on this important point. The absence of Hxk2 impairs the recruitment of Mig1, Snf1 and Reg1 to the *SUC2* promoter and hence the assembly of a functional repressor complex. Since Hxk2 seems to be a key factor for the assembly of *SUC2* repressor complex, it could be a good candidate to operate as a glucose sensor that communicates glucose level information from the environment to the Mig1-regulated genes. A first question unanswered is how Hxk2 phosphorylation affects the Hxk2 recruitment to the *SUC2* promoter. Our data clearly suggest that an impaired Hxk2 phosphorylation at serine-14, as happens in the absence of the Snf1 kinase, does not alter either repressor complex formation or its glucose-dependent regulation. Thus, Hxk2 promotes the assembly of *SUC2*

repressor complex independently of its phosphorylation state. The phosphorylation state of Hxk2 regulates its nucleocytoplasmic shuttling between nucleus and cytoplasm, but not its assembly and disassembly to the repressor complex of the *SUC2* promoter.

A second possibility is that Hxk2 assembly to the *SUC2* promoter could be regulated by conformational changes induced by glucose levels (Kuser et al., 2000). Since measuring dynamic changes of cytosolic glucose by FRET sensors demonstrated that cytosolic glucose accumulation is a direct function of glucose levels in the medium (Bermejo et al., 2011), it seems likely that a Hxk2 conformational change induced by glucose could be the signal that induces Mig1-Hxk2 interaction. The Hxk2 protein is folded into two domains of unequal size called the large and the small domain (Aleshin et al., 1998). These are separated by a deep cleft containing the residues making up the enzyme active site. In low glucose conditions, the Hxk2 structure adopts an open conformation of the cleft between the two domains. In high glucose conditions, a movement of about 8 Å of the domains closing the cleft was observed. The closed active site conformation is probably completed after additional conformational changes that accompany ATP binding. In addition to significant conformational changes of the loops involved in glucose and ATP binding, differences in the conformation of the external loops are also observed (Kuser et al., 2000). Our *in vivo* and *in vitro* results show a striking correlation between a closed conformation (high glucose conditions) of Hxk2 and its binding to the *SUC2* repressor complex, or an open conformation (low glucose conditions, xylose) of Hxk2 and its dissociation from the *SUC2* promoter. Thus, here, we present evidence that supports the hypothesis that Hxk2 acts as a glucose sensor in the

signal transduction pathway mediating glucose-regulated gene expression in yeast; importantly, we document that the signalling activity of Hxk2 is linked to conformational changes induced by the glucose levels which promote its dissociation or reassociation to the *SUC2* promoter to control gene expression.

CONCLUSIONS

CONCLUSIONS

English conclusions.

1. Hxk2 recruitment to the repressor complex of the *SUC2* promoter is a glucose-dependent process. Its union occurs under high glucose conditions, while in low glucose conditions Hxk2 leaves the complex.
2. Mig1 is the main transcriptional repressor that controls *SUC2* gene expression. Its recruitment to the repressor complex of the *SUC2* promoter is a glucose-dependent process, in high glucose conditions Mig1 directly binds to the URSs elements but in low glucose condition a 30% of Mig1 remains bound to the *SUC2* promoter.
3. In high glucose conditions, Hxk2 stabilizes Mig1 binding to the URS elements of *SUC2* promoter but the interaction of Mig1 with DNA in low glucose conditions is stabilized by Hxk1.
4. The SNF1 complex factors Snf1, Snf4 and Gal83 form part of the repressor complex of the *SUC2* promoter. They are constitutively associated with SUC2-URS. Snf1 union with *SUC2* promoter is Hxk2-dependent, and Snf4 and Gal83 recruitment to the SUC2 elements of the *SUC2* promoter is mediated by Snf1.
5. Reg1, the main subunit of Glc7 phosphatase form part of the repressor complex at the *SUC2* promoter in both high and low glucose conditions, and its recruitment to the complex is Hxk2-dependent.
6. In a wild type yeast strain, Mig2 recruitment to the repressor complex of *SUC2* promoter has no role in the *SUC2* gene expression.

However, Mig2 is important to maintain repression in the absence of Mig1 or Hxk2.

7. Hxk2 is an essential component of the repressor complex of *SUC2* promoter. It is a critical factor to maintain the architecture of the complex, by stabilizing Mig1 association with the *SUC2* promoter, and by recruiting the regulator factors Snf1 and Reg1.
8. The SNF1 complex factors Snf1, Snf4 and Gal83, ant Reg1, interact with the classic import system mediated by Kap95 and Kap60. This interaction is detected in both high and low glucose conditions.
9. The SNF1 complex factors Snf1, Snf4 and Gal83, ant Reg1, interact with the export system mediated by Xpo1. This interaction is detected in both high and low glucose conditions, except for Snf1 which only interacts with Xpo1 under low glucose conditions.
10. The *SUC2* promoter is well engaged to the nuclear envelope by its interaction with the nucleoporins Nup84 and Nup120. Nup84 presents a constitutive association with the *SUC2* promoter, and Nup120 union with the *SUC2* promoter occurs in a glucose-dependent manner.
11. Nup120 association with the *SUC2* promoter is highly impaired in the absence of Hxk2 and Mig1.
12. Nup120 is involved in repressing the expression of the *SUC2* gene. In the absence of Nup120 under low glucose conditions, the *SUC2* derepression is twice the derepression observed in a wild-type strain.
13. Nup84 and Nup120 are involved in positioning Hxk2 to the *SUC2* promoter. In the absence of Nup84 or Nup120, Hxk2 is constitutively associated with the *SUC2* elements of the *SUC2* promoter.

14. Phosphorylation state of Hxk2 does not regulate its incorporation to repressor complex of the SUC2 promoter.
15. Hxk2 association with the SUC2 promoter is regulated by its conformation state. During high glucose conditions Hxk2 have a close conformation, which favours its binding to the SUC2 promoter. Under low glucose conditions, Hxk2 have an open conformation which prevents its association with the SUC2 promoter.
16. Hxk2 acts as a glucose sensor in the signal transduction pathway mediating glucose-regulated gene expression in yeast.

Spanish conclusions

1. La unión de Hxk2 con el promotor del gen *SUC2* es dependiente de glucosa. En condiciones de alta glucosa, Hxk2 está unido al promotor del gen *SUC2*, mientras que en baja glucosa Hxk2 abandona el complejo represor.
2. La expresión del gen *SUC2* está regulada principalmente por el represor transcripcional Mig1. La unión de Mig1 con el promotor del gen *SUC2* es dependiente de glucosa; en condiciones de alta glucosa es capaz de unirse directamente a los elementos URS del promotor *SUC2*, y en condiciones de baja glucosa tan solo un 30% de Mig1 permanece unido al promotor *SUC2*.
3. En condiciones de alta glucosa, Hxk2 es capaz de estabilizar la unión de Mig1 con el promotor del gen *SUC2*. En condiciones de baja glucosa la unión de Mig1 con *SUC2* es estabilizada por Hxk1.
4. Los factores del complejo SNF1, Snf1, Snf4 y Gal83, forman parte del complejo represor formado sobre el promotor del gen *SUC2*. Estos factores se encuentran unidos al promotor del gen *SUC2* de manera constitutiva. La unión de Snf1 es dependiente de Hxk2 y la unión de Snf4 y Gal83 se encuentra mediada por Snf1.
5. Reg1, la subunidad principal de la fosfatasa Glc7 forma parte del complejo represor formado en el promotor del gen *SUC2* en condiciones de alta y baja glucosa, y su unión con el complejo es dependiente de Hxk2.
6. En una cepa silvestre, la presencia de Mig2 en el complejo represor del gen *SUC2* no tiene ninguna influencia sobre su nivel de expresión.

Sin embargo, Mig2 es clave para mantener la represión en ausencia de las proteínas Mig1 o Hxk2.

7. Hxk2 es el componente esencial del complejo represor del gen *SUC2*, puesto que ayuda a mantener la estructura de este complejo, estabilizando la unión de Mig1 con el promotor del gen *SUC2* y reclutando a los factores reguladores Snf1 y Reg1.
8. Las subunidades del complejo SNF1, Snf1, Snf4 y Gal83, así como la proteína reguladora Reg1, interaccionan con el sistema clásico de importación nuclear mediado por las carioferinas Kap95/Kap60, tanto en condiciones de alta como de baja glucosa.
9. Las subunidades del complejo SNF1, Snf1, Snf4 y Gal83, así como la proteína reguladora Reg1, interaccionan con el sistema de exportación nuclear mediado por la exportina Xpo1.
10. El promotor del gen *SUC2* se encuentra asociado con la envuelta nuclear por su interacción con la nucleoporinas Nup84 y Nup120. Nup84 presenta una asociación constitutiva con el promotor del gen *SUC2*, mientras que la asociación de la nucleoporina Nup120 con el promotor del gen *SUC2* es dependiente de glucosa.
11. La asociación de Nup120 con el promotor del gen *SUC2* se ve alterada en ausencia de Mig1 o Hxk2.

12.- Nup120 está implicada en la represión del gen *SUC2*. En la ausencia de Nup120 en condiciones de baja glucosa los niveles de desrepresión del gen *SUC2* son el doble que los observados en las mismas condiciones en una cepa silvestre.

13. Nup84 y Nup120 están implicadas en el correcto posicionamiento de Hxk2 sobre el gen *SUC2*. En ausencia de Nup84 y Nup120, Hxk2 se encuentra unido de forma constitutiva al promotor del gen *SUC2*.

14.- El estado de fosforilación de Hxk2 no regula su incorporación y salida del complejo represor.

15. La asociación de Hxk2 al complejo represor está regulado por su estado conformacional. En condiciones de alta glucosa, Hxk2 tiene una conformación cerrada, la cual favorece su unión al promotor del gen *SUC2* y por lo tanto se induce la formación del complejo represor. Sin embargo en condiciones de baja glucosa, Hxk2 adquiere una conformación abierta que impide su unión al promotor del gen *SUC2*.

16.- Hxk2 actúa como un sensor intracelular de glucosa que regula la expresión de los genes regulados por glucosa.

German conclusions

1. Die Hxk2 Rekrutierung zum Repressor-Komplex des *SUC2* Promotors ist ein glukoseabhängige Prozess. Seine Zusammenführung findet statt unter hohem Glukosebedingungen, während bei niedrigen Glukosebedingungen Hxk2 den Komplex verlässt.
2. MIG1 ist der Haupttranskriptionsrepressor, der die *SUC2* Genexpression steuert. Seine Rekrutierung zum Repressor-Komplex des *SUC2* Promotors ist ein glukoseabhängige Prozess, bei hohen Glukosebedingungen bindet Mig1 direkt an die URS-Elemente, doch bei niedrigem Glucoselevel bleiben 30% von Mig1 am *SUC2* Promotors gebunden.
3. Bei hohem Glukoselevel stabilisiert Hxk2 die Bindung von Mig1 an die URS-Elemente des *SUC2*-Promotors, jedoch wird bei niedrigem Glukoselevel die Interaktion von Mig1 mit der DNA durch Hxk1 stabilisiert.
4. Die Faktoren des Snf1-Komplexes Snf1, Snf4 und Gal83 bilden einen Teil des Repressor-Komplexes des *SUC2*-Promotors. Sie sind konstitutiv mit *SUC2-URS* verbunden. Die Bindung von Snf1 mit dem *SUC2*-Promotor ist Hxk2-abhängig und die Rekrutierung von Snf4 und Gal83 zu den *SUC2*-Elementen des *SUC2*-Promotors wird durch Snf1 vermittelt.
5. Reg1, die Hauptuntereinheit der Glc7-Phosphatase bildet einen Teil des Repressor-Komplexes am *SUC2*-Promotor bei hohen und niedrigen Glukosebedingungen, und ihre Rekrutierung zum Komplex ist Hxk2-abhängig.

6. In einem Wildtyp-Hefestamm spielt bei der *SUC2*-Genexpression die Rekrutierung von Mig2 zum Repressorkomplex des *SUC2*-Promotors keine Rolle. Mig2 ist jedoch wichtig, um die Repression in Abwesenheit von Mig1 oder Hxk2 aufrechtzuerhalten.
7. Hxk2 ist eine wesentliche Komponente des Repressorskomplexes des *SUC2*-Promotors. Die Aufrechterhaltung der Architektur des Komplexes ist ein kritischer Faktor. Dies wird durch die Stabilisierung der Interaktion zwischen Mig1 und dem *SUC2*-Promotor erreicht, sowie durch die Rekrutierung der Regulationsfaktoren Snf1 und Reg1.
8. Die Faktoren des Snf1-Komplexes Snf1, Snf4 und Gal83 and Reg1, interagieren mit dem klassischen Importsystem vermittelt durch Kap95 und Kap60. Diese Wechselwirkung wurde bei hohen und niedrigen Glukosebedingungen detektiert.
9. Die Faktoren des Snf1-Komplexes Snf1, Snf4 und Gal83 and Reg1, interagieren mit dem Exportsystem vermittelt durch Xpo1. Diese Wechselwirkung wurde bei hohen und niedrigen Glukosebedingungen festgestellt, mit Ausnahme von Snf1, das mit Xpo1 nur unter niedrigen Glucosebedingungen wechselwirkt.
10. Der *SUC2*-Promotor ist stark mit der Kernhülle durch seine Interaktion mit den Nukleoporinen Nup84 und Nup120 verbunden. Nup84 zeigt eine konstitutive Assoziation mit dem *SUC2*-Promotor und eine Bindung von Nup120 an den *SUC2*-Promotor findet Glukose-abhängig statt.
11. Die Assoziation von Nup120 an den SUC2-Promotor wird stark durch die Abwesenheit von Hxk2 und Mig1 beeinträchtigt.
12. Nup120 ist bei der Repression der Expression des *SUC2*-Gens beteiligt. In Abwesenheit von Nup120 bei niedrigen

Glukosebedingungen ist die Derepression von *SUC2* doppelt so hoch wie es in einem Wildtyp-Stamm beobachtet wurde.

13. Nup84 und Nup120 sind bei der Positionierung von Hxk2 zum *SUC2*-Promotor beteiligt. In Abwesenheit von Nup84 oder Nup120 ist Hxk2 konstitutiv mit den *SUC2*-Elementen des *SUC2*-Promotors assoziiert.
14. Der Phosphorylierungsstatus von Hxk2 reguliert nicht seine Eingliederung in den Repressorkomplex des *SUC2*-Promotors.
15. Die Assoziierung von Hxk2 mit dem *SUC2*-Promotor wird durch seinen Konformationszustand reguliert. Während hoher Glukosebedingungen befindet sich Hxk2 in einer geschlossenen Konformation, die seine Bindung an den *SUC2*-Promotor begünstigt. Bei niedrigen Glukosebedingungen beschreibt Hxk2 eine offene Konformation, die seine Bindung an den *SUC2*-Promotor verhindert.
16. Hxk2 agiert als Glukosesensor in dem Signaltransduktionsweg, der die Glukose-regulierte Genexpression in Hefe vermittelt.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ahmed, S., D.G. Brickner, W.H. Light, I. Cajigas, M. McDonough, A.B. Froyshteter, T. Volpe, and J.H. Brickner. 2010. DNA zip codes control an ancient mechanism for gene targeting to the nuclear periphery. *Nat Cell Biol*. 12:111-118.

Ahuatzi, D., P. Herrero, T. de la Cera, and F. Moreno. 2004. The glucose-regulated nuclear localization of hexokinase 2 in Saccharomyces cerevisiae is Mig1-dependent. *J Biol Chem*. 279:14440-14446.

Ahuatzi, D., A. Riera, R. Pelaez, P. Herrero, and F. Moreno. 2007. Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. *J Biol Chem*. 282:4485-4493.

Alepuz, P.M., K.W. Cunningham, and F. Estruch. 1997. Glucose repression affects ion homeostasis in yeast through the regulation of the stress-activated ENA1 gene. *Mol Microbiol*. 26:91-98.

Aleshin, A.E., C. Zeng, G.P. Bourenkov, H.D. Bartunik, H.J. Fromm, and R.B. Honzatko. 1998. The mechanism of regulation of hexokinase: new insights from the crystal structure of recombinant human brain hexokinase complexed with glucose and glucose-6-phosphate. *Structure*. 6:39-50.

Arts, G.J., M. Fornerod, and I.W. Mattaj. 1998. Identification of a nuclear export receptor for tRNA. *Curr Biol*. 8:305-314.

Ashrafi, K., S.S. Lin, J.K. Manchester, and J.I. Gordon. 2000. Sip2p and its partner snf1p kinase affect aging in S. cerevisiae. *Genes Dev*. 14:1872-1885.

Barrett, L., M. Orlova, M. Maziarz, and S. Kuchin. 2012. Protein kinase A contributes to the negative control of Snf1 protein kinase in Saccharomyces cerevisiae. *Eukaryot Cell*. 11:119-128.

Belinchon, M.M., and J.M. Gancedo. 2007. Different signalling pathways mediate glucose induction of SUC2, HXT1 and pyruvate decarboxylase in yeast. *FEMS Yeast Res*. 7:40-47.

Bermejo, C., F. Haerizadeh, H. Takanaga, D. Chermak, and W.B. Frommer. 2011. Optical sensors for measuring dynamic changes of cytosolic metabolite levels in yeast. *Nat Protoc*. 6:1806-1817.

Bischoff, F.R., C. Klebe, J. Kretschmer, A. Wittinghofer, and H. Ponstingl. 1994. RanGAP1 induces GTPase activity of nuclear Ras-related Ran. *Proc Natl Acad Sci U S A*. 91:2587-2591.

Brickner, D.G., I. Cajigas, Y. Fondufe-Mittendorf, S. Ahmed, P.C. Lee, J. Widom, and J.H. Brickner. 2007. H2A.Z-mediated localization of genes at the nuclear periphery confers epigenetic memory of previous transcriptional state. *PLoS Biol*. 5:e81.

Brickner, J.H. 2009. Transcriptional memory at the nuclear periphery. *Curr Opin Cell Biol*. 21:127-133.

Brickner, J.H., and P. Walter. 2004. Gene recruitment of the activated INO1 locus to the nuclear membrane. *PLoS Biol*. 2:e342.

Bu, Y., and M.C. Schmidt. 1998. Identification of cis-acting elements in the SUC2 promoter of Saccharomyces cerevisiae required for activation of transcription. *Nucleic Acids Res*. 26:1002-1009.

Burnette, W.N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem*. 112:195-203.

Carlson, M., and D. Botstein. 1983. Organization of the SUC gene family in Saccharomyces. *Mol Cell Biol*. 3:351-359.

Carlson, M., B.C. Osmond, and D. Botstein. 1981. Mutants of yeast defective in sucrose utilization. *Genetics*. 98:25-40.

Carlson, M., R. Taussig, S. Kustu, and D. Botstein. 1983. The secreted form of invertase in Saccharomyces cerevisiae is synthesized from mRNA encoding a signal sequence. *Mol Cell Biol*. 3:439-447.

Castermans, D., I. Somers, J. Kriel, W. Louwet, S. Wera, M. Versele, V. Janssens, and J.M. Thevelein. 2012. Glucose-induced posttranslational activation of protein phosphatases PP2A and PP1 in yeast. *Cell Res*. 22:1058-1077.

Cereghino, G.P., and I.E. Scheffler. 1996. Genetic analysis of glucose regulation in saccharomyces cerevisiae: control of transcription versus mRNA turnover. *EMBO J*. 15:363-374.

Ciriacy, M., and V.M. Williamson. 1981. Analysis of mutations affecting Ty-mediated gene expression in Saccharomyces cerevisiae. *Mol Gen Genet*. 182:159-163.

Conti, E., and E. Izaurralde. 2001. Nucleocytoplasmic transport enters the atomic age. *Curr Opin Cell Biol*. 13:310-319.

Conti, E., C.W. Muller, and M. Stewart. 2006. Karyopherin flexibility in nucleocytoplasmic transport. *Curr Opin Struct Biol*. 16:237-244.

Conti, E., M. Uy, L. Leighton, G. Blobel, and J. Kuriyan. 1998. Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. *Cell*. 94:193-204.

Craig, E., Z.K. Zhang, K.P. Davies, and G.V. Kalpana. 2002. A masked NES in INI1/hSNF5 mediates hCRM1-dependent nuclear export: implications for tumorigenesis. *EMBO J*. 21:31-42.

Cronshaw, J.M., A.N. Krutchinsky, W. Zhang, B.T. Chait, and M.J. Matunis. 2002. Proteomic analysis of the mammalian nuclear pore complex. *J Cell Biol*. 158:915-927.

Cullen, P.J., and G.F. Sprague, Jr. 2000. Glucose depletion causes haploid invasive growth in yeast. *Proc Natl Acad Sci U S A*. 97:13619-13624.

Chook, Y.M., and G. Blobel. 1999. Structure of the nuclear transport complex karyopherin-beta2-Ran x GppNHp. *Nature*. 399:230-237.

Chook, Y.M., and G. Blobel. 2001. Karyopherins and nuclear import. *Curr Opin Struct Biol*. 11:703-715.

Chook, Y.M., A. Jung, M.K. Rosen, and G. Blobel. 2002. Uncoupling Kapbeta2 substrate dissociation and ran binding. *Biochemistry*. 41:6955-6966.

Chook, Y.M., and K.E. Suel. 2011. Nuclear import by karyopherin-betas: recognition and inhibition. *Biochim Biophys Acta*. 1813:1593-1606.

De Vit, M.J., J.A. Waddle, and M. Johnston. 1997. Regulated nuclear translocation of the Mig1 glucose repressor. *Mol Biol Cell*. 8:1603-1618.

DelaFuente, G. 1970. Specific inactivation of yeast hexokinase induced by xylose in the presence of a phosphoryl donor substrate. *Eur J Biochem*. 16:240-243.

DeVit, M.J., and M. Johnston. 1999. The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of Saccharomyces cerevisiae. *Curr Biol*. 9:1231-1241.

Dieppois, G., and F. Stutz. 2010. Connecting the transcription site to the nuclear pore: a multi-tether process that regulates gene expression. *J Cell Sci*. 123:1989-1999.

Dingwall, C., S.V. Sharnick, and R.A. Laskey. 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell*. 30:449-458.

Dombek, K.M., V. Voronkova, A. Raney, and E.T. Young. 1999. Functional analysis of the yeast Glc7-binding protein Reg1 identifies a protein phosphatase type 1-binding motif as essential for repression of ADH2 expression. *Mol Cell Biol*. 19:6029-6040.

Dong, X., A. Biswas, K.E. Suel, L.K. Jackson, R. Martinez, H. Gu, and Y.M. Chook. 2009. Structural basis for leucine-rich nuclear export signal recognition by CRM1. *Nature*. 458:1136-1141.

Dubacq, C., A. Chevalier, and C. Mann. 2004. The protein kinase Snf1 is required for tolerance to the ribonucleotide reductase inhibitor hydroxyurea. *Mol Cell Biol*. 24:2560-2572.

Elbing, K., R.R. McCartney, and M.C. Schmidt. 2006. Purification and characterization of the three Snf1-activating kinases of Saccharomyces cerevisiae. *Biochem J*. 393:797-805.

Fang, X., T. Chen, K. Tran, and C.S. Parker. 2001. Developmental regulation of the heat shock response by nuclear transport factor karyopherin-alpha3. *Development*. 128:3349-3358.

Fernandez-Cid, A., M. Vega, P. Herrero, and F. Moreno. 2012. Yeast importin-beta is required for nuclear import of the Mig2 repressor. *BMC Cell Biol*. 13:31.

Fernandez-Garcia, P., R. Pelaez, P. Herrero, and F. Moreno. 2012. Phosphorylation of yeast hexokinase 2 regulates its nucleocytoplasmic shuttling. *J Biol Chem*. 287:42151-42164.

Fernandez, R., P. Herrero, M.T. Fernandez, and F. Moreno. 1986. Mechanism of inactivation of hexokinase PII of Saccharomyces cerevisiae by D-xylose. *J Gen Microbiol*. 132:3467-3472.

Fernandez, R., P. Herrero, and F. Moreno. 1985. Inhibition and inactivation of glucose-phosphorylating enzymes from Saccharomyces cerevisiae by D-xylose. *J Gen Microbiol*. 131:2705-2709.

Flick, K.M., N. Spielewoy, T.I. Kalashnikova, M. Guaderrama, Q. Zhu, H.C. Chang, and C. Wittenberg. 2003. Grr1-dependent inactivation of Mth1 mediates glucose-induced dissociation of Rgt1 from HXT gene promoters. *Mol Biol Cell*. 14:3230-3241.

Gancedo, C., and C.L. Flores. 2008. Moonlighting proteins in yeasts. *Microbiol Mol Biol Rev*. 72:197-210, table of contents.

Gancedo, J.M. 1998. Yeast carbon catabolite repression. *Microbiol Mol Biol Rev*. 62:334-361.

Gascon, S., N.P. Neumann, and J.O. Lampen. 1968. Comparative study of the properties of the purified internal and external invertases from yeast. *J Biol Chem*. 243:1573-1577.

Gertz, J., E.D. Siggia, and B.A. Cohen. 2009. Analysis of combinatorial cis-regulation in synthetic and genomic promoters. *Nature*. 457:215-218.

Golbik, R., M. Naumann, A. Otto, E. Muller, J. Behlke, R. Reuter, G. Hubner, and T.M. Kriegel. 2001. Regulation of phosphotransferase activity of hexokinase 2 from Saccharomyces cerevisiae by modification at serine-14. *Biochemistry*. 40:1083-1090.

Gorlich, D., S. Kostka, R. Kraft, C. Dingwall, R.A. Laskey, E. Hartmann, and S. Prehn. 1995. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr Biol*. 5:383-392.

Gorlich, D., and U. Kutay. 1999. Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol*. 15:607-660.

Grossman, E., O. Medalia, and M. Zwerger. 2012. Functional architecture of the nuclear pore complex. *Annu Rev Biophys*. 41:557-584.

Groves, M.R., and D. Barford. 1999. Topological characteristics of helical repeat proteins. *Curr Opin Struct Biol*. 9:383-389.

Hammell, C.M., S. Gross, D. Zenklusen, C.V. Heath, F. Stutz, C. Moore, and C.N. Cole. 2002. Coupling of termination, 3' processing, and mRNA export. *Mol Cell Biol*. 22:6441-6457.

Hedbacker, K., and M. Carlson. 2006. Regulation of the nucleocytoplasmic distribution of Snf1-Gal83 protein kinase. *Eukaryot Cell*. 5:1950-1956.

Hedbacker, K., and M. Carlson. 2008. SNF1/AMPK pathways in yeast. *Front Biosci*. 13:2408-2420.

Heidrich, K., A. Otto, J. Behlke, J. Rush, K.W. Wenzel, and T. Kriegel. 1997. Autophosphorylation-inactivation site of hexokinase 2 in Saccharomyces cerevisiae. *Biochemistry*. 36:1960-1964.

Henderson, B.R., and A. Eleftheriou. 2000. A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp Cell Res*. 256:213-224.

Herrero, P., J. Galindez, N. Ruiz, C. Martinez-Campa, and F. Moreno. 1995. Transcriptional regulation of the Saccharomyces cerevisiae HXK1, HXK2 and GLK1 genes. *Yeast*. 11:137-144.

Herrero, P., C. Martinez-Campa, and F. Moreno. 1998. The hexokinase 2 protein participates in regulatory DNA-protein complexes necessary for glucose

repression of the SUC2 gene in Saccharomyces cerevisiae. *FEBS Lett*. 434:71-76.

Hong, S.P., and M. Carlson. 2007. Regulation of snf1 protein kinase in response to environmental stress. *J Biol Chem*. 282:16838-16845.

Hong, S.P., F.C. Leiper, A. Woods, D. Carling, and M. Carlson. 2003. Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc Natl Acad Sci U S A*. 100:8839-8843.

Honigberg, S.M., and R.H. Lee. 1998. Snf1 kinase connects nutritional pathways controlling meiosis in Saccharomyces cerevisiae. *Mol Cell Biol*. 18:4548-4555.

Hood, J.K., and P.A. Silver. 1998. Cse1p is required for export of Srp1p/importin-alpha from the nucleus in Saccharomyces cerevisiae. *J Biol Chem*. 273:35142-35146.

Hubner, S., C.Y. Xiao, and D.A. Jans. 1997. The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. *J Biol Chem*. 272:17191-17195.

Jones, S., M.L. Vignais, and J.R. Broach. 1991. The CDC25 protein of Saccharomyces cerevisiae promotes exchange of guanine nucleotides bound to ras. *Mol Cell Biol*. 11:2641-2646.

Jovanovic-Talisman, T., J. Tetenbaum-Novatt, A.S. McKenney, A. Zilman, R. Peters, M.P. Rout, and B.T. Chait. 2009. Artificial nanopores that mimic the transport selectivity of the nuclear pore complex. *Nature*. 457:1023-1027.

Kim, J.H., J. Polish, and M. Johnston. 2003. Specificity and regulation of DNA binding by the yeast glucose transporter gene repressor Rgt1. *Mol Cell Biol*. 23:5208-5216.

Kiseleva, E., T.D. Allen, S. Rutherford, M. Bucci, S.R. Wente, and M.W. Goldberg. 2004. Yeast nuclear pore complexes have a cytoplasmic ring and internal filaments. *J Struct Biol*. 145:272-288.

Klein, C.J., L. Olsson, and J. Nielsen. 1998. Glucose control in Saccharomyces cerevisiae: the role of Mig1 in metabolic functions. *Microbiology*. 144 (Pt 1):13-24.

Komeili, A., and E.K. O'Shea. 1999. Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. *Science*. 284:977-980.

Koshland, D.E. 1958. Application of a Theory of Enzyme Specificity to Protein Synthesis. *Proc Natl Acad Sci U S A*. 44:98-104.

Kosugi, S., M. Hasebe, N. Matsumura, H. Takashima, E. Miyamoto-Sato, M. Tomita, and H. Yanagawa. 2009. Six classes of nuclear localization signals specific to different binding grooves of importin alpha. *J Biol Chem*. 284:478-485.

Kresnowati, M.T., W.A. van Winden, M.J. Almering, A. ten Pierick, C. Ras, T.A. Knijnenburg, P. Daran-Lapujade, J.T. Pronk, J.J. Heijnen, and J.M. Daran. 2006. When transcriptome meets metabolome: fast cellular responses of yeast to sudden relief of glucose limitation. *Mol Syst Biol*. 2:49.

Krishnan, V.V., E.Y. Lau, J. Yamada, D.P. Denning, S.S. Patel, M.E. Colvin, and M.F. Rexach. 2008. Intramolecular cohesion of coils mediated by phenylalanine--glycine motifs in the natively unfolded domain of a nucleoporin. *PLoS Comput Biol*. 4:e1000145.

Kuchin, S., V.K. Vyas, and M. Carlson. 2002. Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate FLO11, haploid invasive growth, and diploid pseudohyphal differentiation. *Mol Cell Biol*. 22:3994-4000.

Kundu, S., P.J. Horn, and C.L. Peterson. 2007. SWI/SNF is required for transcriptional memory at the yeast GAL gene cluster. *Genes Dev*. 21:997-1004.

Kuser, P., F. Cupri, L. Bleicher, and I. Polikarpov. 2008. Crystal structure of yeast hexokinase PI in complex with glucose: A classical "induced fit" example revised. *Proteins*. 72:731-740.

Kuser, P.R., S. Krauchenco, O.A. Antunes, and I. Polikarpov. 2000. The high resolution crystal structure of yeast hexokinase PII with the correct primary sequence provides new insights into its mechanism of action. *J Biol Chem*. 275:20814-20821.

la Cour, T., L. Kiemer, A. Molgaard, R. Gupta, K. Skriver, and S. Brunak. 2004. Analysis and prediction of leucine-rich nuclear export signals. *Protein Eng Des Sel*. 17:527-536.

Lakshmanan, J., A.L. Mosley, and S. Ozcan. 2003. Repression of transcription by Rgt1 in the absence of glucose requires Std1 and Mth1. *Curr Genet*. 44:19-25.

Lui, K., and Y. Huang. 2009. RanGTPase: A Key Regulator of Nucleocytoplasmic Trafficking. *Mol Cell Pharmacol*. 1:148-156.

Lundin, M., J.O. Nehlin, and H. Ronne. 1994. Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein MIG1. *Mol Cell Biol*. 14:1979-1985.

Lutfiyya, L.L., V.R. Iyer, J. DeRisi, M.J. DeVit, P.O. Brown, and M. Johnston. 1998. Characterization of three related glucose repressors and genes they regulate in Saccharomyces cerevisiae. *Genetics*. 150:1377-1391.

Lutfiyya, L.L., and M. Johnston. 1996. Two zinc-finger-containing repressors are responsible for glucose repression of SUC2 expression. *Mol Cell Biol*. 16:4790-4797.

Ma, H., and D. Botstein. 1986. Effects of null mutations in the hexokinase genes of Saccharomyces cerevisiae on catabolite repression. *Mol Cell Biol*. 6:4046-4052.

Matsuura, Y., and M. Stewart. 2004. Structural basis for the assembly of a nuclear export complex. *Nature*. 432:872-877.

Mayer, F.V., R. Heath, E. Underwood, M.J. Sanders, D. Carmena, R.R. McCartney, F.C. Leiper, B. Xiao, C. Jing, P.A. Walker, L.F. Haire, R. Ogrodowicz, S.R. Martin, M.C. Schmidt, S.J. Gamblin, and D. Carling. 2011. ADP regulates SNF1, the Saccharomyces cerevisiae homolog of AMP-activated protein kinase. *Cell Metab*. 14:707-714.

Mayordomo, I., F. Estruch, and P. Sanz. 2002. Convergence of the target of rapamycin and the Snf1 protein kinase pathways in the regulation of the subcellular localization of Msn2, a transcriptional activator of STRE (Stress Response Element)-regulated genes. *J Biol Chem*. 277:35650-35656.

McCartney, R.R., and M.C. Schmidt. 2001. Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. *J Biol Chem*. 276:36460-36466.

Mercado, J.J., R. Smith, F.A. Sagliocco, A.J. Brown, and J.M. Gancedo. 1994. The levels of yeast gluconeogenic mRNAs respond to environmental factors. *Eur J Biochem*. 224:473-481.

Mercado, J.J., O. Vincent, and J.M. Gancedo. 1991. Regions in the promoter of the yeast FBP1 gene implicated in catabolite repression may bind the product of the regulatory gene MIG1. *FEBS Lett*. 291:97-100.

Misteli, T. 2007. Beyond the sequence: cellular organization of genome function. *Cell*. 128:787-800.

Moreno, F., D. Ahuatzi, A. Riera, C.A. Palomino, and P. Herrero. 2005. Glucose sensing through the Hxk2-dependent signalling pathway. *Biochem Soc Trans*. 33:265-268.

Moreno, F., T. Fernandez, R. Fernandez, and P. Herrero. 1986. Hexokinase PII from Saccharomyces cerevisiae is regulated by changes in the cytosolic Mg2+-free ATP concentration. *Eur J Biochem*. 161:565-569.

Moreno, F., and P. Herrero. 2002. The hexokinase 2-dependent glucose signal transduction pathway of Saccharomyces cerevisiae. *FEMS Microbiol Rev*. 26:83-90.

Moriya, H., and M. Johnston. 2004. Glucose sensing and signaling in Saccharomyces cerevisiae through the Rgt2 glucose sensor and casein kinase I. *Proc Natl Acad Sci U S A*. 101:1572-1577.

Mosammaparast, N., and L.F. Pemberton. 2004. Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends Cell Biol*. 14:547-556.

Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol*. 51 Pt 1:263-273.

Nath, N., R.R. McCartney, and M.C. Schmidt. 2003. Yeast Pak1 kinase associates with and activates Snf1. *Mol Cell Biol*. 23:3909-3917.

Nehlin, J.O., M. Carlberg, and H. Ronne. 1991. Control of yeast GAL genes by MIG1 repressor: a transcriptional cascade in the glucose response. *EMBO J*. 10:3373-3377.

Nehlin, J.O., and H. Ronne. 1990. Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *EMBO J*. 9:2891-2898.

Niepel, M., K.R. Molloy, R. Williams, J.C. Farr, A.C. Meinema, N. Vecchietti, I.M. Cristea, B.T. Chait, M.P. Rout, and C. Strambio-De-Castillia. 2013. The

nuclear basket proteins Mlp1p and Mlp2p are part of a dynamic interactome including Esc1p and the proteasome. *Mol Biol Cell*. 24:3920-3938.

Noubhani, A., O. Bunoust, B.M. Bonini, J.M. Thevelein, A. Devin, and M. Rigoulet. 2009. The trehalose pathway regulates mitochondrial respiratory chain content through hexokinase 2 and cAMP in Saccharomyces cerevisiae. *J Biol Chem*. 284:27229-27234.

Ozcan, S., J. Dover, and M. Johnston. 1998. Glucose sensing and signaling by two glucose receptors in the yeast Saccharomyces cerevisiae. *EMBO J*. 17:2566-2573.

Ozcan, S., and M. Johnston. 1995. Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. *Mol Cell Biol*. 15:1564-1572.

Ozcan, S., and M. Johnston. 1996. Two different repressors collaborate to restrict expression of the yeast glucose transporter genes HXT2 and HXT4 to low levels of glucose. *Mol Cell Biol*. 16:5536-5545.

Ozcan, S., and M. Johnston. 1999. Function and regulation of yeast hexose transporters. *Microbiol Mol Biol Rev*. 63:554-569.

Palomino, A., P. Herrero, and F. Moreno. 2005. Rgt1, a glucose sensing transcription factor, is required for transcriptional repression of the HXK2 gene in Saccharomyces cerevisiae. *Biochem J*. 388:697-703.

Palomino, A., P. Herrero, and F. Moreno. 2006. Tpk3 and Snf1 protein kinases regulate Rgt1 association with Saccharomyces cerevisiae HXK2 promoter. *Nucleic Acids Res*. 34:1427-1438.

Pao, S.S., I.T. Paulsen, and M.H. Saier, Jr. 1998. Major facilitator superfamily. *Microbiol Mol Biol Rev*. 62:1-34.

Pasula, S., D. Jouandot, 2nd, and J.H. Kim. 2007. Biochemical evidence for glucose-independent induction of HXT expression in Saccharomyces cerevisiae. *FEBS Lett*. 581:3230-3234.

Patel, S.S., B.J. Belmont, J.M. Sante, and M.F. Rexach. 2007. Natively unfolded nucleoporins gate protein diffusion across the nuclear pore complex. *Cell*. 129:83-96.

Peeters, T., W. Louwet, R. Gelade, D. Nauwelaers, J.M. Thevelein, and M. Versele. 2006. Kelch-repeat proteins interacting with the Galpha protein Gpa2 bypass adenylate cyclase for direct regulation of protein kinase A in yeast. *Proc Natl Acad Sci U S A*. 103:13034-13039.

Pelaez, R., P. Fernandez-Garcia, P. Herrero, and F. Moreno. 2012. Nuclear import of the yeast hexokinase 2 protein requires alpha/beta-importin-dependent pathway. *J Biol Chem*. 287:3518-3529.

Pelaez, R., P. Herrero, and F. Moreno. 2009. Nuclear export of the yeast hexokinase 2 protein requires the Xpo1 (Crm1)-dependent pathway. *J Biol Chem*. 284:20548-20555.

Pelaez, R., P. Herrero, and F. Moreno. 2010. Functional domains of yeast hexokinase 2. *Biochem J*. 432:181-190.

Pemberton, L.F., G. Blobel, and J.S. Rosenblum. 1998. Transport routes through the nuclear pore complex. *Curr Opin Cell Biol*. 10:392-399.

Pemberton, L.F., and B.M. Paschal. 2005. Mechanisms of receptor-mediated nuclear import and nuclear export. *Traffic*. 6:187-198.

Peters, R. 2005. Translocation through the nuclear pore complex: selectivity and speed by reduction-of-dimensionality. *Traffic*. 6:421-427.

Portela, P., and S. Moreno. 2006. Glucose-dependent activation of protein kinase A activity in Saccharomyces cerevisiae and phosphorylation of its TPK1 catalytic subunit. *Cell Signal*. 18:1072-1086.

Portillo, F., J.M. Mulet, and R. Serrano. 2005. A role for the non-phosphorylated form of yeast Snf1: tolerance to toxic cations and activation of potassium transport. *FEBS Lett*. 579:512-516.

Randez-Gil, F., P. Sanz, K.D. Entian, and J.A. Prieto. 1998. Carbon source-dependent phosphorylation of hexokinase PII and its role in the glucose-signaling response in yeast. *Mol Cell Biol*. 18:2940-2948.

Ribbeck, K., and D. Gorlich. 2002. The permeability barrier of nuclear pore complexes appears to operate via hydrophobic exclusion. *EMBO J*. 21:2664-2671.

Riviere, Y., V. Blank, P. Kourilsky, and A. Israel. 1991. Processing of the precursor of NF-kappa B by the HIV-1 protease during acute infection. *Nature*. 350:625-626.

Rodriguez, A., T. De La Cera, P. Herrero, and F. Moreno. 2001. The hexokinase 2 protein regulates the expression of the GLK1, HXK1 and HXK2 genes of Saccharomyces cerevisiae. *Biochem J*. 355:625-631.

Rolland, F., J. Winderickx, and J.M. Thevelein. 2002. Glucose-sensing and -signalling mechanisms in yeast. *FEMS Yeast Res*. 2:183-201.

Rout, M.P., J.D. Aitchison, M.O. Magnasco, and B.T. Chait. 2003. Virtual gating and nuclear transport: the hole picture. *Trends Cell Biol*. 13:622-628.

Rout, M.P., J.D. Aitchison, A. Suprapto, K. Hjertaas, Y. Zhao, and B.T. Chait. 2000. The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J Cell Biol*. 148:635-651.

Rout, M.P., and G. Blobel. 1993. Isolation of the yeast nuclear pore complex. *J Cell Biol*. 123:771-783.

Rubenstein, E.M., R.R. McCartney, C. Zhang, K.M. Shokat, M.K. Shirra, K.M. Arndt, and M.C. Schmidt. 2008. Access denied: Snf1 activation loop phosphorylation is controlled by availability of the phosphorylated threonine 210 to the PP1 phosphatase. *J Biol Chem*. 283:222-230.

Santangelo, G.M. 2006. Glucose signaling in Saccharomyces cerevisiae. *Microbiol Mol Biol Rev*. 70:253-282.

Sanz, P., G.R. Alms, T.A. Haystead, and M. Carlson. 2000. Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. *Mol Cell Biol*. 20:1321-1328.

Sarma, N.J., T.D. Buford, T. Haley, K. Barbara-Haley, G.M. Santangelo, and K.A. Willis. 2011. The nuclear pore complex mediates binding of the Mig1 repressor to target promoters. *PLoS One*. 6:e27117.

Sarma, N.J., T.M. Haley, K.E. Barbara, T.D. Buford, K.A. Willis, and G.M. Santangelo. 2007. Glucose-responsive regulators of gene expression in Saccharomyces cerevisiae function at the nuclear periphery via a reverse recruitment mechanism. *Genetics*. 175:1127-1135.

Schmid, M., G. Arib, C. Laemmli, J. Nishikawa, T. Durussel, and U.K. Laemmli. 2006. Nup-PI: the nucleopore-promoter interaction of genes in yeast. *Mol Cell*. 21:379-391.

Schmidt, M.C., R.R. McCartney, X. Zhang, T.S. Tillman, H. Solimeo, S. Wolfl, C. Almonte, and S.C. Watkins. 1999. Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in Saccharomyces cerevisiae. *Mol Cell Biol*. 19:4561-4571.

Schuurmans, J.M., A. Boorsma, R. Lascaris, K.J. Hellingwerf, and M.J. Teixeira de Mattos. 2008. Physiological and transcriptional characterization of Saccharomyces cerevisiae strains with modified expression of catabolic regulators. *FEMS Yeast Res*. 8:26-34.

Smith, F.C., S.P. Davies, W.A. Wilson, D. Carling, and D.G. Hardie. 1999. The SNF1 kinase complex from Saccharomyces cerevisiae phosphorylates the transcriptional repressor protein Mig1p in vitro at four sites within or near regulatory domain 1. *FEBS Lett*. 453:219-223.

Strawn, L.A., T. Shen, N. Shulga, D.S. Goldfarb, and S.R. Wente. 2004. Minimal nuclear pore complexes define FG repeat domains essential for transport. *Nat Cell Biol*. 6:197-206.

Strom, A.C., and K. Weis. 2001. Importin-beta-like nuclear transport receptors. *Genome Biol*. 2:REVIEWS3008.

Suntharalingam, M., and S.R. Wente. 2003. Peering through the pore: nuclear pore complex structure, assembly, and function. *Dev Cell*. 4:775-789.

Sutherland, C.M., S.A. Hawley, R.R. McCartney, A. Leech, M.J. Stark, M.C. Schmidt, and D.G. Hardie. 2003. Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex. *Curr Biol*. 13:1299-1305.

Taberner, F.J., and J.C. Igual. 2010. Yeast karyopherin Kap95 is required for cell cycle progression at Start. *BMC Cell Biol*. 11:47.

Tanaka, K., M. Nakafuku, T. Satoh, M.S. Marshall, J.B. Gibbs, K. Matsumoto, Y. Kaziro, and A. Toh-e. 1990. S. cerevisiae genes IRA1 and IRA2 encode proteins that may be functionally equivalent to mammalian ras GTPase activating protein. *Cell*. 60:803-807.

Texari, L., G. Dieppois, P. Vinciguerra, M.P. Contreras, A. Groner, A. Letourneau, and F. Stutz. 2013. The nuclear pore regulates GAL1 gene transcription by controlling the localization of the SUMO protease Ulp1. *Mol Cell*. 51:807-818.

Thompson-Jaeger, S., J. Francois, J.P. Gaughran, and K. Tatchell. 1991. Deletion of SNF1 affects the nutrient response of yeast and resembles mutations which activate the adenylate cyclase pathway. *Genetics*. 129:697-706.

Treitel, M.A., and M. Carlson. 1995. Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proc Natl Acad Sci U S A*. 92:3132-3136.

Treitel, M.A., S. Kuchin, and M. Carlson. 1998. Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in Saccharomyces cerevisiae. *Mol Cell Biol*. 18:6273-6280.

Trimble, R.B., and F. Maley. 1977. Subunit structure of external invertase from Saccharomyces cerevisiae. *J Biol Chem*. 252:4409-4412.

Tu, J., and M. Carlson. 1995. REG1 binds to protein phosphatase type 1 and regulates glucose repression in Saccharomyces cerevisiae. *EMBO J*. 14:5939-5946.

Vetter, I.R., A. Arndt, U. Kutay, D. Gorlich, and A. Wittinghofer. 1999. Structural view of the Ran-Importin beta interaction at 2.3 A resolution. *Cell*. 97:635-646.

Vincent, O., R. Townley, S. Kuchin, and M. Carlson. 2001. Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes Dev*. 15:1104-1114.

Wen, W., J.L. Meinkoth, R.Y. Tsien, and S.S. Taylor. 1995. Identification of a signal for rapid export of proteins from the nucleus. *Cell*. 82:463-473.

Winey, M., D. Yarar, T.H. Giddings, Jr., and D.N. Mastronarde. 1997. Nuclear pore complex number and distribution throughout the Saccharomyces cerevisiae cell cycle by three-dimensional reconstruction from electron micrographs of nuclear envelopes. *Mol Biol Cell*. 8:2119-2132.

Yin, Z., S. Wilson, N.C. Hauser, H. Tournu, J.D. Hoheisel, and A.J. Brown. 2003. Glucose triggers different global responses in yeast, depending on the strength of the signal, and transiently stabilizes ribosomal protein mRNAs. *Mol Microbiol*. 48:713-724.

Yoshida, K., and G. Blobel. 2001. The karyopherin Kap142p/Msn5p mediates nuclear import and nuclear export of different cargo proteins. *J Cell Biol*. 152:729-740.

Yun, C.W., H. Tamaki, R. Nakayama, K. Yamamoto, and H. Kumagai. 1997. G-protein coupled receptor from yeast Saccharomyces cerevisiae. *Biochem Biophys Res Commun*. 240:287-292.

Zhao, X., C.Y. Wu, and G. Blobel. 2004. Mlp-dependent anchorage and stabilization of a desumoylating enzyme is required to prevent clonal lethality. *J Cell Biol*. 167:605-611.

PUBLICATIONS

PUBLICATIONS

The following papers have been published during the development of this thesis, and they are attached in the following pages.

Alejandra Fernández-Cid, **Montserrat Vega**, Pilar Herrero, and Fernando Moreno. Yeast importin-β is required for nuclear import of the Mig2 repressor. BMC Cell Biol. 2012 Nov 6;13:31. doi: 10.1186/1471-2121-13-31.

Montserrat Vega, Alberto Riera, Alejandra Fernández-Cid, Pilar Herrero and Fernando Moreno. Hexokinase 2 is an intracellular glucose sensor of yeast cells that maintains the structure and activity of Mig1 repressor complex. (Submitted manuscript)

BMC Cell Biology

RESEARCH ARTICLE Open Access

Yeast importin-β is required for nuclear import of the Mig2 repressor

Alejandra Fernández-Cid[1,2], Montserrat Vega[1], Pilar Herrero[1] and Fernando Moreno[1*]

Abstract

Background: Mig2 has been described as a transcriptional factor that in the absence of Mig1 protein is required for glucose repression of the *SUC2* gene. Recently it has been reported that Mig2 has two different subcellular localizations. In high-glucose conditions it is a nuclear modulator of several Mig1-regulated genes, but in low-glucose most of the Mig2 protein accumulates in mitochondria. Thus, the Mig2 protein enters and leaves the nucleus in a glucose regulated manner. However, the mechanism by which Mig2 enters into the nucleus was unknown until now.

Results: Here, we report that the Mig2 protein is an import substrate of the carrier Kap95 (importin-β). The Mig2 nuclear import mechanism bypasses the requirement for Kap60 (importin-α) as an adaptor protein, since Mig2 directly binds to Kap95 in the presence of Gsp1(GDP). We also show that the Mig2 nuclear import and the binding of Mig2 with Kap95 are not glucose-dependent processes and require a basic NLS motif, located between lysine-32 and arginine-37. Mig2 interaction with Kap95 was assessed *in vitro* using purified proteins, demonstrating that importin-β, together with the GTP-binding protein Gsp1, is able to mediate efficient Mig2-Kap95 interaction in the absence of the importin-α (Kap60). It was also demonstrated, that the directionality of Mig2 transport is regulated by association with the small GTPase Gsp1 in the GDP- or GTP-bound forms, which promote cargo recognition and release, respectively.

Conclusions: The Mig2 protein accumulates in the nucleus through a Kap95 and NLS-dependent nuclear import pathway, which is independent of importin-α in *Saccharomyces cerevisiae*.

Background

In eukaryotic cells the confinement of the genetic material and the transcriptional machinery to the nucleus allows the regulation of central processes including gene expression, signal transduction and control of the cell cycle. One of the key features of such regulation is the selective bi-directional transport of several proteins between the cytoplasm and the nucleus. Such transport happens through large membrane structures termed nuclear pore complexes (NPCs), comprised of approximately 30 proteins collectively termed nucleoporins (Nups) [1]. The mechanism of translocation through the pore is not well understood, but is facilitated by a number of nuclear transport factors or carrier proteins termed karyopherins [2]. In higher eukaryotes, as well as in the yeast *S. cerevisiae*, the karyopherins that mediate nuclear import of proteins and RNAs are generally known as importins [3], whereas those mediating nuclear export are known as exportins [4]. Importins associate with their macromolecular cargo in the cytoplasm, either directly or indirectly via adaptor proteins [5]. Cargo release is achieved by association of the importins with the GTPase Ran (Gsp1 in yeast) in the GTP-bound form (Gsp1-GTP) [6]. Nuclear export is essentially the reciprocal process, with cargo recognition occurring in the nucleus in the presence of Gsp1(GTP) and cargo complexes dissociating in the cytoplasm upon GTP hydrolysis. Gsp1 is present at high concentrations in the nucleus in the GTP-bound form, and mainly in the GDP-bound form in the cytoplasm [7]. This compartmentalization depends on the localization of the proteins that regulate the nucleotide state of Gsp1. GTP hydrolysis requires a GTPase-activating protein (GAP) that is present in the cytoplasm. Conversely, the exchange of

* Correspondence: fmoreno@uniovi.es
[1]Department of Biochemistry and Molecular Biology, University of Oviedo, 33006, Oviedo, Spain
Full list of author information is available at the end of the article

GDP to GTP is catalyzed by a guanine nucleotide exchange factor (GEF) that is bound to the chromatin [1,8,9]. Importin-β (Kap95 in yeast) was first identified as the transport factor for proteins carrying classical nuclear localization signals (NLSs), such as those of nucleoplasmin or the SV40 T antigen [10]. Importin-β hardly ever binds these classical NLSs directly, but binds importin-α (Kap60 in yeast), which in turn binds these NLSs [5]. While this pathway is termed the classical nuclear import pathway, several alternative import pathways have been characterized. Most karyopherins bind their cargos directly, and even importin-β, in some cases, is able to recognize cargo substrates without the need for an importin-α (Kap60) adaptor protein, as in the case of ribosomal proteins [11], SREBP- 2 [12], cyclin B1 [13], SRY [14], PTHrP [15] or yeast phosphatidylinositol 4-kinase [16].

Mig2 was identified several years ago as a repressor that collaborates with Mig1 to cause glucose-induced repression of the *SUC2* gene [17]. A genome wide expression profiling survey of the yeast genome revealed that all the genes regulated by Mig1 are also regulated by Mig2. Thus, it was postulated that Mig2 always works in conjunction with Mig1 [18]. However, Mig1 appears to be the essential component, since its presence is necessary and sufficient to cause full repression of most genes even in the absence of Mig2. Mig1 is a zinc-finger protein that binds to the promoters of many genes repressing their transcription [19]. Mig1 promoter binding is regulated by Hxk2 and Snf1 proteins. Hxk2 has a regulatory role in glucose repression and this is dependent on its interaction with Mig1. Together, they form a repressor complex located in the nucleus of *S. cerevisiae*. In high glucose, nuclear Hxk2 stabilizes the complex by blocking Mig1 S311 phosphorylation by Snf1 protein kinase and this is important for glucose repression signaling [20-22]. In low-glucose, Hxk2 serine-14 and Mig1 serine-311 phosphorylation trigger their export from the nucleus by the Xpo1(Crm1) and Msn5-dependent pathways, respectively [21,23,24]. Thus, Hxk2 and Mig1 become mainly cytoplasmic [20] and this initiates the expression of several genes regulated by glucose repression.

Recently, it has been described that Mig2 moves between the nucleus and mitochondria in a glucose-dependent manner [25]. However, the mechanisms by which Mig2 enters into and leaves the nucleus are largely unknown. Since Mig2 is too large to translocate through the NPC by diffusion, its transport across the nuclear envelope is probably mediated by carrier proteins. In this study, we show that the Mig2 protein is an import cargo of the importin-β (Kap95) carrier in *S. cerevisiae*. We also show that Mig2 interacts with Kap95 in the presence of Gsp1(GDP) without the participation of other auxiliary proteins. Moreover, Mig2-Kap95 interaction depends on a Mig2 NLS motif located between lysine-32 and arginine-37. The observed Mig2-Kap60 interaction is not required for Mig2 entry into the nucleus and does not require the NLS motif of the Mig2 protein. We also demonstrate that Mig2 is recognized by Kap95 in the cytoplasm in the presence of Gsp1-GDP and cargo complexes dissociate in the nucleus upon the GDP/GTP exchange in the Gsp1 protein.

Methods

Strains and growth conditions

The *S. cerevisiae* strains used throughout this study were derived from W303-1A [26] and BY4742 [27] haploid wild-type strains and are listed in Table 1. Strains FMY501, FMY527, FMY528 and FMY535 expressing Mig2-GFP were constructed respectively by homologous recombination in W303-1A *kap95^{ts}*, *kap60^{ts}* and *Δmsn5* strains using a GFP-HIS3 tagging cassette obtained from pFA6a-GFP-HIS3 plasmid [28]. We created two NLS's mutant alleles of *MIG2* by mutating the *MIG2* gene in the pGEMT-MIG2-GFP/HIS3 plasmid to generate *MIG2K32A*,*K33A*,*R37A* (encodes Mig2*nls1* protein) and *MIG2R75A*,*R76A*,*K79A* (encodes Mig2*nls2* protein) genes using a PCR-based mutagenesis protocol. Then, by using these constructs, recombination cassettes were obtained by PCR; these cassettes contained the mutated *MIG2* locus and the *HIS3* marker. These linear DNAs were integrated into the *MIG2* locus of strain FMY501. To confirm the correct insertion, we PCR amplified the *MIG2* locus from strains: FMY523, contains the *MIG2* gene replaced by the *MIG2nls1* gene and FMY524, contains the *MIG2* gene replaced by the *MIG2nls2* gene, to detect the presence of the mutant *MIG2K32A*,*K33A*, *R37A* and *MIG2R75A*,*R76A*, *K79A* alleles by sequence analysis respectively.

Escherichia coli DH5α (*F Ø80dlacZ ΔM15 recA1 endA1 gyrA96 thi-1 hsdR17*(*rk-rk-*) *supE44 relA1 deoRΔ 99U169*) was the host bacterial strain for the recombinant plasmid constructions.

Yeast cells were grown in the following media: YEPD, high-glucose (2% glucose, 2% peptone, and 1% yeast extract), YEPGly, low-glucose (0.05% glucose, 3% glycerol, 2% peptone, and 1% yeast extract) and synthetic media containing the appropriate carbon source and lacking appropriate supplements to maintain selection for plasmids (2% glucose -SD, high glucose- or 3% glycerol and 0.05% glucose -SGly, low glucose-) and 0.67% yeast nitrogen base with ammonium sulfate and without amino acids. Amino acids and other growth requirements were added at a final concentration of 20-150 μg/ml. The solid media contained 2% agar in addition to the components described above.

Table 1 *Saccharomyces cerevisiae* strains used in this study

Name	Relevant genotype	Source/Ref.
W303-1A	*Mat a ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100*	[26]
BY4742	*MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0*	[27]
DBY2052	*Mat a hxk1:: LEU2 hxk2-202 ura3-52 leu2-3,2-112 lys2-801 gal2*	[29]
THG1	MATα *leu2-1 ura3-1 lys1-1 hxk1:: LEU2 hxk2:: LEU2 glk1:: LEU2*	[30]
H174	*MATa ade2-1 canJ-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6 J:: LEU2*	[31]
MAP24	*MATa mig1::loxp mig2::loxp-KAN-lox can1-100 his3-11,15 leu2-3,112 trp1-1; ura 3-1*	[32]
JCY1407	*MATa ade2-1 ura3-1; his3-11,15 trp1-1 leu2-3,112 can1-100 kap95:: HIS3 pSW509/pkap95-L63A*	[33]
JCY1410	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 srp1-31*	[33]
TetR-GFP	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 tetOx200:: URA3 tetR-GFP:: LEU2*	[34]
Y03694	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn5::kanMX4*	Euroscarf
Y14575	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig2::kanMX4*	Euroscarf
FMY501	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 MIG2:: GFP*	This work
FMY523	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 $MIG2_{NLS1}$:: GFP*	This work
FMY524	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 $MIG2_{NLS2}$:: GFP.*	This work
FMY525	*MATa; ade2-1; canJ-100; his3-11,15; leu2-3,112; trpl-J; ura3-1; mig1-6 J:: LEU2; $MIG2_{NLS1}$:: GFP.*	This work
FMY526	*MATa ade2-1 canJ-100 his3-11,15 leu2-3,112 trp1-1; ura3-1; mig1-6 J:: LEU2; $MIG2_{NLS2}$:: GFP.*	This work
FMY527	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 kap95:: HIS3 pSW509/pkap95-L63A MIG2:: GFP.*	This work
FMY528	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 srp1-31 MIG2:: GFP.*	This work
FMY535	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn5::kanMX4 MIG2:: GFP*	This work
FMY536	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 tetOx200:: URA3 tetR-GFP:: LEU2 mig2::kanMX4*	This work

Plasmids

To construct the yeast expression plasmid pGEMT/MIG2-GFP-HIS3 the yeast strain FMY501 was used as template in conjunction with oligonucleotides MIG2-d: TAAGCTGTGGCGATGTGCTG and MIG2-r: CCACC TTATCTCCACGGGAA in a PCR based protocol. To create the *MIG2* mutant alleles *MIG2nls1K32A*,*K33A*, *R37A* and *MIG2nls2R75A*,*R76A*,*K79A* the plasmid pGEMT/MIG2-GFP-HIS3 was used as template in conjunction with oligonucleotides MIG2nls1-d: GTGGTT TCCATCGGTTAGAACA TGCAGCGAGAACACTTG GCAAGACACACTGGGGAAAAACCTC, MIG2nls1-r: GA GGTTTTTCCCCAGTGTGTTGCCAAGTGTCTC GCTGCATGTTCTAACCGATGGAA ACCAC, MIG2 nls2-d: AACGCATACAGGGCAACTCAAGCGGCATT GAAGGCAGC TAGCGTACAGAAACAGGAGTT and MIG2nls2-r: AACTCCTGTTTCTGTACGCTA GCT GCCTTCAATGCCGCTTGAGATTGCCCTGTATGC GTT, in the PCR based site-directed mutagenesis method [35] to generate plasmids pGEMT/MIG2nls1-GFP-HIS3 and pGEMT/MIG2nls2-GFP-HIS3. All nucleotide changes were verified by DNA sequencing. The yeast expression plasmid YEp352/Hxk2*nes2*(Ala)-GFP was constructed as indicated previously [36].

GST fusion vectors pGEX/MIG2 and pGEX/MIG2nls1 were constructed by cloning in the *Sal*I site of pGEX-4 T-1 (GE Healthcare) a previously *Sal*I digested PCR fragment obtained by using pGEMT/MIG2-GFP-HIS3 and pGEMT/MIG2nls1-GFP-HIS3 respectively as templates in conjunction with oligonucleotides MIG2-BD/AD-d: TTCCCGGGTCGACTCATGCCTAAAAAGCA AACGAATTTCCCAGTA and MIG2-BD/AD-r: TTC CCGGGTCGACTCAACTCTTTTGGGACCGTTCAA AACAT. Plasmid pGEX/GSP1 for expression of GST-Gsp1 in *E. coli* was a gift from E. Hurt's laboratory [37] and plasmid pGEX-Kap95 and pGEX-Kap60 for expression of GST-Kap95 and GST-Kap60 in *E. coli* was a gift from M. P. Rout's laboratory [38].

pRS316-Su9-RFP a *CEN-URA3* plasmid expressing RFP fused to the presequence of subunit 9 of the F_o-ATPase of *Neurospora crassa* under control of the *ADH1* promoter was a gift from P. Sanz.

Fluorescence microscopy

Yeast strains expressing the Mig2-GFP, Mig2*nls1*-GFP and Mig2*nls2*-GFP were grown to early-log phase (A_{600nm} of less than 0.8) in YEPD medium. Half of the culture was shifted to YEPGly medium for 1 h. Cells (25 μl) were loaded onto poly L-lysine-coated slides, and the remaining suspension was immediately withdrawn by aspiration. One microliter of DAPI (2.5 μg/ml in 80% glycerol) was added, and a cover slide was placed over the microscope slide. GFP and DAPI localization in cultures was monitored by direct fluorescence using a Leica

DM5000B microscope. To avoid the non-linear range of fluorescent signals, cells highly overexpressing GFP-tagged fusion protein were excluded from further analyses. The localization of proteins was monitored by visual inspection of the images. At least 100 cells were scored in each of at least three independent experiments. The distribution of fluorescence was scored in the following way: N, denotes a nuclear fluorescence signal; M, mitochondrial fluorescent signal without nuclear fluorescence signal. Images representative of the results obtained are shown. Images were processed using Adobe Photoshop CS4.

Statistical analysis

Data were obtained from at least 3 independent experiments. Results are shown as the mean ± standard error (S.E.M.).

Preparation of crude protein extracts

Yeast protein extracts were prepared as follows: yeasts were grown in 10 to 20 ml of YEPD (H-Glc) at 28°C to an optical density at 600 nm of 0.8. Half of the culture was shifted to YEPGly (L-Glc) for 1 h. Cells were collected, washed twice with 1 ml of 1 M sorbitol and resuspended in 200 μl PBS buffer (150 mM NaCl, 100 mM Na_2HPO_4, 18 mM NaH_2PO_4, pH 7.3) containing Roche Protease Inhibitor plus 1 mM DTT and 0.1% Triton X100. The cells were broken using a FastPrep homogenizer (Thermo Electron Co.). Two pulses of 20s at 6.0 m/s were given in the presence of glass beads. Then, 200 μl of PBS buffer were added to the suspension. After centrifugation at 19,000 x g for 30 min at 4°C, the supernatant was used as crude protein extract.

Enzyme assay

Invertase activity was assayed in whole cells as previously described [39] and expressed as micromoles of glucose released per minute and 100 mg of cells (dry weight).

Immunoblot analysis

Mutant or wild-type yeast cells were grown to an optical density at 600 nm of 0.8 in YEPD medium containing high-glucose (2%) and shifted to low-glucose conditions for 1 h. The cells were collected by centrifugation (3,000 g, 4°C, 2 min) and crude extracts were prepared as described above. For Western blotting, 20 to 40 μg of proteins were separated by SDS-12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an enhanced chemiluminescence PVDF transfer membrane (Amersham Biosciences) by electroblotting. The membrane was then incubated with anti-GFP (Invitrogen), anti-Kap60 (Santa Cruz Biotech) or anti-Kap95 (Santa Cruz Biotech) as primary antibodies and the appropriate secondary antibody later. Horseradish peroxidase-conjugated protein-A was used as secondary reactant. West Pico Chemiluminescent system (Pierce) was used for detection.

Co-immunoprecipitation assay

Immunoprecipitation experiments were performed using whole cell extracts from different strains. The extracts were incubated with anti-Kap60, anti-Kap95 or anti-Pho4 polyclonal antibodies for 3 h at 4°C. Protein A-Sepharose beads (Amersham Biosciences) were then added and incubated for 3 h at 4°C in a spinning wheel. After extensive washing with PBS plus 0.5% deoxycholate buffer, immunoprecipitated samples were boiled in SDS-loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The proteins were separated in a 12% SDS-polyacrylamide gel (SDS-PAGE), transferred to an enhanced chemiluminicence PVDF membrane and immunobloted as described above using anti-GFP monoclonal antibodies. Values shown are representative results from individual experiments.

GST pull-down experiments

E. coli cells from the BL21(DE3) pLysS strain were transformed with the fusion protein expression vectors pGEX/MIG2 and pGEX/MIG2nls1. Cells were grown to A_{600nm} 0.5-0.8, induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 3 h, and collected by centrifugation. The pellet was resuspended in PBS buffer (150 mM NaCl, 100 mM Na_2HPO_4, 18 mM NaH_2PO_4, pH 7.3) and sonicated. Insoluble material was removed by centrifugation (17,000 x g for 20 min at 4°C). The soluble extract was incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at 4°C, washed extensively with PBS buffer and resuspended in the same buffer. The Mig2-GST and Mig2*nls*1-GST fusion proteins coupled to glutathione-Sepharose were incubated with yeast whole cell extracts from the wild-type strain (W303-1A) for 1 h at 4°C in PBS buffer. The cell extracts were obtained from yeast cells grown in YEPD medium containing high-glucose (2%) and shifted to 0.05% glucose plus 3% glycerol (low-glucose) for 1 h. Beads were gently washed five times with 2.5 ml of PBS buffer, boiled in 25 μl sample-loading buffer, and analyzed by SDS-PAGE followed by Western blot using anti-Kap95 or anti-Kap60 antibodies and horseradish peroxidase-conjugated protein-A. Bound antibodies were detected using the West Pico Chemiluminescent system (Pierce).

GST fusion protein expression vectors pGEX-Kap60, pGEX-Kap95 and pGEX-GSP1 were transformed into *E. coli* strain BL21(DE3) pLysS. Cells were grown to A_{600nm} 0.5-0.8, induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 3 h, and collected by centrifugation. Cell pellets were resuspended in PBS buffer

and sonicated. The GST-Kap60, GST-Kap95 and GST-Gsp1 fusion proteins coupled to glutathione-Sepharose beads were incubated with 2.5 units of thrombin (2 h at 4°C) for site-specific separation of the GST affinity tag from Kap60, Kap95 and Gsp1 proteins. Affinity-purified Gsp1 was immediately loaded with GTP or GDP by adding 30 mM K_2PO_4 (pH 7.5), 1 mM GTP or 1 mM GDP and 10 mM EDTA (pH 8.0). Then, it was incubated at room temperature for 1 h, supplemented with 20 mM magnesium acetate, incubated on ice for 30 min, and frozen at -80°C [37]. Gsp1-mediated Kap95/Kap60 interactions with Mig2 were analyzed by incubating purified Kap95 or Kap60 with GST-Mig2 bound to glutathione-Sepharose beads in the presence of Gsp1(GTP) or Gsp1 (GDP) for 1 h at 4°C in PBS buffer. Beads were washed and analyzed as described above.

Results

The karyopherin Msn5 (Kap142) is not essential for Mig2 nuclear import

The karyopherin Msn5 is required for nuclear export of Mig1, a transcriptional repressor of *S. cerevisiae* [40]. Since the Msn5 protein functions both as a nuclear importin and exportin carrier [41] and Mig1 and Mig2 proteins have several common structural motifs and functions, we first tested for a possible role of Msn5 in Mig2 nuclear import.

Thus, nuclear accumulation of Mig2-GFP was determined in *Δmsn5* mutant cells by fluorescence microscopy, as compared to wild-type cells. The FMY501 (Mig2-GFP) and FMY535 (*Δmsn5* Mig2-GFP) strains showed identical nuclear distributions of Mig2-GFP growing in high and low glucose conditions. In high glucose conditions, nuclear accumulation of Mig2 was observed, although cytoplasmic background fluorescence was also detected and after a 5 min shift to low glucose-glycerol medium, strong green fluorescence of the characteristic branched tubular mitochondrial network was observed (Figure 1).

These results demonstrate that Mig2-GFP enters and exits from the nucleus both in the presence and absence of the Msn5 protein. This suggests that Msn5 is not implicated in either Mig2 nuclear import or export.

Nuclear import of Mig2 is altered in a *kap95^{ts}* mutant strain but not in *kap60^{ts}* mutant cells

Kap60 normally binds proteins through a highly basic stretch close to its N-terminus, resulting in the recruitment of Kap95 and formation of a heterotrimeric complex that facilitates nuclear import [36]. The carrier Kap60 recognizes two classes of NLSs matching the consensus $K(K/R)X_{1\text{-}3}(K/R)_{1\text{-}2}$ and $(K/R)(K/R)X_{1\text{-}3}(K/R)X_{10\text{-}12}(K/R)_{3\text{-}5}$. The first class, known as monopartite NLS, has a single cluster of basic amino acid residues and the second class, known as bipartite NLS, has two clusters of basic amino acids separated by a 10-12 amino acid linker [42]. Since Mig2 presents two monopartite NLS motives ^{32}KKRHLR^{37} and ^{75}RRLKK^{79}, we hypothesized that the Kap60/Kap95 complex may mediate the nuclear import of Mig2. To test this, we analyzed the subcellular localization of Mig2-GFP in temperature-sensitive *kap60^{ts}* and *kap95^{ts}* mutant cells. The *kap60^{ts}* and *kap95^{ts}* mutants were grown at the permissive temperature (25°C) in high glucose conditions and then shifted to low glucose conditions for 1 h. Then, the high and low glucose cultures were shifted to the nonpermissive temperature (37°C) for 1 h. As can be seen in Figure 2, a similar accumulation of Mig2 was detected in the nuclei and mitochondria of wild-type, *kap60^{ts}* and *kap95^{ts}* mutant cells grown at permissive temperature (Figure 1, Figure 2a and b). In high glucose conditions Mig2 mostly accumulates in the nucleus (80%) but in low-glucose conditions, Mig2 rapidly moves to the mitochondrial network (98%). RFP fused to the presequence of subunit 9 of the mitochondrial F_o-ATPase protein was used as a mitochondrial marker and, as seen in Figure 2a, Mig2-GFP largely colocalized with Su9-RFP protein. Thus, under nutritional stress, Mig2 is essentially a mitochondria associated protein. Moreover, Mig2 translocation from the nucleus to the mitochondria is reversible, because Mig2 localizes back to the nucleus after a shift from low to high glucose conditions [25].

However, in *kap95^{ts}* mutant cells incubated at the non-permissive temperature (37°C) and both in high and low-glucose conditions, 98% of the Mig2 protein was located in the mitochondrial network and no nuclear accumulation of Mig2 was observed in high glucose conditions after 1 h of culture (Figure 2a). In contrast, in *kap60^{ts}* mutant cells incubated at the nonpermissive temperature (37°C) in high glucose conditions, 80% of cells showed nuclear accumulation of the Mig2 protein (Figure 2b), whereas if we used a positive control such as Hxk2 [36], in *kap60^{ts}* mutant cells incubated at the nonpermissive temperature, no nuclear accumulation of Hxk2nes2(Ala) was observed after 1 and 5 h of culture (Figure 2c). These results suggest that the Kap95 protein is necessary for nuclear import of Mig2, but the Kap60 protein does not participate in this process.

Mapping residues necessary for NLS function

We next determined whether the two putative Mig2 NLS sequences are necessary for the entry of Mig2 into the nucleus. In order to investigate this, the putative NLS sequences were mutated and tested for their function. The presence of basic amino acids such as lysine and arginine appears to be an important feature of the NLSs [42]. Thus, we created two *MIG2-GFP* mutants in which three basic residues of either NLS1 or NLS2 were

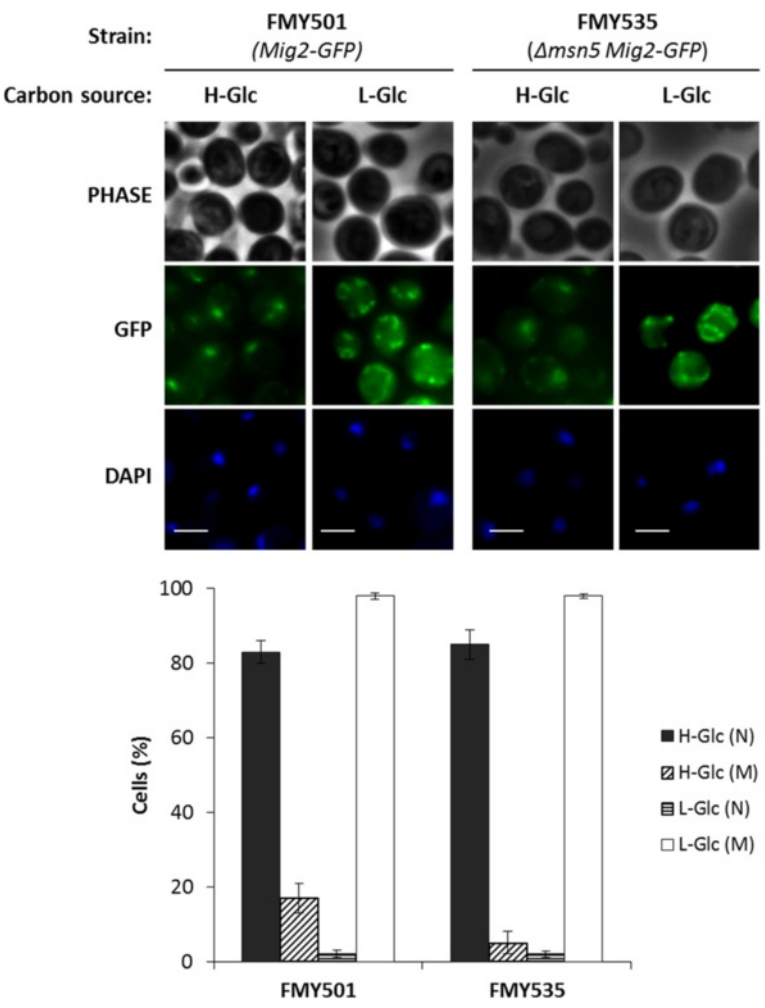


Figure 1 Localization of Mig2-GFP in Δ*msn5* yeast cells. The FMY501(Mig2-GFP) and FMY535 (Δ*msn5* Mig2-GFP) yeast strains, were grown in YEPD high-glucose medium (H-Glc) until an A_{600nm} of 1.0 was reached and then transferred to YEPGly low glucose medium (L-Glc) for 5 min. Cells were stained with DAPI and imaged for GFP and DAPI fluorescence. Scale bar is 10 μm. The nuclear and mitochondrial localization of Mig2-GFP protein was determined in at least 100 cells per growth condition. No statistically significant differences were detected between the wild-type and the mutant strains. N, denotes a nuclear fluorescence signal and M, mitochondrial fluorescence signal.

replaced by alanine residues to generate *MIG2K32A K33AR37A-GFP* (encoding Mig2*nls1*-GFP) and *MIG2R 75AR76AK79A-GFP* (encoding Mig2*nls2*-GFP). Analysis of the intracellular localization of the different Mig2-GFP variants by fluorescence microscopy showed that the Mig2*nls2*-GFP fusion protein accumulated in the nuclei (74%) in high-glucose conditions (Figure 3a). However, in high glucose conditions the Mig2*nls1*-GFP protein was excluded from the nuclei and a mitochondrial (99%) accumulation was detected (Figure 3a). Thus, the *nls1* mutation causes a strong defect in the nuclear import of Mig2.

Accordingly, we hypothesize that consistent with the inability of the mutant Mig2*nls1* protein to enter into the nucleus *in vivo* (Figure 3a); the mutants lacking the NLS1 must be unable to signal glucose repression in a *Δmig1* mutant strain. Thus, we investigated the Mig2-dependent glucose signaling function in the presence and absence of the Mig1 protein (Figure 3b). The cells were grown in YEPD complex medium to an A_{600nm} of 0.8 and the exocellular invertase activity was determined in whole cells as a glucose repression marker. The specific activity of invertase shows identical strong derepression in both *Δmig1Δmig2* and *Δmig1Mig2nls1* mutant strains. However, in the Δ*mig1Mig2nls2* mutant strain a derepression level similar to the *Δmig1* strain was observed. These results confirm the above finding, that the strain expressing Mig2*nls1* has lost nuclear import capacity and thus cannot exert its function as a transcriptional repressor.

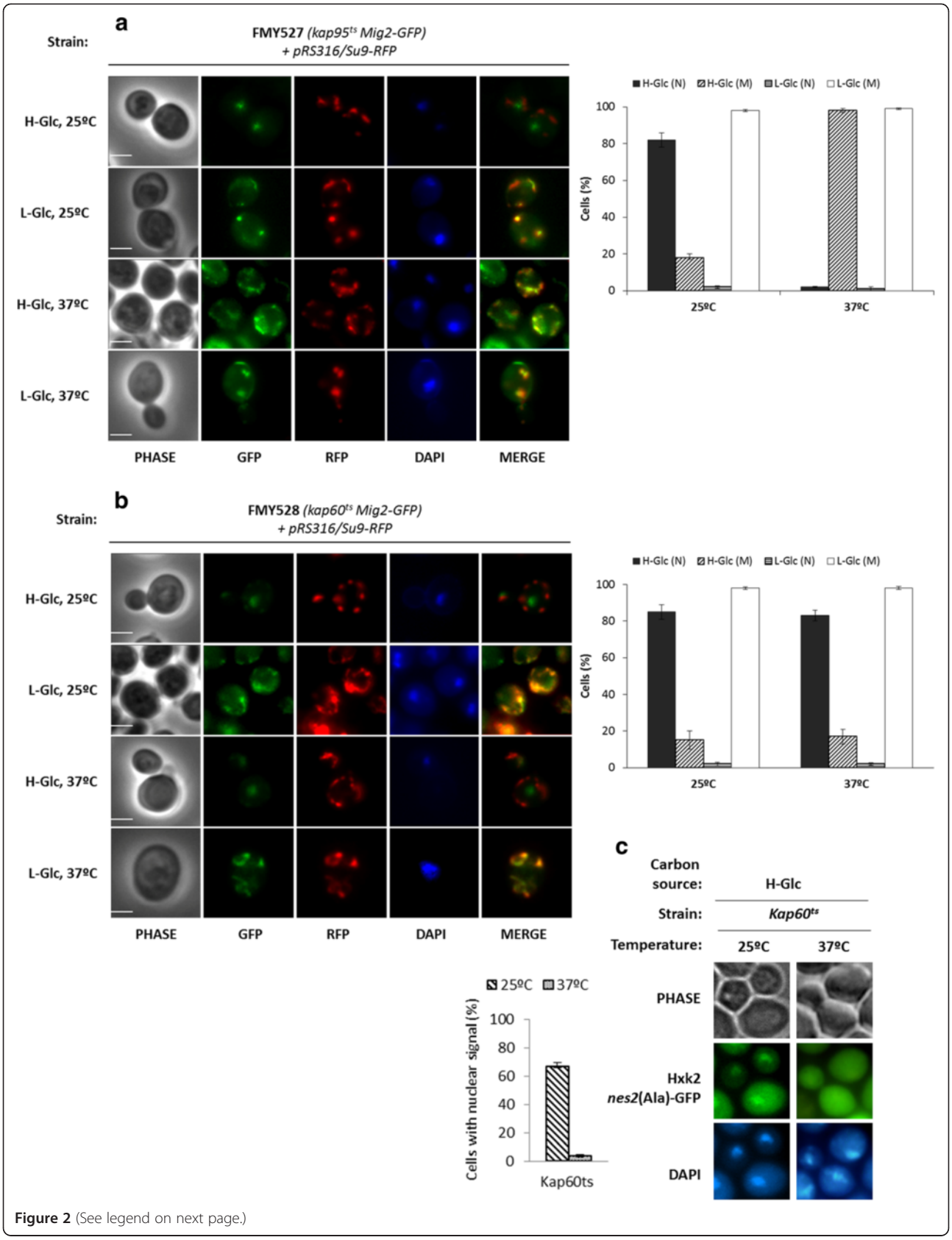


Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 Localization of Mig2-GFP in *kap95*^{ts} and *kap60*^{ts} yeast cells. (**a**) The FMY527 (*kap95*^{ts} Mig2-GFP) and (**b**) FMY528 (*kap60*^{ts} Mig2-GFP) strains were transformed with plasmid pRS316/Su9-RFP. Transformed cells were grown in high-glucose synthetic medium (H-Glc) until an A_{600nm} of 1.0 was reached and then transferred to low glucose synthetic medium (L-Glc) for 5 min. (**c**) The JCY1410 (*kap95*^{ts}) strain transformed with plasmid YEp352/Hxk2*nes2*(Ala)-GFP was used as positive control. Transformed cells were grown in high-glucose synthetic medium (H-Glc) until an A_{600nm} of 1.0 was reached. The cells were grown at 25°C (permissive temperature) and then shifted to 37°C (not permissive temperature) for 1 h. Cells were stained with DAPI and imaged for GFP, RFP and DAPI fluorescence. Scale bar is 10 μm. The nuclear or mitochondrial localization of Mig2-GFP and the nuclear or cytoplasmic localization of Hxk2*nes2*(Ala) proteins was determined in at least 100 cells per growth condition. Error bars represent standard deviations for three independent experiments. N denotes a nuclear fluorescence signal and M mitochondrial fluorescence signal.

Mig2 interacts with Kap60 and Kap95

To test whether Mig2 binds to Kap60 and Kap95 *in vivo* we carried out an immunoprecipitation assay on cells expressing Mig2-GFP and GFP as control. Cell extracts from FMY501 (Mig2-GFP) or TetR-GFP were immunoprecipitated with anti-Kap60 and anti-Kap95 antibodies. The resulting immunoprecipitates were assayed for the presence of Mig2-GFP or GFP by immunoblot analysis with anti-GFP antibodies. As shown in Figure 4a, specific signals of Mig2-GFP were observed with samples immunoprecipitated with both anti-Kap60 and anti-Kap95 antibodies in the strain expressing Mig2-GFP either in

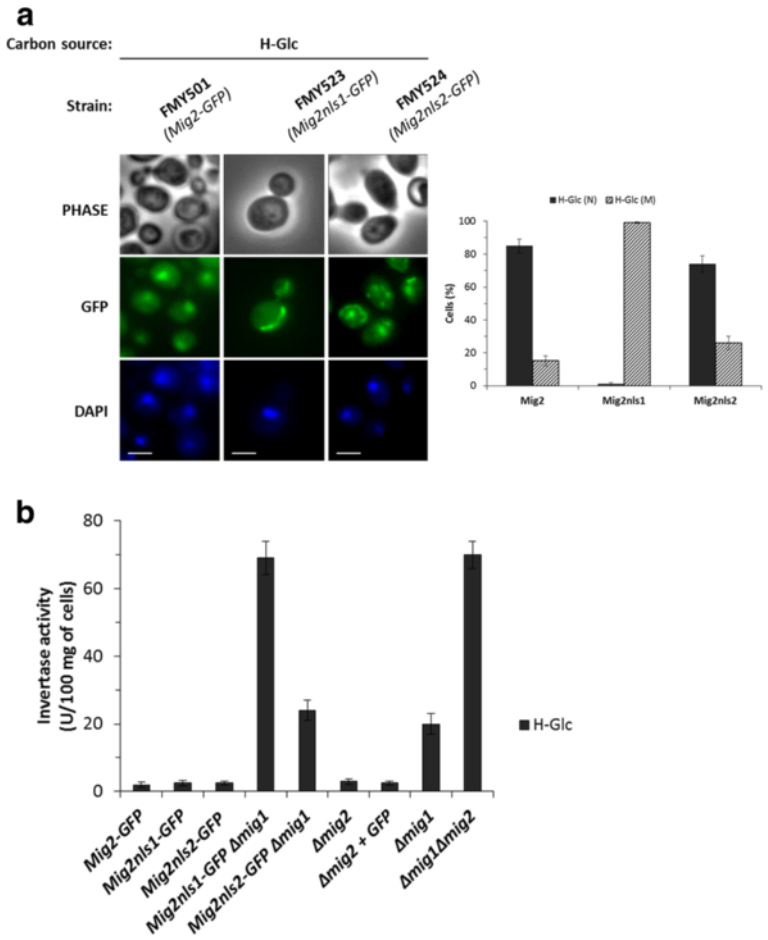


Figure 3 Identification of Mig2 NLSs. (**a**) The FMY501 (Mig2-GFP), FMY523 (Mig2*nls1*-GFP) and FMY524 (Mig2*nls2*-GFP) were grown in YEPD high-glucose medium (H-Glc) until an A_{600nm} of 1.0 was reached and then transferred to YEPGly low glucose medium (L-Glc) for 5 min. The cells were visualized by fluorescence microscopy, DAPI staining revealed nuclear DNA. Scale bar is 10 μm. The nuclear localization of fluorescent reporter proteins was determined in at least 100 cells in three independent experiments. Means and standard deviations are shown for at least three independent experiments. N denotes a nuclear fluorescence signal and M mitochondrial fluorescence signal. (**b**) The FMY501 (Mig2-GFP), FMY523 (Mig2*nls1*-GFP), FMY524 (Mig2*nls2*-GFP), FMY525 (Mig2*nls1*-GFP Δ*mig1*), FMY526 (Mig2*nls2*-GFP Δ*mig1*), Y14575 (Δ*mig2*), FMY536 (Δ*mig2* + GFP), H174 (Δ*mig1*) and MAP24 (Δ*mig1* Δ*mig2*) strains were grown in YEPD high-glucose medium (H-Glc) until an A_{600nm} of 0.8 was reached. Invertase activity was assayed in whole cells. Values are the averages of results obtained in four independent experiments.

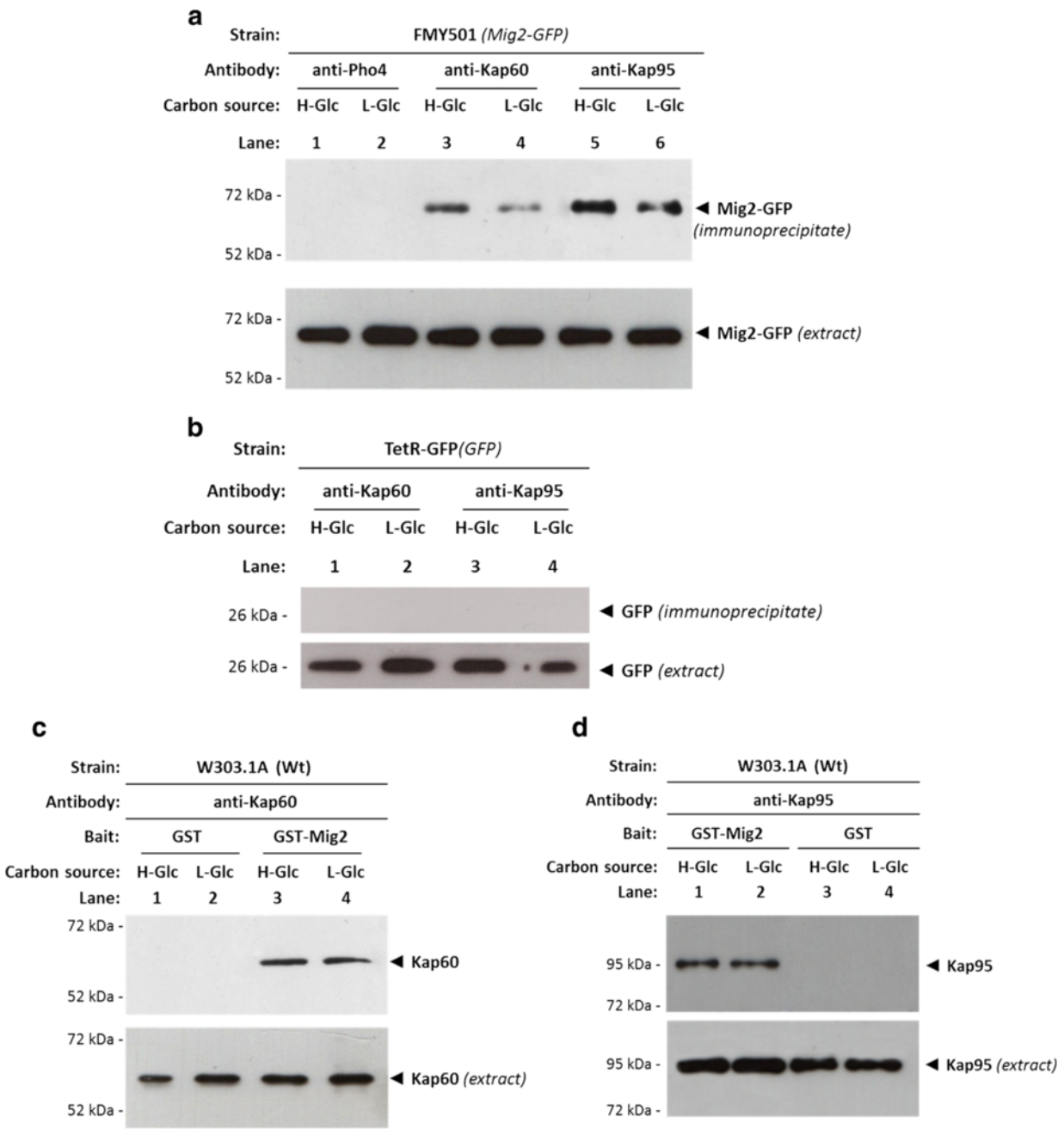


Figure 4 Interaction of Kap60 and Kap95 with Mig2. *In vivo* co-immunoprecipitation of Kap60 and Kap95 with Mig2. The wild-type, FMY501 (Mig2-GFP) (**a**) or a control strain, (TetR-GFP) synthetizing GFP only (**b**) were grown in YEPD-media until an A_{600nm} of 0.8 was reached and then shifted to low glucose (L-Glc) conditions for 1 h. The cell extracts were immunoprecipitated with a polyclonal anti-Kap60 or anti-Kap95 antibody (lanes 3-6) or a polyclonal antibody against Pho4 (lanes 1 and 2). Immunoprecipitates were separated by 12% SDS-PAGE, and co-precipitated Mig2-GFP was visualized by Western blot with polyclonal anti-GFP antibodies. The level of immunoprecipitated Kap60 or Kap95 in the blotted samples was determined by using anti-Kap60 and anti-Kap95 antibodies, respectively. The level of Mig2-GFP and GFP present in the different extracts was determined by Western blot using anti-GFP antibody. The Western blots shown are representative of results obtained from four independent experiments. The GST-Mig2 fusion protein was purified on glutathione-Sepharose columns. Equal amounts of GST-Mig2 were incubated with cell extracts from the wild-type strain W303-1A. The yeasts were grown in YEPD media until an A_{600nm} of 0.8 was reached and then shifted to low (L-Glc) glucose conditions for 1 h. After exhaustive washing the proteins were separated by 12% SDS-PAGE, and retained Kap60 and Kap95 proteins were visualized by Western blot using polyclonal anti-Kap60 (**c**) and anti-Kap95 (**d**) antibodies respectively. For the control samples, GST protein was also incubated with high- (H-Glc) and low-glucose (L-Glc) cell extracts, but no signals were detected. The level of Kap60 and Kap95 proteins present in the different extracts used in Figure 4c and 4d was determined by Western blot using anti-Kap60 and anti-Kap95 antibodies respectively. The Western blots shown are representative of results obtained from four independent experiments.

high and low glucose conditions. However, no signals were observed when the experiment was done using the strain expressing GFP although similar amounts of GFP were detected in the immunoprecipitates when anti-GFP antibody was used (Figure 4b). These results indicated that the glucose levels do not affect the affinity of Mig2, since the Mig2-GFP interaction with Kap60 and Kap95 proteins was similar with samples from high and low glucose-grown cultures. Thus, our data suggest that the glucose levels do not affect the affinity of Mig2 for the importin-α and β.

To confirm the *in vitro* interaction of Mig2 with Kap60 and Kap95, we also performed a GST pull-down assay. We used crude protein extracts from a wild-type strain and a GST-Mig2 fusion protein expressed in *E. coli*. As shown in Figure 4, a clear retention of Kap60 protein (Figure 4c) and Kap95 (Figure 4d) was observed both in high and low-glucose conditions. When a control with GST protein in the reaction mix was used, no Kap60 or Kap95 signal was observed. These results also confirm that the interaction between Mig2 and both importins is similar under high and low-glucose conditions and suggest that both proteins interact in a glucose independent manner.

To analyse the interaction of Mig2*nls1* mutant protein with Kap60 and Kap95, we also performed a GST pull-down assay. We used crude protein extracts from a wild-type strain and purified GST-Mig2*nls1* fusion protein expressed in *E. coli*. As shown in Figure 5a, a clear retention of Kap60 protein was observed both in high and low-glucose conditions in the sample containing GST-Mig2*nls1* and crude extract from the wild-type strain. However, a very weak or no retention of Kap95 protein was observed for the sample containing GST-Mig2*nls1* and crude extract from the wild-type strain (Figure 5b). Together, these data suggest that the Mig2-Kap95 interaction strongly depends on the NLS1 motif, whereas the latter has no influence on the interaction with Kap60.

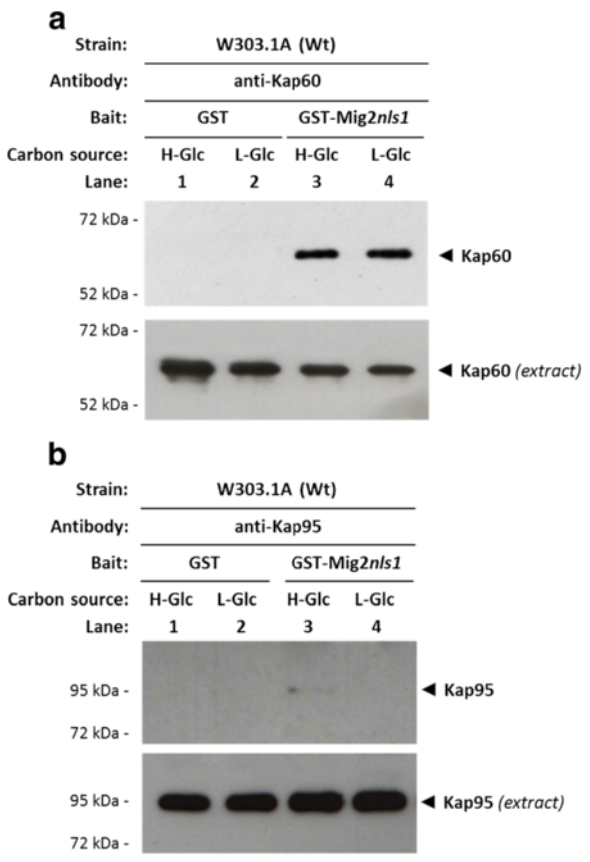


Figure 5 Interaction of Kap60 and Kap95 with Mig2*nls1*. The GST-Mig2*nls1*fusion protein was purified on glutathione-Sepharose columns. Equal amounts of GST-Mig2*nls1* were incubated with cell extracts from the wild-type strain W303-1A. The yeasts were grown in YEPD media until an A_{600nm} of 0.8 was reached and then shifted to low (L-Glc) glucose conditions for 1 h. After exhaustive washing the proteins were separated by 12% SDS-PAGE, and retained Kap60 and Kap95 proteins were visualized on a Western blot with polyclonal anti-Kap60 (**b**) and anti-Kap95 (**c**) antibodies respectively. For the control samples, GST protein was also incubated with high- (H-Glc) and low-glucose (L-Glc) cell extracts, but no signals were detected. The level of Kap60 and Kap95 proteins present in the different extracts used in Figure 5a and 5b was determined by Western blot using anti-Kap60 and anti-Kap95 antibodies respectively. The Western blots shown are representative of results obtained from four independent experiments.

The interaction between Kap95 and Mig2 requires auxiliary factors other than Kap60 protein

As indicated above, Kap95 is required for efficient nuclear import of Mig2. In order to address the question of if this function is direct or indirect, we determined if Kap60 is needed to mediate the interaction with Mig2. For this purpose, again purified proteins from E. coli were employed. We used GST-Mig2, GST-Mig2nls1, GST-Kap60 and GST-Kap95 fusion proteins. The GST fusion proteins were immobilized with glutathione-Sepharose and released with thrombin. First, we immobilized GST-Mig2 on glutathione-Sepharose beads and analysed the binding of purified Kap60 and Kap95 proteins. Our results confirmed that purified Kap60 has a strong affinity for Mig2 (Figure 6a, lane 4) and that this interaction was not NLS1-dependent (Figure 6b). However, no interaction of Mig2 or Mig2*nls1* with Kap95 was detected using purified proteins either in the presence or absence of Kap60 (Figure 6b).

These results indicate that auxiliary factors other than Kap60 protein are required for the Mig2-Kap95 interaction. In order to investigate if the Gsp1-GTP/GDP cycle controls the formation and/or the transport direction of the Mig2-Kap95 complex [43], we tried to reconstruct the import complex *in vitro* using purified proteins. To study how the different factors affect the

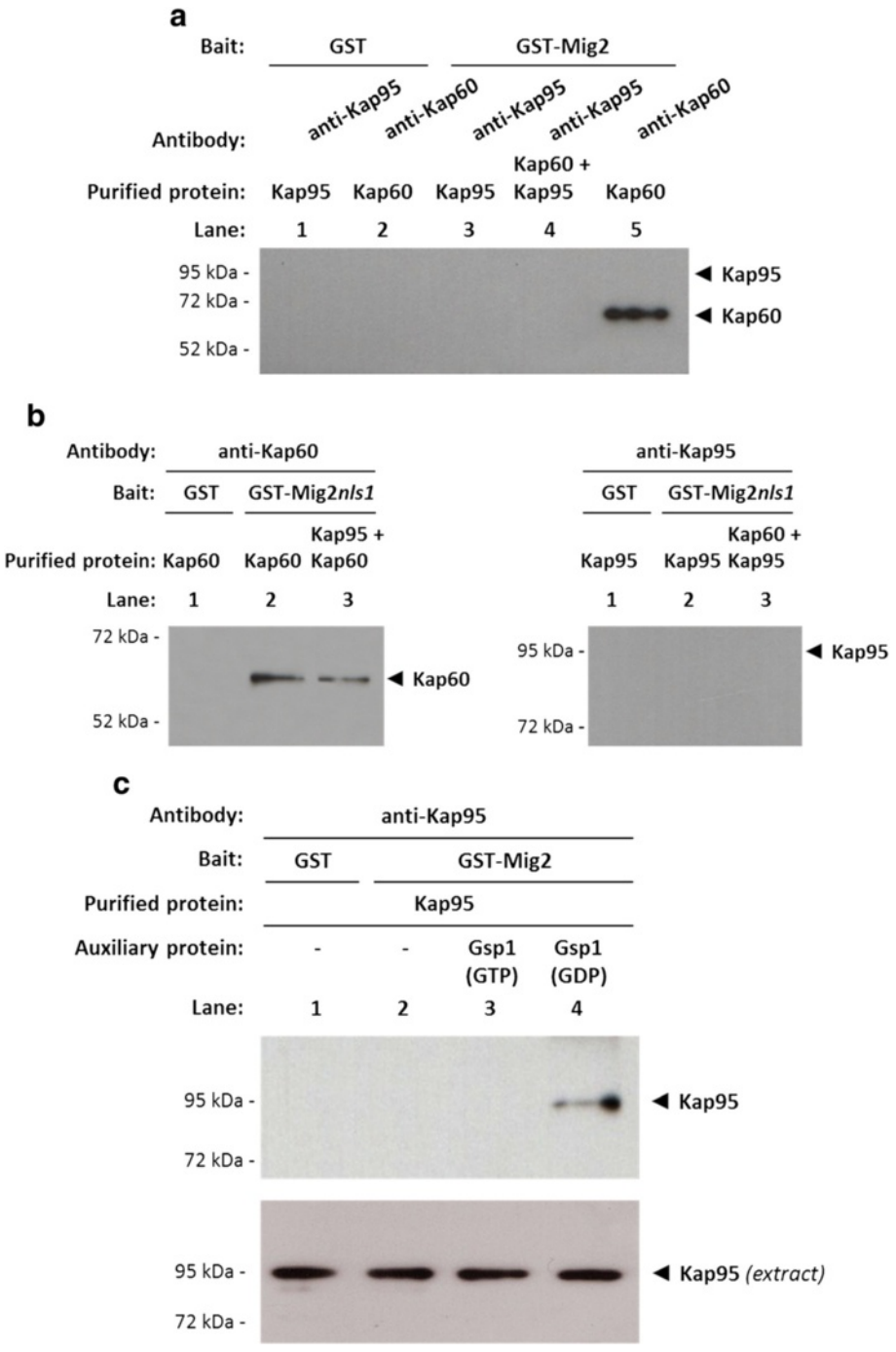


Figure 6 The import Mig2-Kap95 complex formation *in vitro*. GST pull-down analysis of Mig2 (**a**) and Mig2*nls1* (**b**) interaction with Kap60 and Kap95 purified proteins. (**c**) Effect of Gsp1 on the Mig2-Kap95 interaction stability. GST-Mig2, GST-Mig2*nls1*, GST-Gsp1, GST-Kap60 and GST-Kap95 fusion proteins were purified on glutathione-Sepharose columns and incubated with thrombin to release, respectively, Gsp1, Kap60 and Kap95 proteins. The Gsp1 protein was loaded with GTP to generate Gsp1(GTP) or with GDP to generate Gsp1(GDP). The purified proteins were incubated with purified GST-Mig2 or GST-Mig2*nls1* bound to glutathione-Sepharose beads in the absence (**a** and **b**) or in the presence (**c**) of Gsp1(GTP) and Gsp1(GDP). The beads were washed extensively. Co-precipitated proteins were resolved by 12% SDS-PAGE and visualized on a Western blot with polyclonal anti-Kap60 or anti-Kap95 antibodies. The level of Kap95 protein present in the different extracts used in Figure 5c was determined by Western blot using anti-Kap95 antibodies. The Western blots shown are representative of results obtained from four independent experiments.

Mig2-Kap95 interaction affinity, we performed GST pull-down experiments with purified proteins. We used GST-Mig2, GST-Gsp1 and GST-Kap95 fusion proteins from *E. coli* lysates. The GST fusion proteins were immobilized with glutathione-Sepharose and released with thrombin. First, we immobilized GST-Mig2 on glutathione-Sepharose beads and analysed the binding of Kap95. Our results indicate that purified Kap95 has no affinity for Mig2 (Figure 6c, lane 2). However, as expected for an importin-type receptor, the complex formation between Kap95 and Mig2 is regulated by the guanine nucleotide-binding protein Gsp1. Our data indicate that the Mig2-Kap95 complex is not formed in the presence or absence of Gsp1-GTP (Figure 6c lanes 2 and 3). However, the presence of Gsp1-GDP facilitates the formation and increases the stability of the Mig2-Kap95 complex (Figure 6c lane 4).

This experiment suggests that a ternary complex between Kap95, Gsp1 and Mig2 is formed *in vitro*. Moreover, the complex formation is controlled by the GDP/GTP-bound state of Gsp1, because loading of Gsp1 with GTP disrupts the formation of the import complex and Gsp1 loaded with GDP acts as an auxiliary factor required for Mig2-Kap95 interaction and for complex stabilization.

Discussion

Glucose levels regulate Mig2 translocation from the nucleus to the mitochondria [25]. In high-glucose conditions, nuclear Mig2 interacts with Mig1 and Hxk2 to participate in the *SUC2*-Mig1 repressor complex. In low glucose conditions, Mig1 S311 is phosphorylated by Snf1 kinase [21]. Once phosphorylated Mig1 leaves the nucleus [44]. Nuclear Hxk2 loses its anchoring protein and also moves into the cytosol [24]. This process leads to *SUC2* repressor complex dissociation and *SUC2* gene derepression. In these conditions, the vast majority of Mig2 accumulates in the mitochondria. The mitochondrial targeting of Mig2 in low glucose conditions is Ups1-dependent [25]. While it is essential for Mig2 to enter the nucleus to perform its role as transcriptional repressor, the mechanism by which the protein transverses the nuclear envelope has not been investigated until now. In the current study, we have extended our knowledge of the nucleocytoplasmic shuttling of Mig2 by showing that its nuclear translocation is mediated by the importin-β (Kap95) and regulated by the Ran GTPase protein (Gsp1).

In this study, we provide evidence that nuclear import of Mig2 is independent of the yeast Msn5 karyopherin and that Kap95 is the carrier responsible for the nuclear accumulation of Mig2 under high-glucose conditions. We observe that Mig2 protein in *Kap95^{ts}* mutant cells is not efficiently imported into the nucleus and accumulates in the mitochondrial tubules during high-glucose growth. However, in *Kap60^{ts}* mutant cells Mig2 is efficiently imported into the nucleus in high-glucose conditions. These data suggest that Kap95 is the karyopherin responsible for nuclear import of the Mig2 protein, either independently or as part of a larger complex from which Kap60 is excluded.

Taken together, our results demonstrate that the Lys32, Lys33 and/or Arg37 are critical amino acid residues in the Mig2 NLS1 sequence. Moreover, since the NLS2 mutation does not affect Mig2 import because it does not decrease significantly its nuclear accumulation and glucose repression signalling, our data suggest that only the NLS1 motif is necessary for efficient nuclear import of Mig2-GFP *in vivo*. These results are surprising because it has not been previously described that Kap95 directly recognizes a conventional NLS. However, the results are consistent with the idea that only Kap95 protein participates in Mig2 nuclear import. Moreover, the Mig2*nls1* mutant protein lacks repressor function, while, the Mig2*nls2* mutant protein complements the *Δmig2* mutation in a double *Δmig1Δmig2* mutant and exhibits predominantly nuclear localization. Our pull-down assays employing purified proteins suggest, that the Mig2-Kap95 complex is stable and does not require Kap60 for association in the presence of Gsp1(GDP), as happens in the cytoplasm. However, in the nucleus the complex is disrupted in the presence of high levels of Gsp1(GTP). Thus, efficient dissociation of the Mig2-Kap95 complex is mediated primarily by Gsp1(GTP) which displaces Mig2 from the importin-β receptor. These experiments, also exclude the possibility that some unknown protein/s in the yeast extract could mediate the interaction between the Mig2 protein and the Kap95 protein.

Importin-β (Kap95) has two distinct mechanisms by which it associates with transport substrates: indirect and direct. The more conventional import pathway takes place by the indirect association of cargo with the Kap60-Kap95 (importin-α/β) complex. While Kap60 binds to a classical NLS present in the cargo, Kap95 associates with Kap60 and is primarily responsible for translocation of the complex across the nuclear pore. Conversely, direct association involves Kap95 directly binding to its cargo. Several examples of this direct mechanism have been described in mammalian cells [15,45-47], implying that the importin-β (Kap95) may be able to function independently of Kap60 as a nuclear import receptor. The findings of the present study not only establish that Mig2 is similar to the above mammalian proteins in being recognized by Kap95 alone but also demonstrate conclusively for the first time in yeast that Kap95, in concert with Gsp1, is sufficient to mediate nuclear import of Mig2 in the absence of Kap60. The Mig2 protein has been shown to bind directly to Kap95 in the

presence of Gsp1(GDP) without requiring Kap60 for association. Thus, our results are in accordance with previous publications describing Kap95 as an essential karyopherin in the nuclear import of several proteins without requiring importin-α [13,14].

Although the details are not fully understood, there are novel NLS-dependent nuclear import pathways exclusively mediated by Kap95 that are presumably analogous to those mediated by other importin-β homologs. The Kap95 protein has been described as contributing to the nuclear import of cargos by recognizing import signals different from the classical NLS [16,48,49]. Surprisingly, a classical NLS-mediated transport mechanism is required for Mig2-Kap95 binding and Mig2 nuclear accumulation in the absence of a functional Kap60 protein.

Our results also indicate that Kap60 interacts with Mig2-GFP both in high and low-glucose conditions as demonstrated by immunoprecipitation and GST-pull down experiments. However, this interaction is not required for Mig2-GFP nuclear import. Moreover, the NLS1 sequence of Mig2 is not involved in the Kap60-Mig2 interaction. Thus, the physiological significance of Mig2-Kap60 is unknown at the current stage. While a number of nuclear import pathways have been identified, the definition of general rules of what constitutes the targeting signal and the role of Kap60 has only been possible for the classical nuclear import pathway. For other pathways, such rules have not yet been identified probably because an insufficient number of cargos are known, the recognition of cargo by the carrier requires further characterization by structural and interaction analyses or no common rules exist and the recognition is specific to specific cargos. The key to progress is to integrate seemingly disparate types of information, using structural, and sequence information and protein-protein interactions.

Conclusions

The current study has identified a new pathway for Mig2 import into the nucleus. Here we provide data that support a model for Mig2 nuclear import that includes Kap95 as the predominant karyopherin and the NLS1 of Mig2 as essential for efficient Mig2-Kap95 interaction and nuclear import. Mig2, in the presence of Gsp1 (GDP), directly binds importin-β (Kap95), and together they form a complex to allow the passage of Mig2 through the nuclear pore. Thus, we found that Kap95 forms a stable complex with Mig2 in the cytoplasm where the level of Gsp1(GDP) is high. After translocation into the nucleus, where the Gsp1(GDP) level is low and the Gsp1(GTP) level is high, this complex is dissociated via a mechanism that includes GDP/GTP exchange in the import complex.

Abbreviations

NLS: Nuclear localization sequence; GST: Glutathione S-transferase; GFP: Green fluorescent protein; RFP: Red fluorescent protein; DAPI: 4',6-Diamino-2-phenylindole.

Competing interests

The authors declared that they have no competing interests.

Authors' contributions

AF-C carried out the experiments, analysed and interpreted the data. MV purified proteins from bacteria and did *in vitro* experiments. PH made important contributions making several DNA constructs and the design of experiments. FM was involved in the planning, experimental design, data analysis and wrote the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgments

We are grateful to the following people for generously providing yeast strains and plasmids: E. Hurt for the pGEX4T3-GSP1 plasmid, M. P. Rout for the pGEX-Kap95 and pGEX-Kap60 plasmids and J.C. Igual for the *kap95*^{ts} and *kap60*^{ts} strains. A.F-C. was supported by a FPU fellowship provided by the MICINN (Spain) and M.V. was supported by a FPI fellowship provided by the MICINN (Spain). This work was supported by grant BFU2010-19628-C02-01 from the MICINN (Spain).

Author details

[1]Department of Biochemistry and Molecular Biology, University of Oviedo, 33006, Oviedo, Spain. [2]Present Address: MRC Clinical Sciences Centre, Imperial College Faculty of Medicine, London, UK.

Received: 4 July 2012 Accepted: 4 November 2012
Published: 6 November 2012

References

1. Cook A, Bono F, Jinek M, Conti E: **Structural biology of nucleocytoplasmic transport.** *Annu Rev Biochem* 2007, **76:**647–671.
2. Radu A, Blobel G, Moore MS: **Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins.** *Proc Natl Acad Sci U S A* 1995, **92**(5):1769–1773.
3. Gorlich D, Prehn S, Laskey RA, Hartmann E: **Isolation of a protein that is essential for the first step of nuclear protein import.** *Cell* 1994, **79**(5):767–778.
4. Stade K, Ford CS, Guthrie C, Weis K: **Exportin 1 (Crm1p) is an essential nuclear export factor.** *Cell* 1997, **90**(6):1041–1050.
5. Gorlich D, Kostka S, Kraft R, Dingwall C, Laskey RA, Hartmann E, Prehn S: **Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope.** *Curr Biol* 1995, **5**(4):383–392.
6. Pemberton LF, Paschal BM: **Mechanisms of receptor-mediated nuclear import and nuclear export.** *Traffic* 2005, **6**(3):187–198.
7. Weis K: **Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle.** *Cell* 2003, **112**(4):441–451.
8. Kalab P, Weis K, Heald R: **Visualization of a Ran-GTP gradient in interphase and mitotic Xenopus egg extracts.** *Science* 2002, **295**(5564):2452–2456.
9. Smith AE, Slepchenko BM, Schaff JC, Loew LM, Macara IG: **Systems analysis of Ran transport.** *Science* 2002, **295**(5554):488–491.
10. Kalderon D, Roberts BL, Richardson WD, Smith AE: **A short amino acid sequence able to specify nuclear location.** *Cell* 1984, **39**(3 Pt 2):499–509.
11. Jakel S, Gorlich D: **Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells.** *EMBO J* 1998, **17**(15):4491–4502.
12. Nagoshi E, Imamoto N, Sato R, Yoneda Y: **Nuclear import of sterol regulatory element-binding protein-2, a basic helix-loop-helix-leucine zipper (bHLH-Zip)-containing transcription factor, occurs through the direct interaction of importin beta with HLH-Zip.** *Mol Biol Cell* 1999, **10**(7):2221–2233.
13. Moore JD, Yang J, Truant R, Kornbluth S: **Nuclear import of Cdk/cyclin complexes: identification of distinct mechanisms for import of Cdk2/cyclin E and Cdc2/cyclin B1.** *J Cell Biol* 1999, **144**(2):213–224.

14. Harley VR, Layfield S, Mitchell CL, Forwood JK, John AP, Briggs LJ, McDowall SG, Jans DA: **Defective importin beta recognition and nuclear import of the sex-determining factor SRY are associated with XY sex-reversing mutations.** *Proc Natl Acad Sci U S A* 2003, **100**(12):7045–7050.
15. Lam MH, Briggs LJ, Hu W, Martin TJ, Gillespie MT, Jans DA: **Importin beta recognizes parathyroid hormone-related protein with high affinity and mediates its nuclear import in the absence of importin alpha.** *J Biol Chem* 1999, **274**(11):7391–7398.
16. Strahl T, Hama H, DeWald DB, Thorner J: **Yeast phosphatidylinositol 4-kinase, Pik1, has essential roles at the Golgi and in the nucleus.** *J Cell Biol* 2005, **171**(6):967–979.
17. Lutfiyya LL, Johnston M: **Two zinc-finger-containing repressors are responsible for glucose repression of SUC2 expression.** *Mol Cell Biol* 1996, **16**(9):4790–4797.
18. Lutfiyya LL, Iyer VR, DeRisi J, DeVit MJ, Brown PO, Johnston M: **Characterization of three related glucose repressors and genes they regulate in Saccharomyces cerevisiae.** *Genetics* 1998, **150**(4):1377–1391.
19. Ostling J, Carlberg M, Ronne H: **Functional domains in the Mig1 repressor.** *Mol Cell Biol* 1996, **16**(3):753–761.
20. Ahuatzi D, Herrero P, de la Cera T, Moreno F: **The glucose-regulated nuclear localization of hexokinase 2 in Saccharomyces cerevisiae is Mig1-dependent.** *J Biol Chem* 2004, **279**(14):14440–14446.
21. Ahuatzi D, Riera A, Pelaez R, Herrero P, Moreno F: **Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution.** *J Biol Chem* 2007, **282**(7):4485–4493.
22. Moreno F, Ahuatzi D, Riera A, Palomino CA, Herrero P: **Glucose sensing through the Hxk2-dependent signalling pathway.** *Biochem Soc Trans* 2005, **33**(Pt 1):265–268.
23. Treitel MA, Kuchin S, Carlson M: **Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in Saccharomyces cerevisiae.** *Mol Cell Biol* 1998, **18**(11):6273–6280.
24. Pelaez R, Herrero P, Moreno F: **Nuclear export of the yeast hexokinase 2 protein requires the Xpo1 (Crm1)-dependent pathway.** *J Biol Chem* 2009, **284**(31):20548–20555.
25. Fernandez-Cid A, Riera A, Herrero P, Moreno F: **Glucose levels regulate the nucleo-mitochondrial distribution of Mig2.** *Mitochondrion* 2012, **12**(3):370–380.
26. Wallis JW, Chrebet G, Brodsky G, Rolfe M, Rothstein R: **A hyper-recombination mutation in S. cerevisiae identifies a novel eukaryotic topoisomerase.** *Cell* 1989, **58**(2):409–419.
27. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD: **Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications.** *Yeast* 1998, **14**(2):115–132.
28. Longtine MS, 3rd McKenzie A, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR: **Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae.** *Yeast* 1998, **14**(10):953–961.
29. Ma H, Botstein D: **Effects of null mutations in the hexokinase genes of Saccharomyces cerevisiae on catabolite repression.** *Mol Cell Biol* 1986, **6**(11):4046–4052.
30. Belinchon MM, Gancedo JM: **Different signalling pathways mediate glucose induction of SUC2, HXT1 and pyruvate decarboxylase in yeast.** *FEMS Yeast Res* 2007, **7**(1):40–47.
31. Nehlin JO, Ronne H: **Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins.** *EMBO J* 1990, **9**(9):2891–2898.
32. Mercado JJ, Vincent O, Gancedo JM: **Regions in the promoter of the yeast FBP1 gene implicated in catabolite repression may bind the product of the regulatory gene MIG1.** *FEBS Lett* 1991, **291**(1):97–100.
33. Taberner FJ, Quilis I, Igual JC: **Spatial regulation of the start repressor Whi5.** *Cell Cycle* 2009, **8**(18):3010–3018.
34. Michaelis C, Ciosk R, Nasmyth K: **Cohesins: chromosomal proteins that prevent premature separation of sister chromatids.** *Cell* 1997, **91**(1):35–45.
35. Kadowaki H, Kadowaki T, Wondisford FE, Taylor SI: **Use of polymerase chain reaction catalyzed by Taq DNA polymerase for site-specific mutagenesis.** *Gene* 1989, **76**(1):161–166.
36. Pelaez R, Fernandez-Garcia P, Herrero P, Moreno F: **Nuclear import of the yeast hexokinase 2 protein requires alpha/beta-importin-dependent pathway.** *J Biol Chem* 2012, **287**(5):3518–3529.
37. Kunzler M, Gerstberger T, Stutz F, Bischoff FR, Hurt E: **Yeast Ran-binding protein 1 (Yrb1) shuttles between the nucleus and cytoplasm and is exported from the nucleus via a CRM1 (XPO1)-dependent pathway.** *Mol Cell Biol* 2000, **20**(12):4295–4308.
38. Leslie DM, Timney B, Rout MP, Aitchison JD: **Studying nuclear protein import in yeast.** *Methods* 2006, **39**(4):291–308.
39. Gascon S, Lampen JO: **Purification of the internal invertase of yeast.** *J Biol Chem* 1968, **243**(7):1567–1572.
40. DeVit MJ, Johnston M: **The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of Saccharomyces cerevisiae.** *Curr Biol* 1999, **9**(21):1231–1241.
41. Yoshida K, Blobel G: **The karyopherin Kap142p/Msn5p mediates nuclear import and nuclear export of different cargo proteins.** *J Cell Biol* 2001, **152**(4):729–740.
42. Kosugi S, Hasebe M, Matsumura N, Takashima H, Miyamoto-Sato E, Tomita M, Yanagawa H: **Six classes of nuclear localization signals specific to different binding grooves of importin alpha.** *J Biol Chem* 2009, **284**(1):478–485.
43. Quan X, Yu J, Bussey H, Stochaj U: **The localization of nuclear exporters of the importin-beta family is regulated by Snf1 kinase, nutrient supply and stress.** *Biochim Biophys Acta* 2007, **1773**(7):1052–1061.
44. De Vit MJ, Waddle JA, Johnston M: **Regulated nuclear translocation of the Mig1 glucose repressor.** *Mol Biol Cell* 1997, **8**(8):1603–1618.
45. Fornerod M, Ohno M, Yoshida M, Mattaj IW: **CRM1 is an export receptor for leucine-rich nuclear export signals.** *Cell* 1997, **90**(6):1051–1060.
46. Tiganis T, Flint AJ, Adam SA, Tonks NK: **Association of the T-cell protein tyrosine phosphatase with nuclear import factor p97.** *J Biol Chem* 1997, **272**(34):21548–21557.
47. Henderson BR, Percipalle P: **Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta.** *J Mol Biol* 1997, **274**(5):693–707.
48. Fries T, Betz C, Sohn K, Caesar S, Schlenstedt G, Bailer SM: **A novel conserved nuclear localization signal is recognized by a group of yeast importins.** *J Biol Chem* 2007, **282**(27):19292–19301.
49. Belanger KD, Griffith AL, Baker HL, Hansen JN, Kovacs LA, Seconi JS, Strine AC: **The karyopherin Kap95 and the C-termini of Rfa1, Rfa2, and Rfa3 are necessary for efficient nuclear import of functional RPA complex proteins in Saccharomyces cerevisiae.** *DNA Cell Biol* 2011, **30**(9):641–651.

doi:10.1186/1471-2121-13-31
Cite this article as: Fernández-Cid *et al.*: Yeast importin-β is required for nuclear import of the Mig2 repressor. *BMC Cell Biology* 2012 **13**:31.

Hexokinase 2 is an intracellular glucose sensor of yeast cells that maintains the structure and activity of Mig1 repressor complex

Montserrat Vega, Alberto Riera‡, Alejandra Fernández-Cid‡, Pilar Herrero and Fernando Moreno[1]

From the Department of Biochemistry and Molecular Biology. University of Oviedo. 33006-Oviedo. Spain.

‡ Present address: MRC Clinical Sciences Centre. Imperial College Faculty of Medicine. Hammersmith Hospital Campus. London. UK.

Running head: Hxk2 as intracellular glucose sensor

[1] To whom correspondence may be addressed: Departamento de Bioquímica y Biología Molecular, Universidad de Oviedo, Campus de "El Cristo", 33006-Oviedo. Spain.
Tel.: +34 985 103 567; Email: fmoreno@uniovi.es

Keywords: Glucose signalling, Hxk2, Snf1, Snf4, Gal83, Reg1, Mig1, Mig2.

ABSTRACT

Hexokinase 2 (Hxk2) from *Saccharomyces cerevisiae* is a bi-functional enzyme, being both a catalyst in the cytosol and an important regulator of glucose repression signal in the nucleus. Despite considerable recent progress, little is known about the regulatory mechanism that controls nuclear Hxk2 association with the *SUC2* promoter chromatin and how this association is necessary for *SUC2* gene repression. Our data indicate that in the *SUC2* promoter context, Hxk2 functions through a variety of structurally unrelated factors, mainly the DNA-binding Mig1 and Mig2 repressors and the regulatory Snf1 and Reg1 factors. Hxk2 sustains the repressor complex architecture maintaining transcriptional repression at *SUC2* gene. Using chromatin immunoprecipitation assays, we discovered that the Hxk2 in its open configuration, at low glucose conditions, leaves the repressor complex which induces its dissociation and promotes *SUC2* gene expression. In high glucose conditions, Hxk2 adopts a close conformation that promotes Hxk2 binding to Mig1 protein and the reassembly of the *SUC2* repressor complex. Additional findings highlight the possibility that Hxk2 constitutes an intracellular glucose sensor which operates by changing its conformation in response to cytoplasmic glucose levels that regulate its interaction with Mig1 and thus its recruitment to the repressor complex of the *SUC2* promoter. Thus, our data indicate that Hxk2 is more intimately involved in gene regulation than previously thought.

Transcriptional repressors bind to *cis*-acting elements of gene promoter regions to repress transcription by altering chromatin structure and regulating RNA polymerase II accumulation in the promoter (1). Thus, transcriptional repression involves the coordinated binding of several proteins in the promoter region at specific upstream repressing sequences (URS) to form a repressor complex. This repressor complex also interacts with other corepressors to regulate gene expression (2).

The *Saccharomyces cerevisiae* Tup1 protein is a global corepressor with WD40 repeats that can interact with Ssn6 (3,4) and has been suggested to be a potential yeast orthologue of the Groucho family of corepressors (5). The Tup family transcriptional corepressors are conserved between yeast and humans, and regulate gene expression during stress response and cellular differentiation. *S. cerevisiae* Tup1 represses several genes regulated by glucose, oxidative stress, DNA damage, and other cellular stress responses (6). In the case of several genes regulated by glucose repression, Tup1 is recruited specifically to different promoters by the DNA-binding proteins Mig1 and Mig2 (7-9), to generate a glucose repression state of gene expression. Tup1 represses the expression of genes via distinct mechanisms: by establishing a repressive chromatin structure around the target gene promoter, by recruiting histone deacetylases, and by directly interfering with the general transcription machinery (10,11). However, the precise molecular mechanism of action of Tup1 family proteins in gene repression induced by high glucose levels has not been fully resolved. This is probably because the mechanism depends on the kinetics of formation and dissociation of the repressor complex, which in the case of glucose repression signalling is not well characterized.

Glucose is not only a fuel that serves as a preferential substrate for energy yielding metabolism but also functions as a signalling molecule that regulates the central pathways of carbohydrate metabolism. Although regulation of glucose repression is relatively well understood and the proteins that carry out this regulation have been studied extensively (12), the glucose signalling mechanism and the factors involved in the repressor complexes structure at high and low glucose conditions are scarcely characterized. In high glucose conditions, the repressor protein Mig1 is the main transcription factor responsible for the repression of genes needed for utilization of alternative fermentable carbon sources (7,12). Mig1 binds to DNA and inhibits transcription of *SUC2*, plus approximately 350 other genes (13,14), but it has been demonstrated that in several cases this process also requires the Hxk2 protein (15-17). Expression of *HXK2* gene is controlled by glucose availability and is mediated by the Rgt1 and Med8 transcription factors, which repress *HXK2* expression in low-glucose containing media (18,19). Transcriptional analysis of a *Δhxk2* mutant strain showed significant upregulation of genes with binding sites for Mig1 and/or Cat8 (15,20,21). In addition, Hxk2 also participates in the control of genes encoding sugar transporters (22) and genes modulating mitochondrial cytochrome content and respiratory activity (23). This is also supported by a diminished Crabtree effect in *Δhxk2* mutants, resulting in a nearly complete respiratory metabolism at high glucose concentration (20). Thus, Hxk2 protein can be regarded as a global regulator of carbon metabolism that is essential for mediating the glucose repression signal.

In *S. cerevisiae*, Hxk2 is the predominant glucose-kinase in cells growing in high-glucose conditions and has dual functions (24,25). It is a glycolytic enzyme, essential for cell energy metabolism in the cytoplasm, but also acts as a regulator of gene transcription in the nucleus (26,27). In order to carry out their functions, Hxk2 has to shuttle in and out of the nucleus but the protein is too large to translocate through the nuclear pore complex by diffusion. Thus, the Hxk2 transport across the nuclear envelope must be a mediated and regulated process. Recently, the carrier proteins involved in the Hxk2 transport across the nuclear envelope have been identified. The mechanisms by which Hxk2 enters and exits the nucleus is mediated by the α/β-importin (Kap60/Kap95) pathway (28) and the Xpo1 (Crm1) carrier protein (29), respectively. The directionality of transport is regulated by the guanine nucleotide binding protein Gsp1 (28,29) and the Snf1 kinase-Glc7 phosphatase protein pair works together to control the phosphorylation state of serine-14 of Hxk2 and thus its nucleocytoplasmic distribution (30).

Snf1 kinase, a homologue of mammalian AMP-activated kinase, also plays a central role in regulating the glucose repression signalling pathway. The Snf1 kinase, under low glucose conditions, is

activated and modifies the phosphorylation state of Hxk2 (30) and Mig1 (31). Snf1 kinase forms a complex with an activating subunit Snf4 and one of the three Snf1-interacting proteins Sip1, Sip2 or Gal83, which target Snf1 to distinct subcellular locations (32). Regulation of Snf1 activity involves phosphorylation of the Snf1 catalytic subunit at threonine-210 by one of the three kinases, Sak1, Tos3 or Elm1 (33-35). ADP also appears to play a significant role in Snf1 activation in response to glucose limitation by protecting the enzyme against dephosphorylation by the Glc7-Reg1 phosphatase (36). In addition, high glucose levels inactivate Snf1 by stimulating the activity of the Glc7-Reg1 phosphatase, located in the cytosol (37-39). The activated Snf1 protein kinase phosphorylates at least four serine residues of Mig1 protein in low glucose-growth conditions (37). Nevertheless, only phosphorylation of the serine-311 in Mig1 has been shown to be enough to inhibit Mig1 repressor activity and to induce translocation of the protein from the nucleus to the cytoplasm (40). Thus, the activity of Mig1 is regulated by serine-311 phosphorylation and subcellular localization. In yeast, the regulatory subunit that targets the Glc7 protein phosphatase to Mig1 (41) and Hxk2 (30) in the glucose repression pathway is Reg1. In high glucose, Mig1 and Hxk2 are dephosphorylated by the Glc7-Reg1 protein phosphatase complex (30,42) and are found in the nucleus, where they can repress transcription.

The *SUC2* system provides a good model to understand how Mig1 and Hxk2 proteins control gene expression and how these regulatory factors, together with others, participate in the structure and dynamics of the repressor complex. Here, we demonstrate that under high glucose conditions Hxk2 is a critical factor for the stabilization of the *SUC2* repressor complex. The presence of Hxk2 in the repressor complex is required to inhibit Mig1 phosphorylation by Snf1 kinase (30). In low glucose conditions, the Hxk2 interaction with Mig1 is abolished, perhaps by Snf1-dependent Hxk2 phosphorylation (40), and a transient increment in Snf1 and Mig1 interaction is detected (37). These interaction patterns stimulate Mig1 and Hxk2 phosphorylation by Snf1 kinase at serine-311 (40) and serine-14 (30), respectively. Phosphorylation of Hxk2 and Mig1 results in the export of these proteins from nucleus to cytoplasm and the disassembly of the repressor complex at the *SUC2* promoter. In this paper we also establish that besides Mig1 and Hxk2, the regulatory proteins Mig2, Snf1, Snf4, Gal83 and Reg1 are also part of the repressor complex at the *SUC2* gene promoter both at high and low glucose conditions.

However, the two questions: how glucose level is detected and how this information is transmitted to the nucleus to generate a coordinated cellular response, have not been answered in an appropriate manner until now. Recently, FRET sensor assays have proven to be useful in determining the cytosolic glucose levels in yeast. Quantitative *in vivo* measurement of cytosolic glucose shows a concentration of approximately 10mM in yeast cells growing in complex or synthetic media containing 50-100mM glucose (43). Thus, in a high glucose-containing media Hxk2 will be saturated with cytosolic glucose and thereby in a closed conformation (44). On the other hand, in low glucose media Hxk2 has an open conformation (44). We describe here that the entry or exit of the Hxk2 protein to the *SUC2* repressor complex is not regulated by phosphorylation, but is regulated by the cytosolic glucose levels which ultimately determine the open or closed active site conformation of Hxk2 protein. Thus, we present Hxk2 as an intracellular glucose sensor involved in signalling glucose levels to generate a coordinated transcriptional response.

EXPERIMENTAL PROCEDURES

Strains and growth conditions – The *S. cerevisiae* strains used throughout this study were derived from W303-1A (45), DBY1315 (46) and BY4741 (Euroscarf) haploid wild-type strains and are listed in Table 1. Strains FMY350 and FMY351 expressing Snf1-HA were constructed respectively by homologous recombination in Y14403 and Y04620 strains using an HA-HIS3 tagging cassette obtained from pFA6a-3HA-HIS3MX6 plasmid (47). Strains

FMY403 and FMY833 expressing respectively Snf4-HA and Gal83-HA were constructed by homologous recombination in W303-1A strain using an HA-TRP1 tagging cassette obtained from pFA6a-3HA-HIS3MX6 plasmid (47). Strains FMY320, FMY321 and FMY322 expressing Mig1-GFP were constructed respectively by homologous recombination in W303-1A, Y04620 (Δ*hxk2*) and DBY2052 (Δ*hxk1*Δ*hxk2*) strains using a GFP-HIS3 tagging cassette obtained from pFA6a-GFP-HIS3MX6 plasmid in the two first strains and a GFP-KanMX6 tagging cassette obtained from pFA6a-GFP-KanMX6 plasmid in the last (47). Strains FMY501, FMY507 and FMY509 expressing Mig2-GFP were constructed respectively by homologous recombination in W303-1A, H174 (Δ*mig1*) and Y04620 (Δ*hxk2*) strains using a GFP-HIS3 tagging cassette obtained from pFA6a-GFP- HIS3MX6 plasmid (47). Strains FMY901, FMY902 and FMY903 expressing Reg1-GFP were constructed respectively by homologous recombination in W303-1A, Y14403 (Δ*mig1*) and Y04620 (Δ*hxk2*) strains using a GFP-HIS3 tagging cassette obtained from pFA6a-GFP-HIS3MX6 plasmid (47).

Escherichia coli DH5*α* (*F Ø80dlacZ ΔM15 recA1 endA1 gyrA96 thi-1 hsdR17(rk-rk-) supE44 relA1 deoRΔ 99U169*) was the host bacterial strain for the recombinant plasmid constructions. Fusion protein expression was performed in *E. coli* BL21(DE3)pLysS (Promega).

Yeast cells were grown in the following media: YEPD, high-glucose (2% glucose, 2% peptone, and 1% yeast extract), YEPE, low-glucose (0.05% glucose, 3% ethanol, 2% peptone, and 1% yeast extract), YEPX, xylose medium (2% xylose, 2% peptone, and 1% yeast extract) and synthetic media containing the appropriate carbon source and lacking appropriate supplements to maintain selection for plasmids (2% glucose (SD) or 3% ethanol and 0.05% glucose (SE); and 0.67% yeast nitrogen base without amino acids). Amino acids and other growth requirements were added at a final concentration of 20-150 μg/ml. The solid media contained 2% agar in addition to the components described above.

Plasmids– GST fusion vectors pGEX/MIG1 and pGEX/HXK2 for expression respectively of GST-Mig1 and GST-Hxk2 were constructed as indicated (27). Plasmid pGEX-*XPO1* for expression of GST-Xpo1 in *E. coli* was a gift from C.N. Cole laboratory (48). Plasmids pWS93/Mig1-HA and pWS93/Snf1-HA were generated by cloning a 1.5-kb and 1.9-kb PCR products containing respectively the *MIG1* and *SNF1* genes into the BamH1 site of vector pWS93, which expresses a triple HA epitope from *ADH1* promoter (a gift of P. Sanz, Valencia). The DNA sequence of all PCR-generated constructs was verified by sequencing.

Statistical analysis– Data were obtained from at least 3 independent experiments. Results are shown as the mean ± standard error (S.E.M.).

Enzyme assay– Cells were grown on 2% glucose (H-Glc) and then half of the cells were shifted to 0.05% glucose plus 3% ethanol (L-Glc) or to 2% xylose medium (Xyl). Samples were harvested by centrifugation and washed three times with ice cold saline buffer. Invertase activity was assayed in whole cells as previously described (49) and expressed as micromoles of glucose released per minute per 100 mg of cells (dry weight).

Preparation of crude protein extracts– Yeast protein extracts were prepared as follows: yeasts were grown in 10 to 20 ml of YEPD (H-Glc) at 28ºC to an optical density at 600 nm of 0.8. Half of the culture was shifted to YEPE (L-Glc) or YEPX (Xyl) for 1 h. Cells were collected, washed twice with 1ml of 1M sorbitol and resuspended in 200μl PBS buffer (150mM NaCl, 100mM Na_2HPO_4, 18mM NaH_2PO_4, pH 7.3) containing Roche Protease Inhibitor plus 1mM DTT and 0.1% Triton X100. The cells were broken using a FastPrep homogenizer (Thermo Electron Co.). Two pulses of 20s at 6.0 m/s were given in the presence of glass beads. Then, 200 μl of PBS buffer were added to the suspension. After centrifugation at 19,000 x g for 30 min at 4ºC, the supernatant was used as crude protein extract.

Immunoblot analysis– Mutant or wild-type yeast cells were grown to an optical density at 600 nm of 0.8-1.0 in selective medium containing high-glucose (2%) and shifted to low-glucose conditions for 1h. The cells were collected by centrifugation (3,000 g, 4ºC, 2 min), and crude extracts were prepared as described above. For Western blotting, 20 to 40 µg of proteins were separated by SDS-12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to enhanced chemiluminescence PVDF transfer membrane (Amersham Hybond™-P, GE Healthcare) by electroblotting, which was then incubated with anti-Hxk2, anti-GFP (Santa Cruz Biotech), or anti-HA (Cell Signaling Tech.) as primary antibodies and then the appropriate secondary antibody. Horseradish peroxidase-conjugated protein-A was used as secondary reactant. West Pico Chemiluminescent system (Pierce) was used for detection.

Co-immunoprecipitation assay– Immunoprecipitation experiments were performed using whole cell extracts from different strains. The extracts were incubated with anti- anti-HA, anti-Hxk2, anti-GFP or anti-Pho4 polyclonal antibodies for 3 h at 4ºC. Protein A-Sepharose beads (GE Healthcare) were then added and incubated for 3h at 4ºC in a spinning wheel. After extensive washing with PBS buffer, immunoprecipitated samples were boiled in SDS-loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The supernatant was subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to an enhanced chemiluminiscence PVDF membrane and immunobloted as described above using anti-GFP or anti-Hxk2 polyclonal antibodies. Values shown are representative results from individual experiments.

GST pull-down experiments– *E. coli* cells from the BL21(DE3) pLysS strain were transformed with the fusion protein expression vectors pGEX-MIG1, pGEX-HXK2 or pGEX-*XPO1*. Cells were grown to an OD at 600 nm of 0.6-0.8, induced with 0.5mM isopropyl-1-thio-β-D-galactopyranoside at 37ºC for 3 h, and collected by centrifugation. The pellet was resuspended in PBS buffer (150mM NaCl, 100mM Na_2HPO_4, 18mM NaH_2PO_4, pH 7.3) and sonicated. Insoluble material was removed by centrifugation (17,000 x g for 20 min at 4ºC). The soluble extract was incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at 4ºC, washed extensively with PBS buffer and resuspended in the same buffer. The GST-Hxk2 fusion protein coupled to glutathione-Sepharose beads was incubated with 2.5 units of thrombin (2h at 4ºC) for site specific separation of the GST affinity tag from Hxk2 protein. Identical amounts of Hxk2 affinity purified protein were added to 20 µl of assay buffer (150mM NaCl, 100mM Na_2HPO_4, 18mM NaH_2PO_4, pH 7.3 containing 8mM magnesium acetate, 0.5mM EDTA and 0.5mM DTT) and incubated in the presence or absence of 1mM ATP, 1mM AMP-PNP or different amounts of D-glucose and D-xylose for 30 min at 30ºC. The GST-Mig1 fusion protein coupled to glutathione-Sepharose was incubated with the assay mixture for 1h at 4ºC in the assay buffer. The GST-Xpo1 fusion protein coupled to glutathione-Sepharose was incubated with whole cell extracts from the FMY901 strain. The cell extracts were obtained from yeast cells grown in high-glucose and shifted to low-glucose medium for 60 min. Beads were gently washed five times with 2.5 ml of PBS buffer, boiled in 25 µl sample-loading buffer, and analysed by SDS-PAGE followed by Western blot. Proteins were separated by SDS-12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an enhanced chemiluminescence PVDF transfer membrane (Amersham Biosciences) by electroblotting. The membrane was then incubated with anti-Hxk2 antibody as primary antibody and anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as secondary antibody. Horseradish peroxidase-conjugated protein-A was used as secondary reactant. West Pico Chemiluminescent system (Pierce) was used for detection.

Chromatin immunoprecipitation assay – Chromatin immunoprecipitation (ChIP) assays were performed essentially as

described previously (19). Cells were cultured in 100 ml of YEPD medium to reach an OD at 600 of 0.8. Cells from half of the culture were collected by centrifugation (3.000 g, 4ºC, 2 min) at room temperature. Then, the cell pellet was washed two times with YEP medium, resuspended in YEPE or YEPX medium and incubated for 1h at 28ºC with shaking. 1 ml 37% formaldehyde was added and samples were incubated for additional 30 min. Finally, cells were extensively washed, broken and sonicated. The cross-linked chromatin solution was collected, Hxk2 and GFP or HA-tagged proteins were immunoprecipitated by incubating respectively the chromatin solution with anti-Hxk2 (26), anti-GFP or anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) pre-bound to protein A-Sepharose beads (General Electric) for 4h at 4ºC. Precipitated complexes were washed 5 times with PBS buffer, immunoprecipitants were eluted from the beads by heating to 65ºC for 10 min in elution buffer (50mM Tris-HCl, pH 8.0, 10mM EDTA, 1% SDS). Formaldehyde cross-link was reverted by heating to 65ºC overnight and incubation with proteinase K for 1h at 37ºC. DNA was precipitated after phenol-chloroform extraction. Amounts of specific DNA target present in input and immunoprecipitated samples were analysed by PCR using primers: OL-d, 5'-AGCTCGAGTTATTACTCTGAACAGG A-3' (sense) and OL-r, 5'-TAGTCGACA AGTCGTCAAATCTTTCT-3' (antisense), specific to the SUC2B-URS element of the *SUC2* promoter. Quantitative PCR analysis was performed by triplicate with the same primers indicated above. *ACT1* (OL_{ACT1}-d, GCCTTCTACGTTTCCATCCA and OLACT1-r, GGCCAAATCGATTCTCAA AA on the ORF of the *ACT1* gene) and anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used as negative controls. The linearity of PCR reactions was shown by multiple template dilutions of input and immunoprecipitated DNA and varying the number of PCR cycles. Gene-specific PCR was usually 18–22 PCR cycles. In general, immunoprecipitated DNA samples were initially diluted 1:10 and inputs were diluted 1:50 with 10 mM Tris HCl pH 8.5. Control PCR for *ACT1* (not shown, expression was not influenced by glucose induced nutritional stress), anti-rabbit antibody (unspecific antibody) and extracts prior to immunoprecipitation (input, whole-cell extract) were used as internal controls at 30–35 cycles, in order to detect a background signal for normalization. Experiments were performed using three independent chromatin preparations, and quantitative PCR analysis was performed in real time using an Applied Biosystems 7300 sequence detector. Data is presented as fold immunoprecipitation over the unspecific antibody precipitated DNA.

RESULTS

Interdependent binding of Mig1 and Hxk2 at the SUC2 promoter repressor complex– Repression of the *SUC2* gene is carried mainly by Mig1, a zinc-finger DNA-binding protein (7). The Mig1 protein represses transcription by binding to two upstream repressing sequences (URS) of the *SUC2* promoter (SUC2A: 5'-A_{-499}A TAAAAATGCGGGGAA$_{-484}$-3' and SUC2B: 5'-G_{-449}GAAATTATCCGGGGG C_{-431}-3') and recruiting the general corepressors Tup1 and Ssn6 (13). Either SUC2A or SUC2B (Fig. 1A) is sufficient for Mig1-mediated repression, indicating that these two elements are functionally redundant with regard to Mig1 repressor function (50), hereafter we referred to them as SUC2 elements. Previous experiments established that Mig1 makes a large contribution toward the glucose-induced repression of *SUC2* but there is a lack of detailed information about the transmission mechanism of glucose signal from the environment to the Mig1 repressor complex. To answer this question, we employed a system that measures the extent to which the SUC2 elements can be cross-linked to a specific protein *in vivo* (51). We therefore investigated the interaction of Mig1, and several other factors significantly related to glucose repression signalling, with the *SUC2* promoter by chromatin immunoprecipitation (ChIP). Cross-linked protein-DNA complexes were immunoabsorbed, and selected stretches of co-precipitated DNA were amplified by PCR and analysed by gel electrophoresis. Moreover, to quantify the amount of each factor associated with the SUC2 elements region we used RT-PCR. As

a control gene we used *ACT1* and as a control antibody we used anti-rabbit. As it can be seen in our results, there is little or no detectable DNA precipitation in the control lanes, demonstrating the specificity of our ChIP assay.

Cells expressing a functional GFP-tagged form of the Mig1 protein at its chromosomal locus were exposed to high and low glucose conditions and used for ChIP assays. The results shown in Fig. 1B and C indicate that Mig1-GFP binding to SUC2 elements of the *SUC2* promoter occurs in a glucose-dependent manner, with peak Mig1-binding activity in cells grown in high glucose conditions (Fig. 1B-a and C). Glucose starvation decreases the association of Mig1 with SUC2-URS region by 68% (Fig. 1B-a and C). Our finding is consistent with Mig1 subcellular localization in high glucose conditions, but not in low glucose conditions (40,52). It was previously reported that upon glucose removal, Mig1-GFP rapidly translocates to the cytoplasm (52). However, our results suggest that 32% of the protein remains associated with the *SUC2* promoter after glucose starvation. This indicates that the interaction of Mig1 with the *SUC2* promoter might be regulated by both the phosphorylation state of Mig1 (38) and an independent mechanism. Since the *HXK2* gene is expressed in cells grown in high glucose (25), but after shifting the cells to a low glucose medium the *HXK2* gene is repressed and the *HXK1* gene is rapidly expressed (15). A possible explanation for the independent mechanism is that, in low glucose conditions, Hxk1 partially mimics the function performed by Hxk2 during glucose repression signalling in high glucose. In order to avoid the synthesis of Hxk1 in low glucose conditions, we used a *Δhxk1Δhxk2* double mutant strain, with a functional GFP-tagged form of the Mig1 protein at its chromosomal locus, to perform ChIP assay. The results for Mig1-GFP *SUC2* chromatin interactions are shown in Figures 1B-b and C. In high and low glucose conditions, less than 5% of the protein became cross-linked with the SUC2-URS of the *SUC2* promoter (Fig. 1C), a much lower degree of binding than found in a wild-type strain at low glucose conditions. Since the Hxk2 protein is necessary for glucose-induced repression of the *HXK1* gene (15) and only 30% of *SUC2* repression is lost in *Δhxk2* mutant cells (Fig. 2), it seems likely that Hxk1 protein could mimic Hxk2 function. To further address this possibility, we used a *Δhxk2* mutant strain, with a functional GFP-tagged form of the Mig1 protein at its chromosomal locus, to perform the ChIP assay. We found that in the absence of Hxk2, the recruitment of Mig1 to the *SUC2* promoter is significantly decreased in high glucose conditions (Fig. 1B-c and C). However, the recruitment of Mig1 to the *SUC2* promoter in low glucose conditions is identical to that found in the wild-type strain in glucose starved cells. Together, these results support that in high glucose conditions, Mig1 recruitment to SUC2-URS of the *SUC2* promoter is Hxk2-dependent and the presence of Hxk1 is important to modulate the Mig1 SUC2-DNA binding in low glucose conditions.

Since it is known that the Hxk2 protein interacts directly with Mig1 *in vivo* and *in vitro* (27), firstly we investigated the interaction of Hxk2 with the *SUC2* promoter in high and low glucose conditions. In second place, we investigated how Mig1 affects this interaction. We tested direct interactions *in vivo* by ChIP experiments using a specific anti-Hxk2 antibody. A comparison between high and low glucose growing cells revealed that nuclear Hxk2 was well engaged with the SUC2 elements of the *SUC2* promoter in high glucose, but less than 8% of Hxk2 was bound to the SUC2 elements in low glucose conditions (Fig. 3A-a, B). The occupation rate of the SUC2 regions of the *SUC2* promoter by Hxk2 in high glucose conditions is affected by the absence of the Mig1 repressor. In *Δmig1* mutant cells growing in high or low glucose conditions, less than 2% of Hxk2 is associated to the *SUC2* promoter, demonstrating that binding of Hxk2 to the *SUC2* promoter is Mig1-dependent (Fig. 3A-b and B). These results indicate that Mig1 is required to capture the Hxk2 protein to the repressor complex of the *SUC2* promoter and that Hxk2 do not interact directly with DNA.

In *Δmig1* mutant cells growing in high glucose conditions, only 27% of *SUC2*

repression is lost (Fig. 2) so it seems likely that other DNA binding proteins could mimic Mig1 function. Since like Mig1, Mig2 represses transcription in response to glucose through Tup1 and Ssn6 corepressors (9), we have investigated the role of Mig2 in the SUC2 repressor complex and how Mig1 and Hxk2 affect Mig2 *SUC2* promoter interaction. A wild-type strain with a functional GFP-tagged form of the Mig2 protein at its chromosomal locus was used to determine by ChIP the SUC2-URS localization of Mig2. In wild-type cells grown in high and low glucose conditions about 26-28% of Mig2 was recruited to the SUC2 elements of the *SUC2* promoter (Fig. 4A-a and B). The level of Mig2 associated with the *SUC2* promoter reaches a maximum at low glucose conditions in a *Δhxk2* mutant strain (100%) (Fig. 4A-c and B). The level of Mig2 associated with the *SUC2* promoter also increases at high and low glucose conditions in a *Δmig1* mutant strain with respect to the wild-type (Fig.4A-b and B). Thus, in high glucose conditions in the absence of Mig1 protein, Mig2 is actively recruited to the *SUC2* promoter (95%) contributing to maintain 70% of *SUC2* gene repression. In low glucose conditions 45% of the Mig2 protein was associated with the SUC2 elements of the *SUC2* promoter (Fig. 4A-b and B). Although Mig2 appears to play no role in *SUC2* repression when Mig1 is present because its deletion has no effect on *SUC2* expression in a *MIG1* strain (Fig. 2), yeast cells are only fully derepressed in a *Δmig1Δmig2* double mutant strain (Fig. 2). These data support the idea that Mig2 may bind to the *SUC2* promoter mainly in the absence of Mig1 protein as happens in *Δmig1* or *Δhxk2* mutant strains. It also appears that the role of Mig2 in the yeast cell is as a redundant transcriptional repressor.

It is also tempting to speculate that Mig2 could function as a structural protein which, by interacting with other factors associated with the *SUC2* promoter, could stabilize the repressor complex structure. To address this possibility, we examined the ability of Mig2 to interact with Mig1, Hxk2 and Snf1 by using an immunoprecipitation assay. Cell extracts from a strain with a modified *MIG2* gene, which encodes for a C-terminal Mig2 protein tagged with GFP, were immunoprecipitated with the antibodies indicated in Fig. 5. Following immune precipitation, the Mig2-GFP protein was detected in a complex with Mig1 (Fig. 5A), Hxk2 (Fig. 5B) and Snf1 (Fig. 5C), from both glucose-starved cells and glucose-rich medium grown cells. When an anti-Pho4 antibody was used to detect unspecific immunoprecipitation no signals were observed. Similar amounts of the Mig2-GFP protein were detected in the different protein extracts used by immunoblot analysis with an anti-GFP antibody. We conclude that Mig2 associates with Mig1, Hxk2 and Snf1 *in vivo* and that the association is independent of the glucose levels in the culture medium.

Hxk2 recruits SNF1 complex and Reg1-Glc7 phosphatase to the repressor complex of the SUC2 promoter – Because it has been previously described that the Snf1 kinase is constitutively associated with Hxk2 both at high and low glucose conditions and that Hxk2 accumulates in the nucleus upon *SNF1* gene disruption, we considered a model in which the main regulatory proteins of the phosphorylation state of Mig1 and Hxk2 act by stable association with SUC2-URS on the *SUC2* promoter. We used ChIP analysis to test this model. Similar chromatin binding experiments to that described above were pursued with Snf1, Snf4, Gal83 and Reg1 proteins all of them characterized as important regulatory factors in glucose repression signalling. Thus, we investigated if the SNF1 complex proteins interact with the SUC2-URS of the *SUC2* promoter in high and low glucose conditions and how Mig1 and Hxk2 affect this interaction. Cells expressing functional HA-tagged forms of Snf1, Snf4 and Gal83 proteins at their chromosomal loci were first exposed to high glucose conditions until an OD_{600} of 0.8 was reached and then shifted for 60 min to low glucose conditions. Proteins were then cross-linked to DNA with formaldehyde, followed by cell lysis and DNA fragmentation. Snf1-HA, Snf4-HA and Gal83-HA were immunoprecipitated from extracts, and coprecipitating SUC2-URS of the *SUC2* promoter sequence was detected by PCR and RT-PCR.

As shown in Fig. 6A-a, b, c and B, Snf1, Snf4 and Gal83 bind specifically to the SUC2 elements sequence both at high and low glucose conditions. We find that Snf4 recruitment (100%) to the *SUC2* promoter, at high glucose conditions, was more pronounced than Snf1 (43%) and Gal83 (36%). In low glucose conditions, a 72% of Snf4 remains at the *SUC2* promoter and a similar amount to that found at high glucose conditions of Snf1 (48%) and Gal83 (35%). To determine if Snf1-HA association with the *SUC2* promoter was dependent on the Mig1 and Hxk2 proteins, the *SNF1* gene was HA-tagged at its chromosomal locus in *Δmig1* and *Δhxk2* mutant cells. As can be seen in Fig. 6A-d, e and B, Snf1-HA was not detected in association with the *SUC2* promoter both at high and low glucose conditions in *Δmig1* and *Δhxk2* mutant cells.

To analyse the specific requirements for incorporation of Snf1, Snf4 and Gal83 to the repressor complex at the *SUC2* promoter, we conducted GST pull-down experiments to characterize physical interactions of these proteins with Hxk2. As was observed in Fig. 7A, and previously (30), a strong and specific signal of Snf1-HA was observed both with samples from high and low glucose-grown cells. We next tested the interactions of Snf4 and Gal83 with Hxk2 by GST pull-down experiments. We used extracts from the Snf4-HA and Gal83-HA producing strains and GST-Hxk2 expressed in bacteria. As shown in Fig. 7B and D, weak signals of Snf4 and Gal83 proteins, retained by GST-Hxk2, were detected in extracts from either high or low glucose-grown cells. When a bacterial produced GST protein was used as bait in these experiments in order to detect nonspecific protein binding, no signals were observed. To confirm that the same amount of Snf1-HA, Snf4-HA and Gal83-HA fusion proteins are present in each sample used in these experiments, cell extracts from high and low glucose grown cells were subjected to western blot analysis. Since in the extracts we keep the amount of each SNF1 complex factor constant, and the Snf4 and Gal83 signal intensity in western blot experiments was greatly reduced in comparison with Snf1, we believe that these results suggest that the interaction takes place at the level of the Snf1 subunit and the weak detection of Gal83 and Snf4 proteins could be due to indirect Snf1-Snf4 and Gal83 interactions. To test this hypothesis, we used GST pull-down assay in a *Δsnf1* mutant strain with a functional HA-tagged form of the Snf4 protein at its chromosomal locus. Our results show that in cells grown in high and low glucose medium, Snf4 protein was not detected in complex with Hxk2 (Fig. 7C). We conclude that Snf4 association with Hxk2 is Snf1-dependent and that Snf4 does not interact directly with Hxk2.

Given these results, it could be tempting to propose that Hxk2 could act as an anchor point for the proteins that are part of the *SUC2* gene repressor complex. To address this possibility, we examined the ability of Reg1 to interact *in vivo* with Hxk2 at the *SUC2* promoter level by using a ChIP assay. The wild-type, *Δmig1* and *Δhxk2* strains with functional GFP-tagged forms of the Reg1 protein at its chromosomal locus were also used to determine by ChIP the SUC2-URS localization of Reg1 in the absence of Mig1 and Hxk2 proteins. The cells were first exposed to high glucose conditions until an OD_{600} of 0.8 was reached and then shifted for 60 min to low glucose conditions. In FMY901 cells expressing the Reg1-GFP fusion protein, Reg1 was detected in a complex with SUC2-URS element of the *SUC2* promoter both at high and low glucose conditions (Fig. 8A-a and B). However, less than 5% of the Reg1-GFP fusion protein bound to the *SUC2* promoter in a FMY901 cell was detected in *Δmig1* and *Δhxk2* mutants, both in high and low glucose conditions (Fig. 8A-b,c and B). Thus, the repressor complex of the *SUC2* promoter is an assembly of the proteins that directly bind to DNA (Mig1 and Mig2) as well as the machinery regulating the Mig1 repressing activity.

To confirm these results we also determined the ability of Reg1 to interact with Hxk2 by co-immunoprecipitation assay. Cell extracts from a strain with a modified *REG1* gene, which encodes for a C-terminal Reg1 protein tagged with GFP, were immunoprecipitated with anti-GFP antibody. Following immune precipitation, the Reg1-GFP protein was detected in a complex with Hxk2 (Fig. 9A), from both

glucose-starved cells and glucose-rich medium cells. When an anti-Pho4 antibody was used to detect unspecific immunoprecipitation no signals were observed. Similar amounts of the Hxk2 protein was detected in the different protein extracts used by immunoblot analysis with an anti-Hxk2 antibody. We conclude that Reg1 associates with Hxk2 *in vivo* and that the association is independent of the glucose levels in the culture medium.

Since it has been described that Reg1 is predominantly cytoplasmic (53), while our results indicate that Reg1 also has nuclear localization, we examined the ability of the protein to interact with the import (Kap60-Kap95) and export (Xpo1) machinery used by Hxk2 to enter and exit the nucleus (28,29). To test whether Reg1 binds to Kap60, Kap95 and Xpo1 *in vivo* we used immunoprecipitation and GST pull-down assays in cells expressing Reg1-GFP. First, cell extracts from FMY901 (Reg1-GFP) strain were immunoprecipitated with anti-Kap60 and anti-Kap95 antibodies. The resulting immunoprecipitates were assayed for the presence of Reg1-GFP by immunoblot analysis with anti-GFP antibodies. As shown in Fig. 9B, a strong and specific Reg1-GFP signal was observed with samples immunoprecipitated with an anti-Kap60 antibody in cells grown both in high and low-glucose conditions. However, no or very weak interaction was observed when the experiment was done using samples immunoprecipitated with anti-Kap95 antibody, although similar amounts of Kap60 and Kap95 proteins were detected in the immunoprecipitates. Similarly, the same amount of the Reg1-GFP protein was detected by immunoblot analysis with anti-GFP antibody from the protein extract used for immunoprecipitation (Fig. 9B; lane 7). These results suggest that Reg1 enters the nucleus via the canonical route by directly binding to the α-importin Kap60 and the β-importin Kap95 is recruited to the ternary complex mainly during high-glucose growth conditions. Then, the tripartite protein complex could be recognized by the nuclear pore for transport into the nucleus.

To investigate the Reg1-Xpo1 interaction, we performed GST pull-down experiments with raw protein extracts from a strain expressing a functional Reg1-GFP protein at its chromosomal locus and purified GST-Xpo1 fusion protein produced from bacteria. As shown in Fig. 9C, a clear retention of Reg1 protein was observed in the sample containing GST-Xpo1 and raw extract from the FMY901 strain grown in high and low-glucose conditions. However, the interaction between Reg1-GFP and Xpo1 was systematically stronger with samples from low glucose-grown cultures than from high glucose cultures, our data suggest that the low glucose condition increases the affinity of Reg1 for the export receptor. When a control with GST protein alone in the reaction mixture was used, no signal was observed. These results suggest that Reg1 is a cargo of Xpo1 to exit the nucleus in *S. cerevisiae*. Since results from numerous studies support a direct substrate interaction mechanism to explain the ability of Reg1 to direct Glc7 to its substrates of interest, it is tempting to speculate that the Glc7 phosphatase could be recruited by Reg1 to the repressor complex of the *SUC2* promoter to act over its substrates Snf1, Mig1 and Hxk2.

Regulation of the Hxk2 incorporation to the repressor complex of the SUC2 promoter – Hxk2 protein shuttles in and out the repressor complex of the *SUC2* promoter in response to glucose availability. Since the shuttling back and forth between the nucleus and the cytoplasm of Hxk2 is also controlled by glucose availability and glucose determines the phosphorylation state of Hxk2 by controlling Snf1 kinase and Reg1-Glc7 phosphatase activities (30), an attractive hypothesis could be that the phosphorylation state of Hxk2 regulates its incorporation and dissociation from the repressor complex of the *SUC2* promoter. To address this possibility, we used a *Δsnf1* mutant strain to perform ChIP assays using a specific polyclonal antiserum against Hxk2. As shown in Fig. 10A and B, ChIP analysis indicates that Hxk2 is able to interact with the *SUC2* promoter in high glucose (95%) but less than 8% of Hxk2 was bound to the SUC2 elements in low glucose conditions, which is a similar result to that obtained using a wild-type strain. Thus, this result supports that phosphorylation of Hxk2 by

the Snf1 kinase is not essential to regulate Hxk2-*SUC2* promoter binding.

We then tested whether conformational changes in the Hxk2 protein affect the binding of Hxk2 to both Mig1 and the *SUC2* promoter. D-Xylose binding in the presence of ATP promotes a conformational change in Hxk2 and its inactivation by autophosphorylation of serine-158 (54-57). Additionally, glucose protects against xylose-induced inactivation with efficiencies closely related to their respective glucose-*Km* or xylose-*Ki* values (57). Thus, it seems likely that xylose, a glucose non phosphorylable analogue, could induce an open conformation of the protein that mimics the low glucose conditions. To address this possibility we first used GST pull-down assays to determine the effect of xylose on the interaction between Hxk2 and Mig1. GST and GST-Mig1 were purified from *Escherichia coli* with the help of glutathione beads, and equal amounts of purified Hxk2 were incubated with the beads in the presence of different metabolites. After precipitation, the beads were washed and bound Hxk2 was detected by western blot with the help of a specific anti-Hxk2 antibody. Fig. 11A shows that GST-Mig1, but not GST alone, was able to precipitate the purified Hxk2 protein either in the absence or in the presence of substrates as glucose and ATP or the non-hydrolysable ATP analogue AMP-PNP. However, no signal was observed when the assay was done in the presence of 4mM xylose (Fig. 11A, lanes 7-9). This experiment supports that the Hxk2-Mig1 interaction is abrogated by xylose and does not require neither ATP nor AMP-PNP. This result suggest that the efficiency of xylose as inactivating agent of Hxk2-Mig1 interaction could be related to its ability to bind to Hxk2 and not to inhibit competitively the hexokinase reaction for which a metal-nucleotide complex is also required (57). In order to confirm that the xylose-Hxk2 binding is critical for suppression of Hxk2-Mig1 interaction, we measured it at a fixed glucose concentration (0.5mM) and increasing xylose concentrations. Fig. 11B shows that increasing xylose concentration in the reaction mixture promotes the abrogation of Hxk2-Mig1 interaction, with total suppression at 4mM xylose. A similar study was carried out with variable concentrations of glucose at a fixed (2mM) concentration of xylose. The results shown in Fig. 11C support that glucose acts as a protecting agent with high efficiency to revert the changes induced by xylose. Taken together, these results show that the regulation of Hxk2 interaction with Mig1 involves a conformational change of the protein induced by the sugar substrate glucose and its analogue xylose. Thus, it could be assumed that the same flexibility of the Hxk2 active site which allows a "induced fit" (58) can promote an inactive conformation.

To confirm these results obtained *in vitro* with purified proteins, we performed ChIP assays in the presence of xylose to characterize *in vivo* Hxk2 binding to the *SUC2* promoter. Fig. 12A-a and B shows that the binding of Hxk2 to the *SUC2* promoter was affected by the presence of xylose and less than 6% of Hxk2 was bound to the SUC2-URS of the *SUC2* promoter in these growth conditions, supporting that in the presence of xylose an inactivating Hxk2 conformational change is induced. As can be seen in Fig. 12A-b and B, the occupation rate of the SUC2 elements of the *SUC2* promoter by Hxk2 in *Δsnf1* mutant cells is also affected by the presence of xylose. A comparison between high glucose and xylose conditions revealed that nuclear Hxk2 was well engaged with the SUC2 elements of the *SUC2* promoter in high glucose, but only 14% of Hxk2 was bound to the SUC2 elements in xylose conditions. In a *Δsnf1* mutant strain Mig1 and Hxk2 are dephosphorylated and cells are repressed both at high and low glucose conditions (Fig. 2). Although in this mutant strain an inactivating Hxk2 conformational change induced by xylose was observed (Fig. 12B), *SUC2* gene expression was not promoted (Fig. 12C). A possible explanation is that although Hxk2 has adopted an inactive structure, since only 14% of the protein remains associated with the *SUC2* promoter (Fig. 12B), Mig1 is not phosphorylated and stays associated with the SUC2-URS element repressing gene transcription. Conversely, in a wild-type strain Hxk2 is removed from the repressor complex,

favouring the phosphorylation of Mig1 by Snf1 kinase (30, 40), which promotes increased invertase activity (Fig. 12C). Therefore, Hxk2 phosphorylation does not affect the interaction of Hxk2 with Mig1, but affects the regulation of Hxk2 nucleocytoplasmic shuttling between the nucleus and the cytoplasm (30). Taken together these results strongly suggest that hexokinase 2 acts as an intracellular glucose sensor and that the structural conformation of Hxk2 regulates its interaction with Mig1 and it binding to the repressor complex of the *SUC2* promoter.

DISCUSSION

Although previous studies (27,30,40) pointed to a role of Hxk2 in transcription it was not clear how Hxk2 affects this process. Here we show that Hxk2 is recruited to the SUC2-URS of the Mig1-dependent gene, *SUC2*, as a component of a repressor complex by direct physic interaction with Mig1. Subsequently, it promotes the formation of a functional repressor complex by recruiting the SNF1 complex proteins Snf1, Snf4 and Gal83, together with the Reg1 subunit of Glc7 protein phosphatase. This complex is disassembled during low glucose conditions and is reassembled in high glucose conditions. In this work, we tried to readdress two main questions: how Hxk2 promotes the formation and stabilization of a repressor complex at the *SUC2* promoter, and how glucose level regulates this process.

Mig1 and Mig2 have similar DNA-binding zinc fingers and recognize identical DNA-binding sequences (59). However, Mig1 protein is more important than Mig2 in *SUC2* repression, since it is sufficient to achieve complete repression. To investigate the importance of Hxk2 in the Mig1 binding to the SUC2-URS we used a ChIP assay and cells from both *Δhxk1Δhxk2* double mutant and *Δhxk2* single mutant strains expressing a Mig1 GFP-tagged protein. We found that in the absence of both Hxk1 and Hxk2 proteins Mig1 cannot bind to SUC2-URS region. In these mutant cells less than 5% of Mig1 was found associated with the *SUC2* promoter both at high and low glucose conditions, a level as low as that measured in wild-type cells growing in low glucose. The most straightforward interpretation of these results could be that, in the absence of both Hxk1 and Hxk2 proteins, the Snf1 kinase is activated (39,60), Mig1 is phosphorylated and is exported to the cytoplasm by binding to the Msn5 transporter (61). However, in the absence of Hxk2 protein the recruitment of Mig1 to SUC2-URS is very similar to that found in a wild-type strain grown in low glucose conditions. A possible interpretation of these results could be that, since the *HXK1* gene expression is highest during growth on low-glucose and glucose-induced repression involves Hxk2, the Hxk1 protein could mimic, at least partially, the Hxk2 function at the *SUC2* promoter repressor complex both in low glucose conditions and in *Δhxk2* mutant cells. In this regard, Hxk1 and Hxk2 proteins have more than 77% identity, and the first 24 amino acids of the N-terminal region are identical in both. Since Hxk2-Mig1 interaction is mediated by a 10-amino acid motif in Hxk2 located between lysine-6 and methionine-15 (27,62) (Hxk2 is numbered from residues 1 to 485; residue 1 is a valine because the initiator methionine is cleaved off from the primary translation product) and Hxk1 has in its N-terminal region the same 10-amino acid motif, we can supposed that Hxk1 could also interact with Mig1 protein in these metabolic conditions to prevent, at least in part, the Snf1 kinase-mediated phosphorylation of Mig1 at serine-311. Thus, in low glucose conditions 32% of the Mig1 protein is retained in the nucleus associated to the SUC2-URS and 70% *SUC2* repression is maintained. This behaviour has not been previously described because the amount of Mig1-GFP retained in the nucleus is probably not detectable by conventional fluorescent microscopy, so in these conditions it has been claimed that Mig1 is localized exclusively in the cytoplasm (52). These results explain why fully derepression of the *SUC2* gene is only achieved in the absence of both Hxk1 and Hxk2 proteins (Fig. 2).

Moreover, the binding of the regulatory factors Snf1 and Reg1 to the SUC2 elements of the *SUC2* promoter is Hxk2-dependent. In the absence of either Mig1 or Hxk2 proteins, recruitment of both Snf1 and Reg1 proteins to the *SUC2* promoter is abolished. Since in

an *Δhxk2* mutant strain about 30% of Mig1 protein remains associated with the *SUC2* promoter, we should detect a similar amount of Snf1 and Reg1 proteins associated with the SUC2-URS element if Mig1 where the critical factor for this association. However, less than 5% of these proteins where detected associated with the *SUC2* promoter in an *Δhxk2* strain, suggesting that the interaction of Snf1 and Reg1 proteins with the *SUC2* repressor complex is Hxk2-dependent. These results are also supported by the fact that Hxk2 interacts with Snf1 and Reg1 proteins both at high and low glucose. Although Reg1 has been described as a predominantly cytoplasmic protein (53), our data for the first time support that a fraction of the Reg1 and Snf1 proteins present in the cell are constitutively associated with SUC2-URS element of the *SUC2* promoter, together with their substrates of interest, Hxk2 and Mig1, that exhibit the same nuclear localization.

Snf4 and Gal83 proteins, the regulatory units of the SNF1 complex, have also been detected associated with SUC2-URS elements at a similar level both in high and low glucose conditions. On the other hand, GST pull-down experiments show a weak interaction of Snf4 and Gal83 proteins with Hxk2, and in the absence of Snf1 no interaction of Snf4 with Hxk2 was observed. Thus, SNF1 complex regulatory proteins Snf4 and Gal83 binding to *SUC2* repressor complex appears to be Snf1-dependent.

Here, we show that Hxk2 is recruited to the SUC2-URS sequences of the *SUC2* promoter by its interaction with the DNA-bound Mig1 protein and subsequently it promotes the formation of a functional repressor complex. Snf1 and Reg1, whose interaction with Hxk2 was confirmed *in vitro* and *in vivo*, are also components of it. Mig2 appears to play no role in *SUC2* repression when Mig1 is present, since in these conditions the absence of Mig2 has no effect on *SUC2* expression. However, in wild-type cells grown in high and low glucose conditions approximately 25% of the Mig2 detected in the absence of Mig1 was recruited to the *SUC2* promoter. The level of Mig2 associated with the *SUC2* promoter increases in the absence of either Mig1 or Hxk2 proteins, which contributes to maintain approximately 70% of *SUC2* repression, as observed in *Δmig1* and *Δhxk2* mutant cells (Fig. 2). Taking into account that Mig2 interacts with Mig1, Hxk2 and Snf1 proteins both in high and low glucose conditions, our results suggest that Mig2 could be a structural component of *SUC2* repressor complex in high glucose. In the absence of Mig1 or Hxk2 proteins an increased amount of Mig2 is dragged to the *SUC2* promoter, and thus 70% of *SUC2* repression is observed. In these conditions, SNF1 complex components would remain associated with the *SUC2* promoter due to their interaction with Mig2. A fully derepression of the *SUC2* gene, in high and low glucose conditions, is only achieved in the absence of either Mig1-Mig2 or Hxk1-Hxk2 protein pairs, because in these conditions the *SUC2* promoter repressor complex is completely disassembled.

Although our ChIP experiments support that the *SUC2* repressor complex dynamically disassembles and reassembles in a glucose-dependent manner, they do not resolve the problem of how glucose signal is transmitted from the environment to the nucleus to regulate *SUC2* expression. The results reported here provide for the first time a much clearer picture on this important point. The absence of Hxk2 impairs the recruitment of Mig1, Snf1 and Reg1 to the *SUC2* promoter and hence the assembly of a functional repressor complex. Since Hxk2 seems to be a key factor for the assembly of *SUC2* repressor complex, it could be a good candidate to operate as a glucose sensor that communicates glucose level information from the environment to the Mig1-regulated genes. A first question unanswered is how Hxk2 phosphorylation affects the Hxk2 recruitment to the *SUC2* promoter. Our data clearly suggest that an impaired Hxk2 phosphorylation at serine-14, as happens in the absence of the Snf1 kinase, does not alter either repressor complex formation or its glucose-dependent regulation. Thus, Hxk2 promotes the assembly of *SUC2* repressor complex independently of its phosphorylation state. The phosphorylation state of Hxk2 regulates its nucleocytoplasmic shuttling between nucleus and cytoplasm, but not its assembly

and disassembly to the repressor complex of the *SUC2* promoter.

A second possibility is that Hxk2 assembly to the *SUC2* promoter could be regulated by conformational changes induced by glucose levels (44). Since measuring dynamic changes of cytosolic glucose by FRET sensors demonstrated that cytosolic glucose accumulation is a direct function of glucose levels in the medium (43), it seems likely that a Hxk2 conformational change induced by glucose could be the signal that induces Mig1-Hxk2 interaction. The Hxk2 protein is folded into two domains of unequal size called the large and the small domain (63). These are separated by a deep cleft containing the residues making up the enzyme active site. In low glucose conditions, the Hxk2 structure adopts an open conformation of the cleft between the two domains. In high glucose conditions, a movement of about 8 Å of the domains closing the cleft was observed. The closed active site conformation is probably completed after additional conformational changes that accompany ATP binding. In addition to significant conformational changes of the loops involved in glucose and ATP binding, differences in the conformation of the external loops are also observed (44). Our *in vivo* and *in vitro* results show a striking correlation between a close conformation (high glucose conditions) of Hxk2 and its binding to the *SUC2* repressor complex, or an open conformation (low glucose conditions, xylose) of Hxk2 and its dissociation from the *SUC2* promoter. Thus, here, we present evidence that supports the hypothesis that Hxk2 acts as a glucose sensor in the signal transduction pathway mediating glucose-regulated gene expression in yeast; importantly, we document that the signalling activity of Hxk2 is linked to conformational changes induced by the glucose levels which promote its dissociation or reassociation to the *SUC2* promoter to control gene expression.

Acknowledgements: We are grateful to following people for generously providing yeast strains and plasmids: C.N. Cole for the pGEX-XPO1 plasmid and P. Sanz for the pWS93 plasmid. M. V. was supported by a FPI fellowship associated to the grant BFU2010-19628-C02-01 and provided by the MICINN (Spain).

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions: Conception and design was by F. M. Development and methodology was designed by P. H. and F. M. Acquisition of data was by M. V., A. F-C., A. R. and P. H. Analysis and interpretation was by M. V., A. F-C., A. R., P. H. and F. M. Writing and review of the manuscript was by A. F-C., A. R., P. H. and F.M. Study supervision and design was by F.M.

REFERENCES

1. Struhl, K. (1995) *Annu Rev Genet* **29**, 651-674
2. Malave, T. M., and Dent, S. Y. (2006) *Biochem Cell Biol* **84**, 437-443
3. Redd, M. J., Arnaud, M. B., and Johnson, A. D. (1997) *J Biol Chem* **272**, 11193-11197
4. Varanasi, U. S., Klis, M., Mikesell, P. B., and Trumbly, R. J. (1996) *Mol Cell Biol* **16**, 6707-6714
5. Flores-Saaib, R. D., and Courey, A. J. (2000) *Nucleic Acids Res* **28**, 4189-4196
6. Roth, S. Y. (1995) *Curr Opin Genet Dev* **5**, 168-173
7. Nehlin, J. O., and Ronne, H. (1990) *EMBO J* **9**, 2891-2898
8. Lutfiyya, L. L., Iyer, V. R., DeRisi, J., DeVit, M. J., Brown, P. O., and Johnston, M. (1998) *Genetics* **150**, 1377-1391
9. Lutfiyya, L. L., and Johnston, M. (1996) *Mol Cell Biol* **16**, 4790-4797

10. Zhang, Z., and Reese, J. C. (2004) *EMBO J* **23**, 2246-2257
11. Zhang, Z., and Reese, J. C. (2004) *J Biol Chem* **279**, 39240-39250
12. Gancedo, J. M. (1998) *Microbiol Mol Biol Rev* **62**, 334-361
13. Treitel, M. A., and Carlson, M. (1995) *Proc Natl Acad Sci U S A* **92**, 3132-3136
14. Gertz, J., Siggia, E. D., and Cohen, B. A. (2009) *Nature* **457**, 215-218
15. Rodriguez, A., De La Cera, T., Herrero, P., and Moreno, F. (2001) *Biochem J* **355**, 625-631
16. Moreno, F., and Herrero, P. (2002) *FEMS Microbiol Rev* **26**, 83-90
17. Moreno, F., Ahuatzi, D., Riera, A., Palomino, C. A., and Herrero, P. (2005) *Biochem Soc Trans* **33**, 265-268
18. Palomino, A., Herrero, P., and Moreno, F. (2005) *Biochem J* **388**, 697-703
19. Palomino, A., Herrero, P., and Moreno, F. (2006) *Nucleic Acids Res* **34**, 1427-1438
20. Schuurmans, J. M., Boorsma, A., Lascaris, R., Hellingwerf, K. J., and Teixeira de Mattos, M. J. (2008) *FEMS Yeast Res* **8**, 26-34
21. Schuurmans, J. M., Rossell, S. L., van Tuijl, A., Bakker, B. M., Hellingwerf, K. J., and Teixeira de Mattos, M. J. (2008) *FEMS Yeast Res* **8**, 195-203
22. Ozcan, S., and Johnston, M. (1995) *Mol Cell Biol* **15**, 1564-1572
23. Noubhani, A., Bunoust, O., Bonini, B. M., Thevelein, J. M., Devin, A., and Rigoulet, M. (2009) *J Biol Chem* **284**, 27229-27234
24. Pelaez, R., Herrero, P., and Moreno, F. (2010) *Biochem J* **432**, 181-190
25. Herrero, P., Galindez, J., Ruiz, N., Martinez-Campa, C., and Moreno, F. (1995) *Yeast* **11**, 137-144
26. Randez-Gil, F., Herrero, P., Sanz, P., Prieto, J. A., and Moreno, F. (1998) *FEBS Lett* **425**, 475-478
27. Ahuatzi, D., Herrero, P., de la Cera, T., and Moreno, F. (2004) *J Biol Chem* **279**, 14440-14446
28. Pelaez, R., Fernandez-Garcia, P., Herrero, P., and Moreno, F. (2012) *J Biol Chem* **287**, 3518-3529
29. Pelaez, R., Herrero, P., and Moreno, F. (2009) *J Biol Chem* **284**, 20548-20555
30. Fernandez-Garcia, P., Pelaez, R., Herrero, P., and Moreno, F. (2012) *J Biol Chem* **287**, 42151-42164
31. Carlson, M. (1999) *Curr Opin Microbiol* **2**, 202-207
32. Vincent, O., Townley, R., Kuchin, S., and Carlson, M. (2001) *Genes Dev* **15**, 1104-1114
33. Nath, N., McCartney, R. R., and Schmidt, M. C. (2003) *Mol Cell Biol* **23**, 3909-3917
34. Hong, S. P., Leiper, F. C., Woods, A., Carling, D., and Carlson, M. (2003) *Proc Natl Acad Sci U S A* **100**, 8839-8843
35. Sutherland, C. M., Hawley, S. A., McCartney, R. R., Leech, A., Stark, M. J., Schmidt, M. C., and Hardie, D. G. (2003) *Curr Biol* **13**, 1299-1305
36. Mayer, F. V., Heath, R., Underwood, E., Sanders, M. J., Carmena, D., McCartney, R. R., Leiper, F. C., Xiao, B., Jing, C., Walker, P. A., Haire, L. F., Ogrodowicz, R., Martin, S. R., Schmidt, M. C., Gamblin, S. J., and Carling, D. (2011) *Cell Metab* **14**, 707-714
37. Treitel, M. A., Kuchin, S., and Carlson, M. (1998) *Mol Cell Biol* **18**, 6273-6280
38. McCartney, R. R., and Schmidt, M. C. (2001) *J Biol Chem* **276**, 36460-36466
39. Sanz, P., Alms, G. R., Haystead, T. A., and Carlson, M. (2000) *Mol Cell Biol* **20**, 1321-1328
40. Ahuatzi, D., Riera, A., Pelaez, R., Herrero, P., and Moreno, F. (2007) *J Biol Chem* **282**, 4485-4493
41. Tu, J., and Carlson, M. (1995) *EMBO J* **14**, 5939-5946
42. Alms, G. R., Sanz, P., Carlson, M., and Haystead, T. A. (1999) *EMBO J* **18**, 4157-4168
43. Bermejo, C., Haerizadeh, F., Takanaga, H., Chermak, D., and Frommer, W. B. (2011) *Nat Protoc* **6**, 1806-1817
44. Kuser, P. R., Krauchenco, S., Antunes, O. A., and Polikarpov, I. (2000) *J Biol Chem* **275**, 20814-20821
45. Wallis, J. W., Chrebet, G., Brodsky, G., Rolfe, M., and Rothstein, R. (1989) *Cell* **58**, 409-419

46. Ma, H., and Botstein, D. (1986) *Mol Cell Biol* **6**, 4046-4052
47. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) *Yeast* **14**, 953-961
48. Hammell, C. M., Gross, S., Zenklusen, D., Heath, C. V., Stutz, F., Moore, C., and Cole, C. N. (2002) *Mol Cell Biol* **22**, 6441-6457
49. Gascon, S., and Lampen, J. O. (1968) *J Biol Chem* **243**, 1567-1572
50. Bu, Y., and Schmidt, M. C. (1998) *Nucleic Acids Res* **26**, 1002-1009
51. Hecht, A., Strahl-Bolsinger, S., and Grunstein, M. (1999) *Methods Mol Biol* **119**, 469-479
52. De Vit, M. J., Waddle, J. A., and Johnston, M. (1997) *Mol Biol Cell* **8**, 1603-1618
53. Dombek, K. M., Voronkova, V., Raney, A., and Young, E. T. (1999) *Mol Cell Biol* **19**, 6029-6040
54. Fernandez, R., Herrero, P., Fernandez, M. T., and Moreno, F. (1986) *J Gen Microbiol* **132**, 3467-3472
55. Fernandez, R., Herrero, P., and Moreno, F. (1985) *J Gen Microbiol* **131**, 2705-2709
56. Heidrich, K., Otto, A., Behlke, J., Rush, J., Wenzel, K. W., and Kriegel, T. (1997) *Biochemistry* **36**, 1960-1964
57. DelaFuente, G. (1970) *Eur J Biochem* **16**, 240-243
58. Koshland, D. E. (1958) *Proc Natl Acad Sci U S A* **44**, 98-104
59. Lundin, M., Nehlin, J. O., and Ronne, H. (1994) *Mol Cell Biol* **14**, 1979-1985
60. Mayordomo, I., Estruch, F., and Sanz, P. (2002) *J Biol Chem* **277**, 35650-35656
61. DeVit, M. J., and Johnston, M. (1999) *Curr Biol* **9**, 1231-1241
62. Herrero, P., Martinez-Campa, C., and Moreno, F. (1998) *FEBS Lett* **434**, 71-76
63. Aleshin, A. E., Zeng, C., Bourenkov, G. P., Bartunik, H. D., Fromm, H. J., and Honzatko, R. B. (1998) *Structure* **6**, 39-50
64. Mercado, J. J., Vincent, O., and Gancedo, J. M. (1991) *FEBS Lett* **291**, 97-100
65. Fernandez-Cid, A., Vega, M., Herrero, P., and Moreno, F. (2012) *BMC Cell Biol* **13**, 31

FOOTNOTES: This work was supported in whole or part by Ministerio de Ciencia e Innovación (MICINN), Spain, grants BFU2007-66063-C02-02 and BFU2010-19628-C02-01 to F. M.

TABLE 1. *S. cerevisiae* strains used in this study

Name	Relevant genotype	Source/Ref
W303-1A	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100*	(45)
DBY1315	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2*	(46)
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
DBY2052	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 hxk1::LEU2 hxk2-202*	(46)
DBY2053	*MATα ura3-52 leu2-3,2-112 lys2-801 gaI2 hxk1 ::LEU2*	(46)
MAP24	*MATα can1-100 his3-11,15 leu2-3,112 trp1-1 ura 3-1 mig1::loxp mig2::loxp-KAN-lox*	(64)
H174	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6J::LEU2*	(7)
FMY301	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 mig1::LEU2 hxk2-202*	(27)
FMY303	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 SNF1-HA*	(40)
FMY350	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig1::kanMX4 SNF1-HA*	This work
FMY351	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 SNF1-HA*	This work

FMY403	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 SNF4-HA*	This work
FMY481	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 snf1::kanMX4 SNF4-HA*	This work
FMY833	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 GAL83-HA*	This work
FMY320	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 MIG1-GFP*	This work
FMY321	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 MIG1-GFP*	This work
FMY322	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 hxk1::LEU2 hxk2-202 MIG1-GFP*	This work
FMY501	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 MIG2-GFP*	(65)
FMY507	*MATα ade2-1 canJ-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-6J::LEU2 MIG2-GFP*	This work
FMY509	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 MIG2-GFP*	This work
FMY901	*MATα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 REG1-GFP*	This work
FMY902	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig1::kanMX4 REG1-GFP*	This work
FMY903	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4 REG1-GFP*	This work
Y04620	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxk2::kanMX4*	Euroscarf
Y14403	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig1::kanMX4*	Euroscarf
Y14575	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mig2::kanMX4*	Euroscarf
Y14311	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 snf1::kanMX4*	Euroscarf
Y14482	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf4::kanMX4*	Euroscarf
Y16694	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gal83::kanMX4*	Euroscarf
Y13967	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 reg1::kanMX4*	Euroscarf

LEGENDS FOR FIGURES

FIGURE 1. ***Association of Mig1 repressor with the SUC2 promoter is glucose and Hxk2 dependent.*** (A) Schematic diagram showing the location of primer pair at the *SUC2* promoter used for the ChIP analysis. The numbers are presented with respect to the position of the first nucleotide of the initiation codon (+1). (B) Association of Mig1-GFP with the *SUC2* promoter as measured by ChIPs. Strains FMY320 (a), FMY322 (*Δhxk1Δhxk2*) (b) and FMY321 (*Δhxk2*) (c) expressing a GFP-tagged Mig1 protein were grown in high glucose conditions (2% glucose, H-Glc) until an OD_{600} of 0.8 was reached. Afterwards half of the culture was exposed to low glucose conditions (0.05% glucose plus 3% ethanol, L-Glc) for 60 min. Mig1 and the *SUC2* promoter association was determined by ChIP assays. Results were analysed by PCR. At least three independent experiments were performed with *ACT1* (not shown, expression was not influenced by glucose induced nutritional stress), anti-rabbit antibody (unspecific antibody) and extracts prior to immunoprecipitation (input, whole-cell extract) as internal controls. (C) Quantification of Mig1-GFP association in wild-type (wt), *Δhxk1Δhxk2* and *Δhxk2* mutant strains with the *SUC2* promoter. Cells were treated as described for panel B, but ChIPs were analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

FIGURE 2. ***Quantitative invertase assays were performed on cells with the indicated mutations.*** Whole cells from the wild-type strain, W303-1A, and the mutant strains *Δhxk1*, *Δhxk2*,

Δhxk1Δhxk2, *Δmig1*, *Δmig2*, *Δmig1Δmig2*, *Δsnf1*, *Δsnf4*, *Δgal83* and *Δreg1* were used for invertase activity determination. Invertase was assayed using cells grown in high-glucose medium (H-Glc, black bars) until an A_{600nm} of 0.8 was reached and then transferred to low-glucose medium for 60 min (L-Glc, white bars). Error bars represent the standard error of the mean for three independent determinations using three colonies of each strain.

FIGURE 3. ***Association of Hxk2 with the SUC2 promoter is glucose and Mig1 dependent.*** (A) Association of Hxk2 with the *SUC2* promoter as measured by ChIPs. The wild-type strain W303-1A (a) and the mutant strain H174 (*Δmig1*) (b) were grown in high glucose conditions (2% glucose, H-Glc) until an OD_{600} of 0.8 was reached. Afterwards half of the culture was exposed to low glucose conditions (0.05% glucose plus 3% ethanol, L-Glc) for 60 min. Hxk2 and the *SUC2* promoter association was determined by ChIP. Results were analysed by PCR. At least three independent experiments were performed with *ACT1* (not shown, expression was not influenced by glucose induced nutritional stress), anti-rabbit antibody (unspecific antibody) and extracts prior to immunoprecipitation (input, whole-cell extract) as internal controls. The agarose electrophoresis shown are representative of results obtained from three independent experiments. (B) Quantification of Hxk2 association in wild-type (wt) and *Δmig1* mutant strain with the *SUC2* promoter. Cells were treated as described for panel A (H-Glc, black bars; L-Glc, white bars), but ChIPs were analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

FIGURE 4. ***Association of Mig2-GFP with the SUC2 promoter is Mig1 and Hxk2-dependent.*** (A) Association of Mig2-GFP with the *SUC2* promoter as measured by ChIPs. The FMY501 strain expressing a GFP-tagged Mig2 protein (a) and the mutant strains FMY507 (*Δmig1*) (b) and FMY509 (*Δhxk2*) (b), both expressing a GFP-tagged Mig2 protein, were grown in high glucose conditions (2% glucose, H-Glc) until an OD_{600} of 0.8 was reached. Afterwards half of the culture was exposed to low glucose conditions (0.05% glucose plus 3% ethanol, L-Glc) for 60 min. Mig2 and the *SUC2* promoter association was determined by ChIP. Results were analysed by PCR. At least three independent experiments were performed with *ACT1* (not shown, expression was not influenced by glucose induced nutritional stress), anti-rabbit antibody (unspecific antibody) and extracts prior to immunoprecipitation (input, whole-cell extract) as internal controls. The agarose electrophoresis shown are representative of results obtained from three independent experiments. (B) Quantification of Mig2 association in FMY501, FMY507 (*Δmig1*) and FMY509 (*Δhxk2*) strains with the *SUC2* promoter. Cells were treated as described for panel A (H-Glc, black bars; L-Glc, white bars), but ChIPs were analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

FIGURE 5. ***Interaction of Mig1, Hxk2 and Snf1 with Mig2.*** *In vivo* co-immunoprecipitation of Mig2-GFP with Mig1-HA (A), Hxk2 (B) and Snf1-HA (C) is shown. The FMY507 strain, expressing a Mig2 GFP-tagged fusion protein, was transformed with plasmids pWS93/Mig1 and pWS93/Snf1, which encode an HA-tagged Mig1 and Snf1 proteins respectively. The cells were grown in SG-media, lacking appropriate supplement to maintain selection for plasmid, until an A_{600nm} of 0.8 was reached and then shifted to high (H-Glc) and low (L-Glc) glucose conditions for 1 h. The cell extracts were immunoprecipitated with a monoclonal anti-HA, a polyclonal anti-Hxk2 antibodies, or a polyclonal antibody to Pho4 (lanes 3, 4). Immunoprecipitates were separated by 12% SDS-PAGE, and the level of immunoprecipitated Mig2-GFP in the blotted samples was determined by using anti-GFP antibody. The level of Mig2-GFP present in the different extracts was determined by western blot using anti-GFP antibody. All western blots shown are representative of results obtained from three independent experiments.

FIGURE 6. ***Association of Snf1-HA, Snf4-HA and Gal83-HA with the SUC2 promoter is Mig1 and Hxk2-dependent.*** (A) Association of Snf1, Snf4 and Gal83 with the *SUC2* promoter as measured by ChIPs. The FMY303 (a), FMY403 (b) and FMY833 (c) strains expressing HA-

tagged Snf1, Snf4 and Gal83 protein, respectively, and the mutant strains FMY350 (*Δmig1*; Snf1-HA) (d) and FMY351 (*Δhxk2*; Snf1-HA) (b), both expressing a HA-tagged Snf1 protein, were grown in high glucose conditions (2% glucose, H-Glc) until an OD_{600} of 0.8 was reached. Afterwards half of the culture was exposed to low glucose conditions (0.05% glucose plus 3% ethanol, L-Glc) for 60 min. Snf1 and the *SUC2* promoter association was determined by ChIP in the presence or absence of Mig1 (d) and Hxk2 (e) proteins. Results were analysed by PCR. At least three independent experiments were performed with *ACT1* (not shown, expression was not influenced by glucose induced nutritional stress), anti-rabbit antibody (unspecific antibody) and extracts prior to immunoprecipitation (input, whole-cell extract) as internal controls. The agarose electrophoresis shown are representative of results obtained from three independent experiments. (B) Quantification of Snf1, Snf4 and Gal83 association in the presence of Mig1 and Hxk2 proteins with the *SUC2* promoter was analysed by quantitative real-time PCR (RT-PCR). Snf1 association in the absence of Mig1 and Hxk2 proteins with the *SUC2* promoter was also analysed by RT-PCR. Cells were treated as described for panel A (H-Glc, black bars; L-Glc, white bars). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

FIGURE 7. ***GST pull-down assays of the interaction of Snf1, Snf4 and Gal83 with Hxk2.*** A GST-Hxk2 fusion protein was purified on glutathione-sepharose columns. Equal amounts of GST-Hxk2 were incubated with cell extracts from FMY303, FMY403, FMY481 and FMY833 strains expressing Snf1-HA (A), Snf4-HA (B), Snf4-HA in the absence of Snf1 (C) and Gal83-HA (D) fusion proteins, respectively. The yeast strains were grown in YEPD media until an A_{600nm} of 0.8 was reached and then shifted to high (H-Glc) and low (L-Glc) glucose conditions for 1 h. For the control samples, GST protein was also incubated with the H-Glc and L-Glc cell extracts but no signals were detected (lanes 1 and 2). The level of Snf1, Snf4 and Gal83 present in the different extracts used were determined by western blot using a monoclonal anti-HA antibody. The western blots shown are representative of results obtained from three independent experiments.

FIGURE 8. ***Association of Reg1-GFP with the SUC2 promoter is Mig1 and Hxk2-dependent.*** (A) Association of Reg1-GFP with the *SUC2* promoter as measured by ChIPs. The FMY901 strain expressing a GFP-tagged Reg1 protein (a) and the mutant strains FMY902 (*Δmig1*) (b) and FMY903 (*Δhxk2*) (b), both also expressing a GFP-tagged Reg1 protein, were grown in high glucose conditions (2% glucose, H-Glc) until an OD_{600} of 0.8 was reached. Afterwards half of the culture was exposed to low glucose conditions (0.05% glucose plus 3% ethanol, L-Glc) for 60 min. Reg1 and the *SUC2* promoter association was determined by ChIP. Results were analysed by PCR. At least three independent experiments were performed with *ACT1* (not shown, expression was not influenced by glucose induced nutritional stress), anti-rabbit antibody (unspecific antibody) and extracts prior to immunoprecipitation (input, whole-cell extract) as internal controls. The agarose electrophoresis shown are representative of results obtained from three independent experiments. (B) Quantification of Reg1 association in FMY901, FMY902 (*Δmig1*) and FMY903 (*Δhxk2*) strains with the *SUC2* promoter. Cells were treated as described for panel A (H-Glc, black bars; L-Glc, white bars), but ChIPs were analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

FIGURE 9. ***In vivo interaction of Reg1 with Hxk2, Kap60, Kap95 and Xpo1.*** The FMY901 strain, expressing a Reg1-GFP fusion protein, was grown in high-glucose YEPD-medium (H-Glc) until an A_{600nm} of 0.8 was reached and then transferred to low glucose medium (L-Glc) for 60 min. (A) The cell extracts were immunoprecipitated with a polyclonal anti-GFP antibody (lanes 1 and 2), or a polyclonal antibody to Pho4 (lanes 3 and 4). (B) The cell extracts were immunoprecipitated with polyclonal anti-Kap95 and anti-Kap60 antibodies (lanes 3 to 6), or a polyclonal antibody to Pho4 (lanes 1 and 2). Imunoprecipitates were separated by 12% SDS-

PAGE, and co-precipitated Hxk2 or Reg1-GFP was visualized on a Western blot with polyclonal anti-Hxk2 or anti-GFP antibodies. The level of Hxk2 or Reg1 present in the different extracts was determined by western blot using anti-Hxk2 or anti-GFP antibodies. (C) A GST-Xpo1 fusion protein was purified on glutathione-sepharose columns. Equal amounts of GST-Xpo1 were incubated with cell extracts from FMY901 strain. The yeast strain was grown in YEPD media until an A_{600nm} of 0.8 was reached and then shifted to high (H-Glc) and low (L-Glc) glucose conditions for 1 h. For the control samples, GST protein was also incubated with the H-Glc and L-Glc cell extracts but no signals were detected (lanes 1 and 2). The level of Reg1present in the different extracts used were determined by western blot using an anti-GFP antibody. The western blots shown are representative of results obtained from three independent experiments.

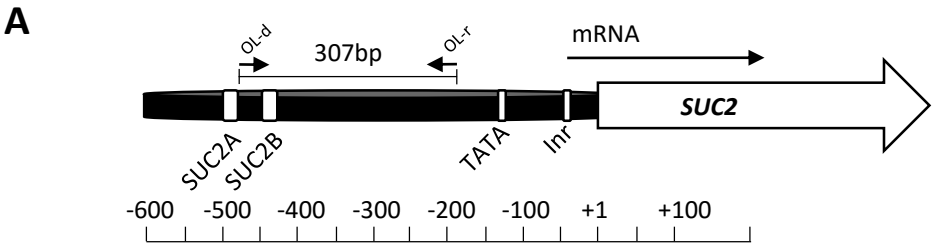
FIGURE 10. ***Association of Hxk2 with the SUC2 promoter is not regulated by phosphorylation.*** (A) Association of Hxk2 with the *SUC2* promoter as measured by ChIPs. The wild-type strain W303-1A (a) and the mutant strain Y14311 (*Δsnf1*) (b) were grown in high glucose conditions (2% glucose, H-Glc) until an OD_{600} of 0.8 was reached, afterwards half of the culture was exposed to low glucose conditions (0.05% glucose plus 3% ethanol, L-Glc) for 60 min. Hxk2 and the *SUC2* promoter association was determined by ChIP. Results were analysed by PCR. At least three independent experiments were performed with *ACT1* (not shown, expression was not influenced by glucose induced nutritional stress), anti-rabbit antibody (unspecific antibody) and extracts prior to immunoprecipitation (input, whole-cell extract) as internal controls. The agarose electrophoresis shown are representative of results obtained from three independent experiments. (B) Quantification of Hxk2 association in wild-type (wt) and *Δsnf1* mutant strain with the *SUC2* promoter. Cells were treated as described for panel A (H-Glc, black bars; L-Glc, white bars), but ChIPs were analysed by quantitative real-time PCR (RT-PCR). Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

FIGURE 11. ***In vitro conformational changes in Hxk2 affect its interaction with the Mig1 repressor.*** (A) Identical amounts of Hxk2 affinity purified protein from bacteria were incubated in the presence of 2mM glucose, 4mM D-xylose, 1mM $MgATP^{2-}$ and 1mM MgAMP-PNP for 30 min at 20ºC in PBS buffer. (B) Time course of D-xylose inhibition of Hxk2-Mig1 interaction. Identical amounts of Hxk2 affinity purified protein from bacteria were incubated in the presence of 0.5mM glucose and increasing amounts of D-xylose, from 0.5 to 4mM (lanes 4-7) for 30 min at 20ºC in PBS buffer. (C) Time course of glucose activation of Hxk2-Mig1 interaction. Identical amounts of Hxk2 affinity purified protein from bacteria were incubated in the presence of 2mM D-xylose and increasing amounts of glucose, from 0.5 to 8mM (lanes 4-6) for 30 min at 20ºC in PBS buffer. Then, the GST-Mig1 fusion protein coupled to glutathione-Sepharose was incubated with the assay mixtures for 90 min at 4ºC in PBS buffer. Beads were gently washed five times with 2.5 ml of PBS buffer, boiled in 25 µl sample-loading buffer, and analysed by SDS-PAGE followed by Western blot using anti-Hxk2 antibodies. The western blot shown is representative of results obtained from three independent experiments. *In vitro* kinase assay.

FIGURE 12. ***In vivo conformational changes of Hxk2 regulate its interaction with the Mig1 repressor.*** (A) Association of Hxk2 with the *SUC2* promoter as measured by ChIPs. The wild-type strain W303-1A (a) and the mutant strain Y14311 (*Δsnf1*) (b) were grown in high glucose conditions (2% glucose, H-Glc) until an OD_{600} of 0.8 was reached. Afterwards half of the culture was exposed to low glucose conditions (0.05% glucose plus 3% ethanol, L-Glc) for 60 min. Hxk2 and the *SUC2* promoter association was determined by ChIP. Results were analysed by PCR. At least three independent experiments were performed with *ACT1* (not shown, expression was not influenced by glucose induced nutritional stress), anti-rabbit antibody (unspecific antibody) and extracts prior to immunoprecipitation (input, whole-cell extract) as internal controls. The agarose electrophoresis shown are representative of results obtained from three independent experiments. (B) Quantification of Hxk2 association in wild-type (wt) and *Δsnf1* mutant strain with the *SUC2* promoter. Cells were treated as described for panel A (H-Glc, black bars; L-Glc, white bars), but ChIPs were analysed by quantitative real-time PCR (RT-PCR).

Data are expressed as signal normalized to the untreated sample. Error bars represent the standard error of the mean for three independent experiments.

Figure 1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OL-r
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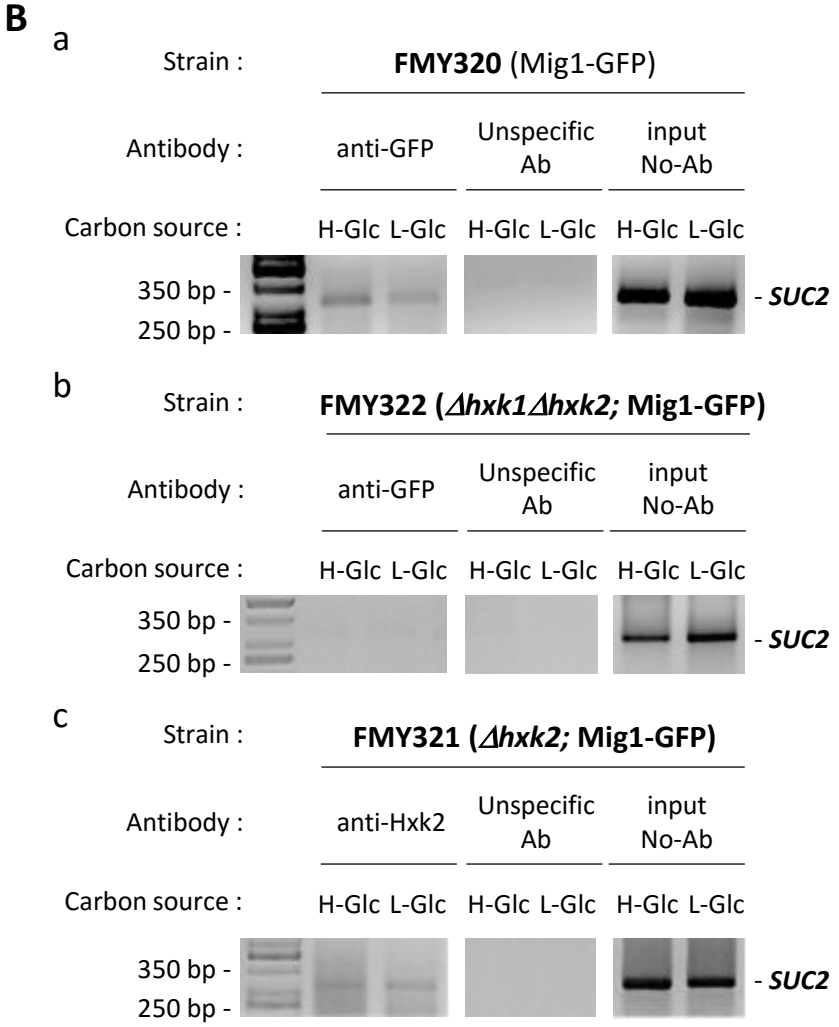
-100
+1
+100


B
a
Strain :
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Antibody :
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250 bp -
- SUC2
b
Strain :
FMY322 (Δhxk1Δhxk2; Mig1-GFP)
Antibody :
anti-GFP
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input No-Ab
Carbon source :
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350 bp -
250 bp -
- SUC2
c
Strain :
FMY321 (Δhxk2; Mig1-GFP)
Antibody :
anti-Hxk2
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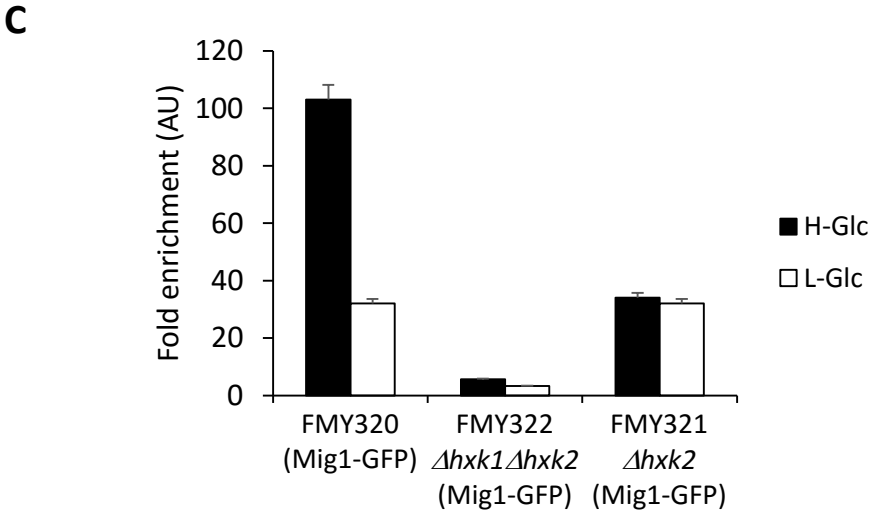
250 bp -
- SUC2


C
Fold enrichment (AU)
120
100
80
60
40
20
0
H-Glc
L-Glc
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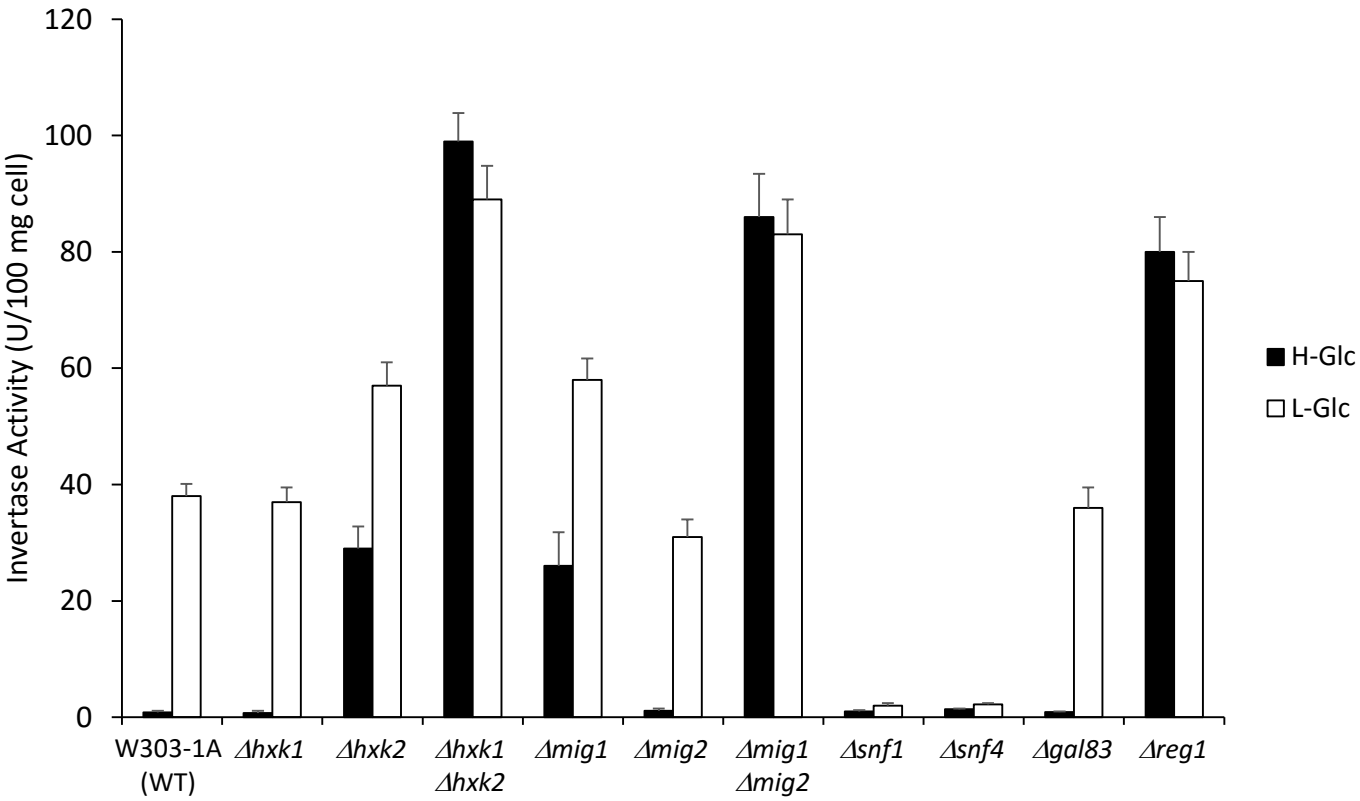
FMY322 Δhxk1Δhxk2 (Mig1-GFP)
FMY321 Δhxk2 (Mig1-GFP)

Figure 2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80
60
40
20
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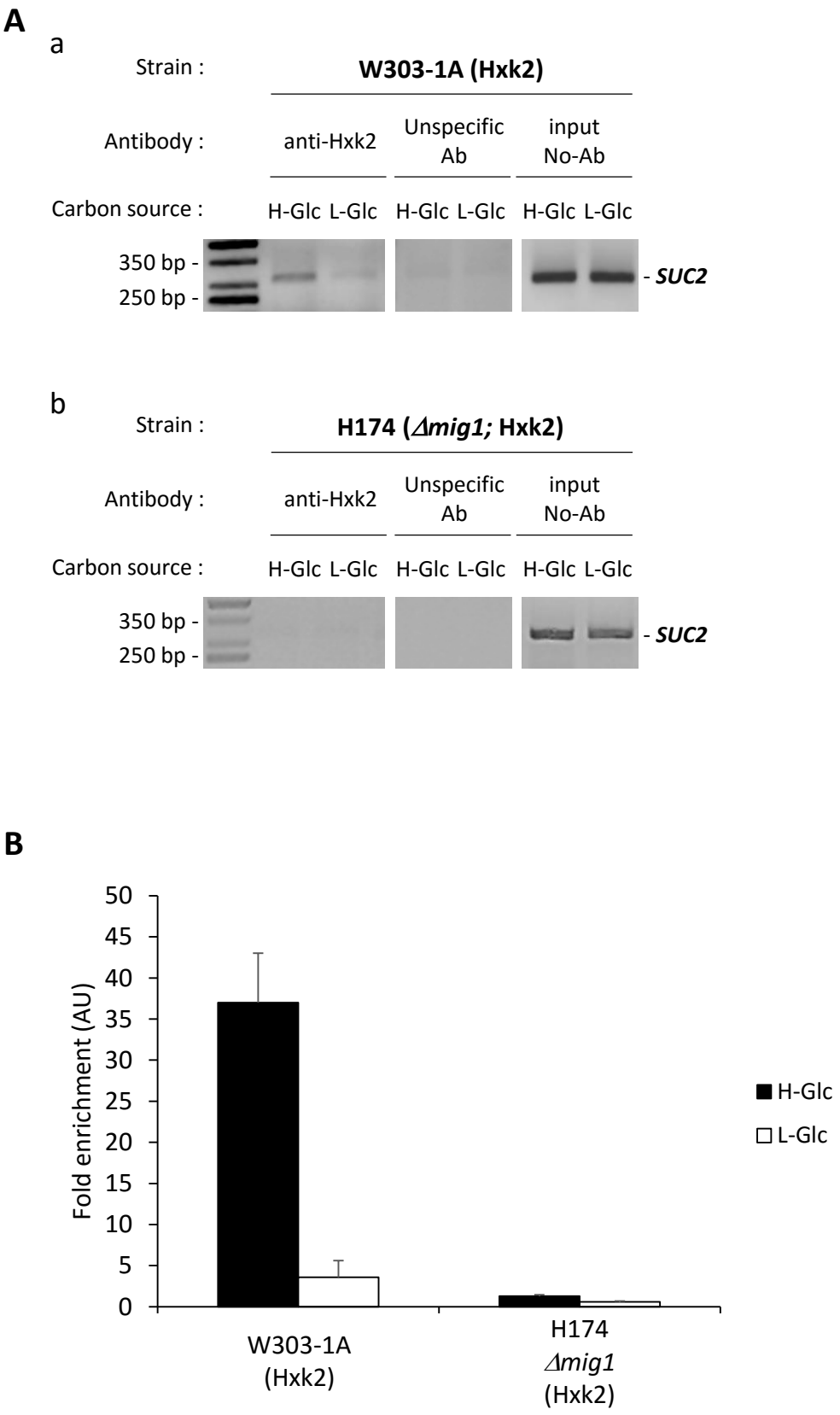
L-Glc
W303-1A (WT)
Δhxk1
Δhxk2
Δhxk1 Δhxk2
Δmig1
Δmig2
Δmig1 Δmig2
Δsnf1
Δsnf4
Δgal83
Δreg1

Figure 3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W303-1A (Hxk2)
Antibody :
anti-Hxk2
Unspecific Ab
input No-Ab
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H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2
b
Strain :
H174 (Δmig1; Hxk2)
Antibody :
anti-Hxk2
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2
B
Fold enrichment (AU)
50
45
40
35
30
25
20
15
10
5
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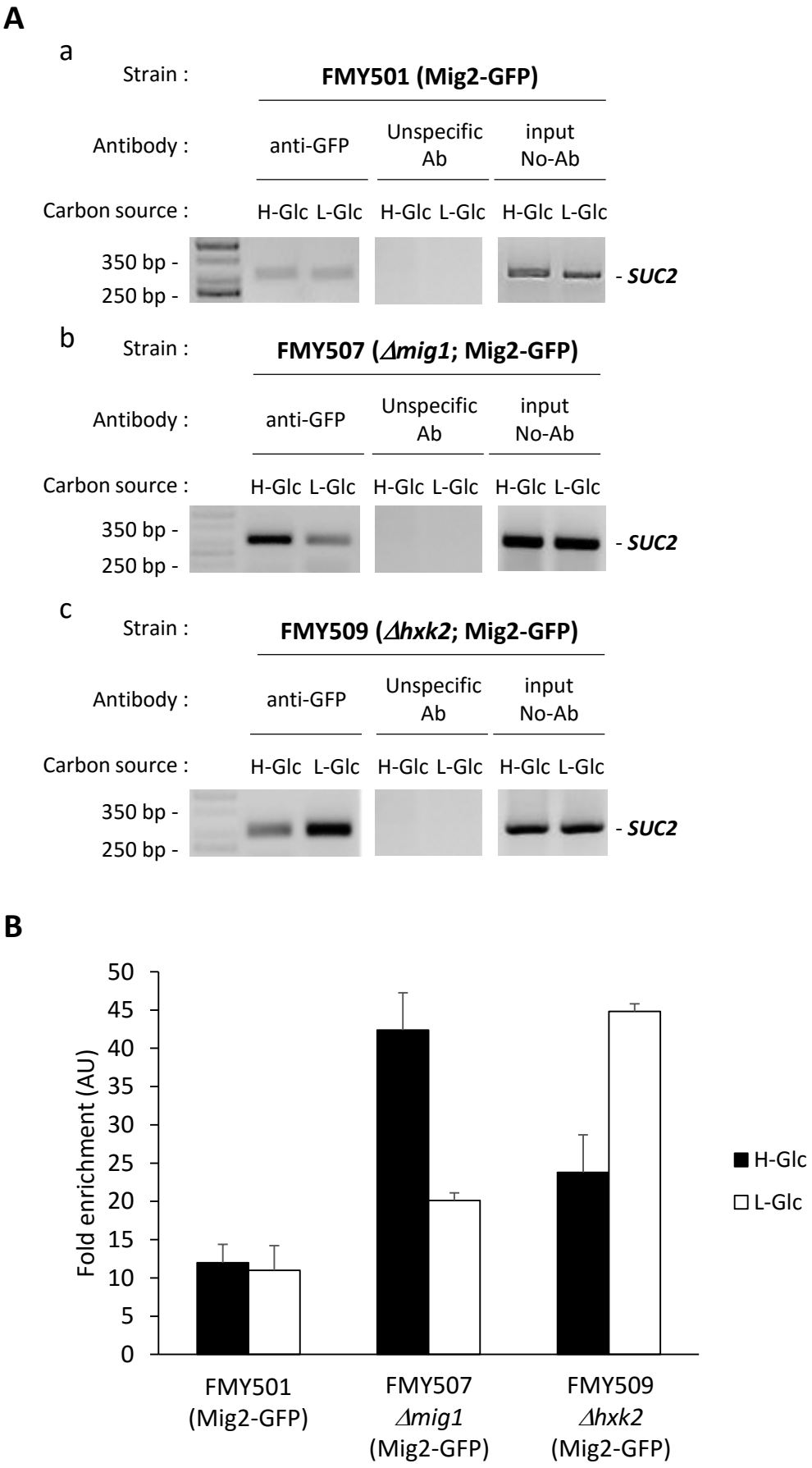
H174 Δmig1 (Hxk2)
H-Glc
L-Glc

Figure 4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a
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FMY501 (Mig2-GFP)
Antibody :
anti-GFP
Unspecific Ab
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Carbon source :
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350 bp -
250 bp -
- SUC2
b
Strain :
FMY507 (Δmig1; Mig2-GFP)
Antibody :
anti-GFP
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2
c
Strain :
FMY509 (Δhxk2; Mig2-GFP)
Antibody :
anti-GFP
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2
B
Fold enrichment (AU)
50
45
40
35
30
25
20
15
10
5
0
H-Glc
L-Glc
FMY501 (Mig2-GFP)
FMY507 Δmig1 (Mig2-GFP)
FMY509 Δhxk2 (Mig2-GFP)

Figure 5

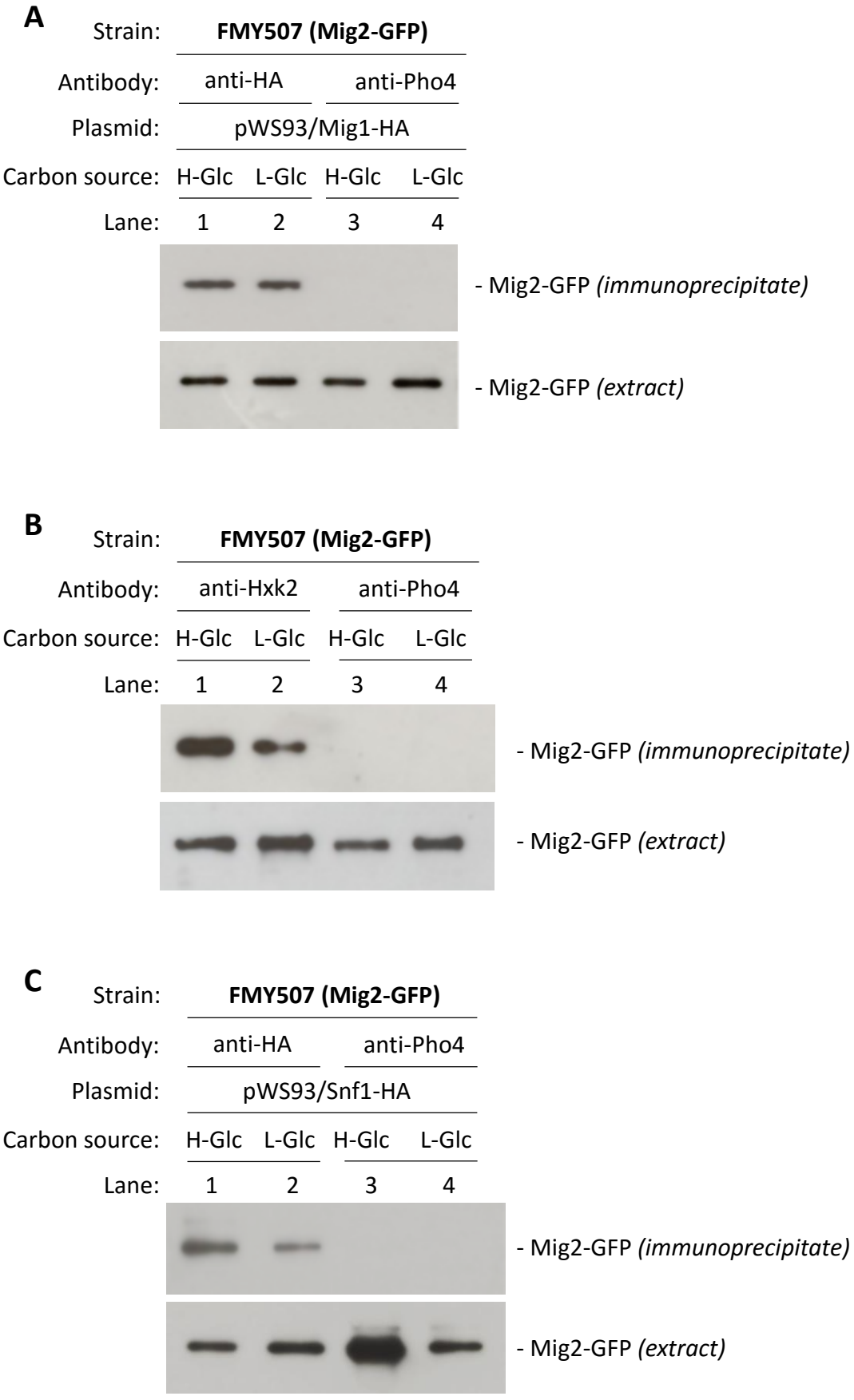

A
Strain:
FMY507 (Mig2-GFP)
Antibody:
anti-HA
anti-Pho4
Plasmid:
pWS93/Mig1-HA
Carbon source:
H-Glc
L-Glc
H-Glc
L-Glc
Lane:
1
2
3
4
- Mig2-GFP (immunoprecipitate)
- Mig2-GFP (extract)
B
Strain:
FMY507 (Mig2-GFP)
Antibody:
anti-Hxk2
anti-Pho4
Carbon source:
H-Glc
L-Glc
H-Glc
L-Glc
Lane:
1
2
3
4
- Mig2-GFP (immunoprecipitate)
- Mig2-GFP (extract)
C
Strain:
FMY507 (Mig2-GFP)
Antibody:
anti-HA
anti-Pho4
Plasmid:
pWS93/Snf1-HA
Carbon source:
H-Glc
L-Glc
H-Glc
L-Glc
Lane:
1
2
3
4
- Mig2-GFP (immunoprecipitate)
- Mig2-GFP (extract)

Figure 6

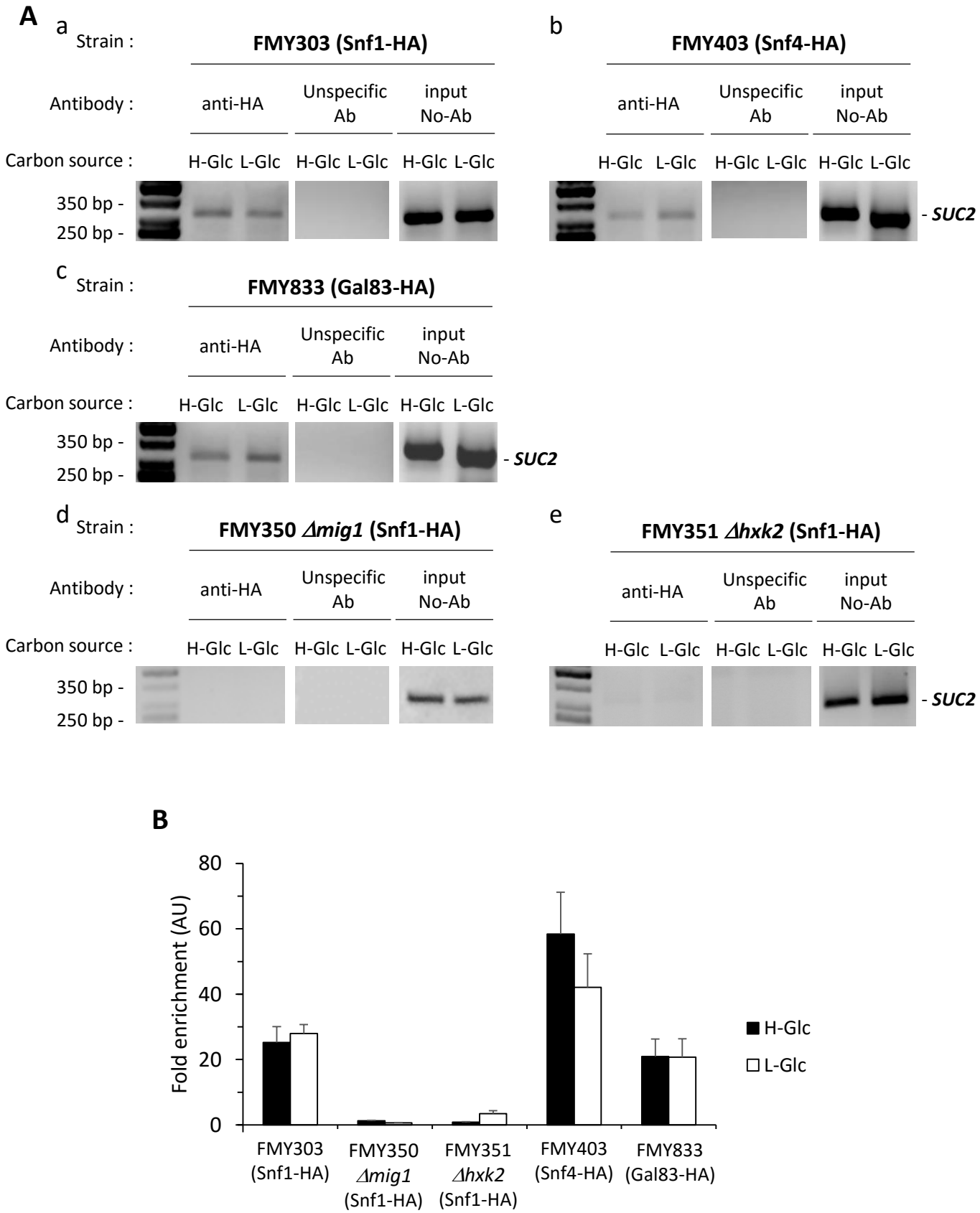

A
a
Strain :
FMY303 (Snf1-HA)
Antibody :
anti-HA
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
b
FMY403 (Snf4-HA)
anti-HA
Unspecific Ab
input No-Ab
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
- SUC2
c
Strain :
FMY833 (Gal83-HA)
Antibody :
anti-HA
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2
d
Strain :
FMY350 Δmig1 (Snf1-HA)
Antibody :
anti-HA
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
e
FMY351 Δhxk2 (Snf1-HA)
anti-HA
Unspecific Ab
input No-Ab
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
- SUC2
B
Fold enrichment (AU)
80
60
40
20
0
FMY303 (Snf1-HA)
FMY350 Δmig1 (Snf1-HA)
FMY351 Δhxk2 (Snf1-HA)
FMY403 (Snf4-HA)
FMY833 (Gal83-HA)
H-Glc
L-Glc

Figure 7

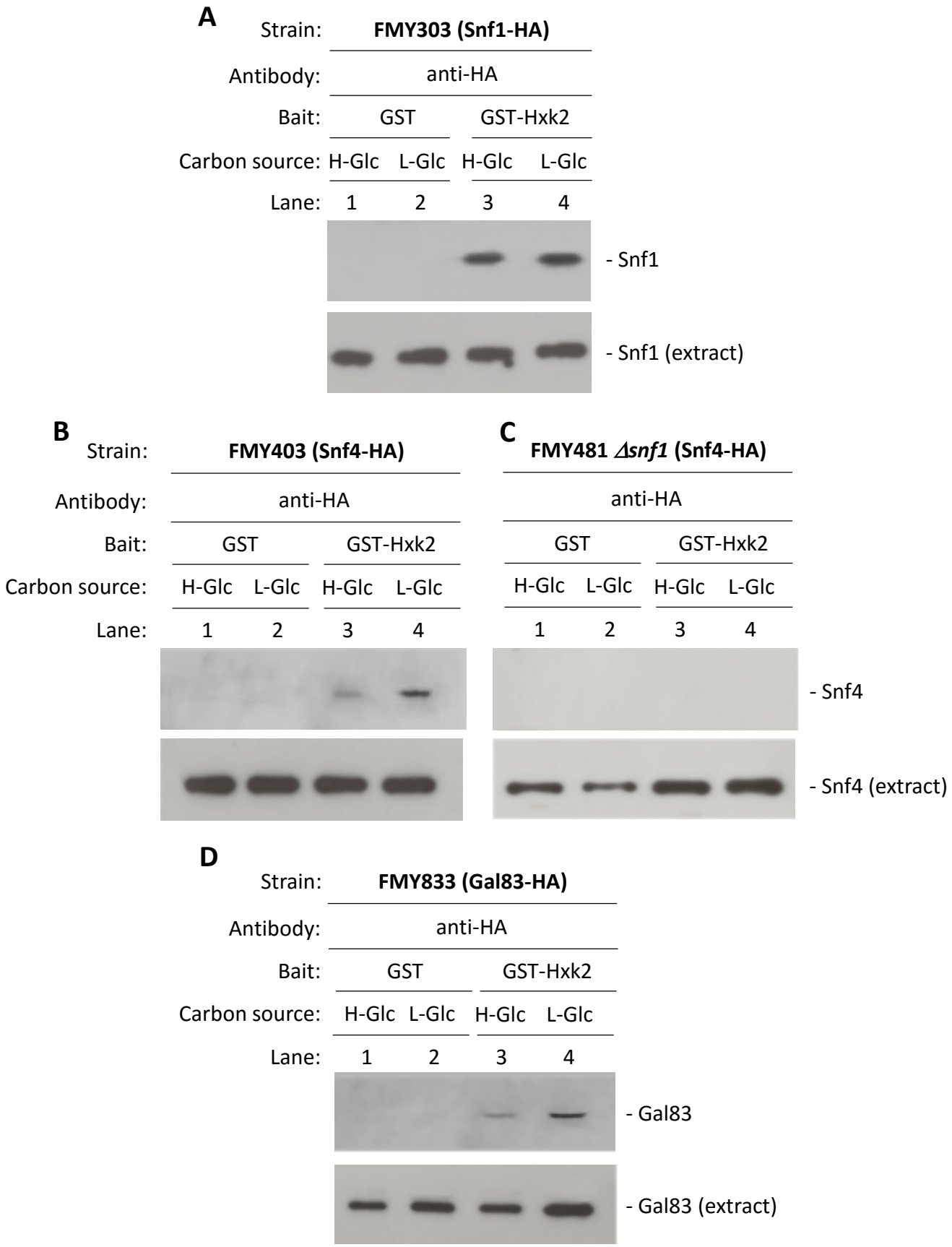

A
Strain:
FMY303 (Snf1-HA)
Antibody:
anti-HA
Bait:
GST
GST-Hxk2
Carbon source:
H-Glc
L-Glc
H-Glc
L-Glc
Lane:
1
2
3
4
- Snf1
- Snf1 (extract)
B
Strain:
FMY403 (Snf4-HA)
Antibody:
anti-HA
Bait:
GST
GST-Hxk2
Carbon source:
H-Glc
L-Glc
H-Glc
L-Glc
Lane:
1
2
3
4
C
FMY481 Δsnf1 (Snf4-HA)
anti-HA
GST
GST-Hxk2
H-Glc
L-Glc
H-Glc
L-Glc
1
2
3
4
- Snf4
- Snf4 (extract)
D
Strain:
FMY833 (Gal83-HA)
Antibody:
anti-HA
Bait:
GST
GST-Hxk2
Carbon source:
H-Glc
L-Glc
H-Glc
L-Glc
Lane:
1
2
3
4
- Gal83
- Gal83 (extract)

Figure 8

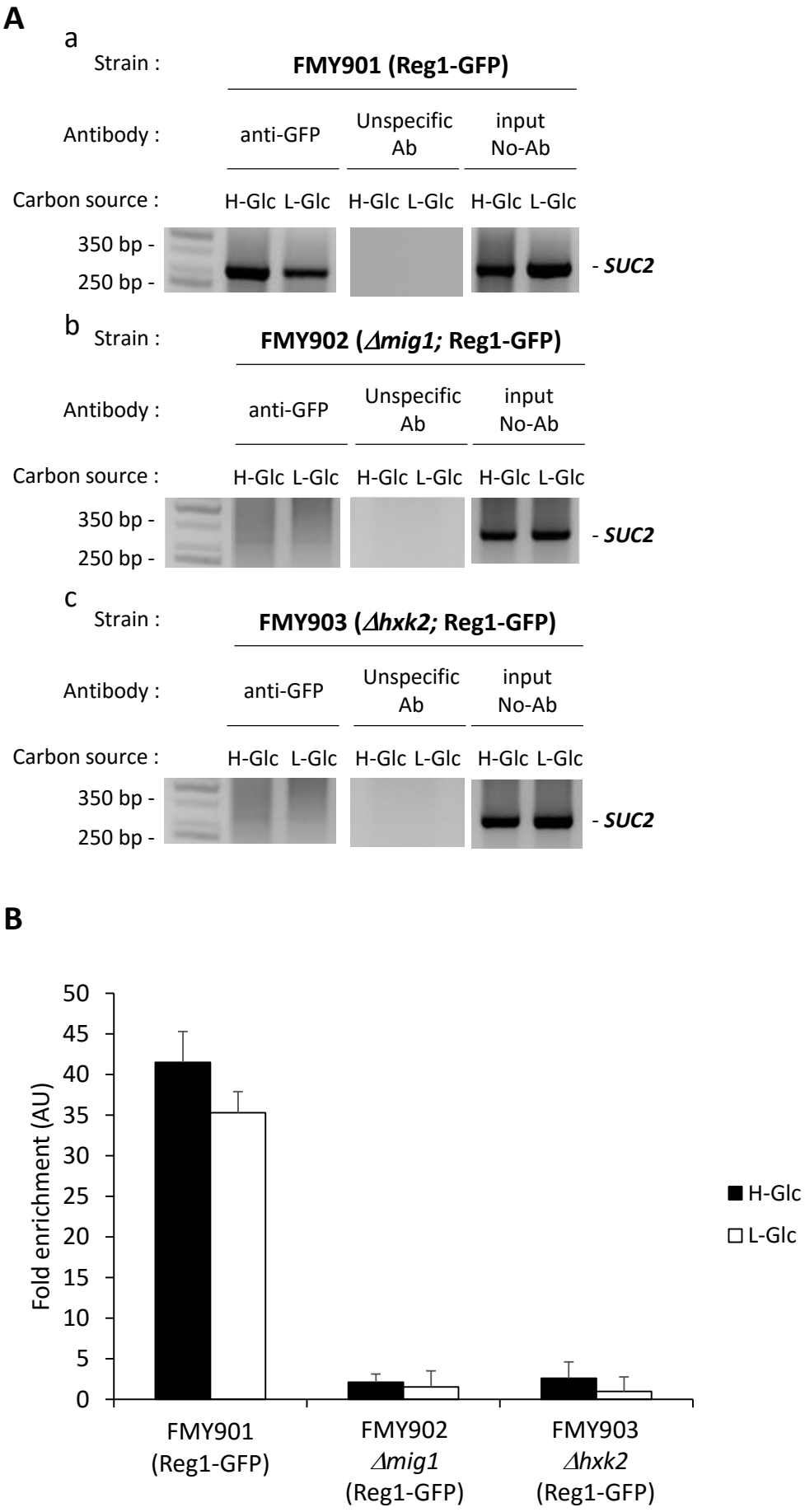

A
a
Strain :
FMY901 (Reg1-GFP)
Antibody :
anti-GFP
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2
b
Strain :
FMY902 (Δmig1; Reg1-GFP)
Antibody :
anti-GFP
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2
c
Strain :
FMY903 (Δhxk2; Reg1-GFP)
Antibody :
anti-GFP
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2
B
Fold enrichment (AU)
50
45
40
35
30
25
20
15
10
5
0
FMY901 (Reg1-GFP)
FMY902 Δmig1 (Reg1-GFP)
FMY903 Δhxk2 (Reg1-GFP)
H-Glc
L-Glc

Figure 9

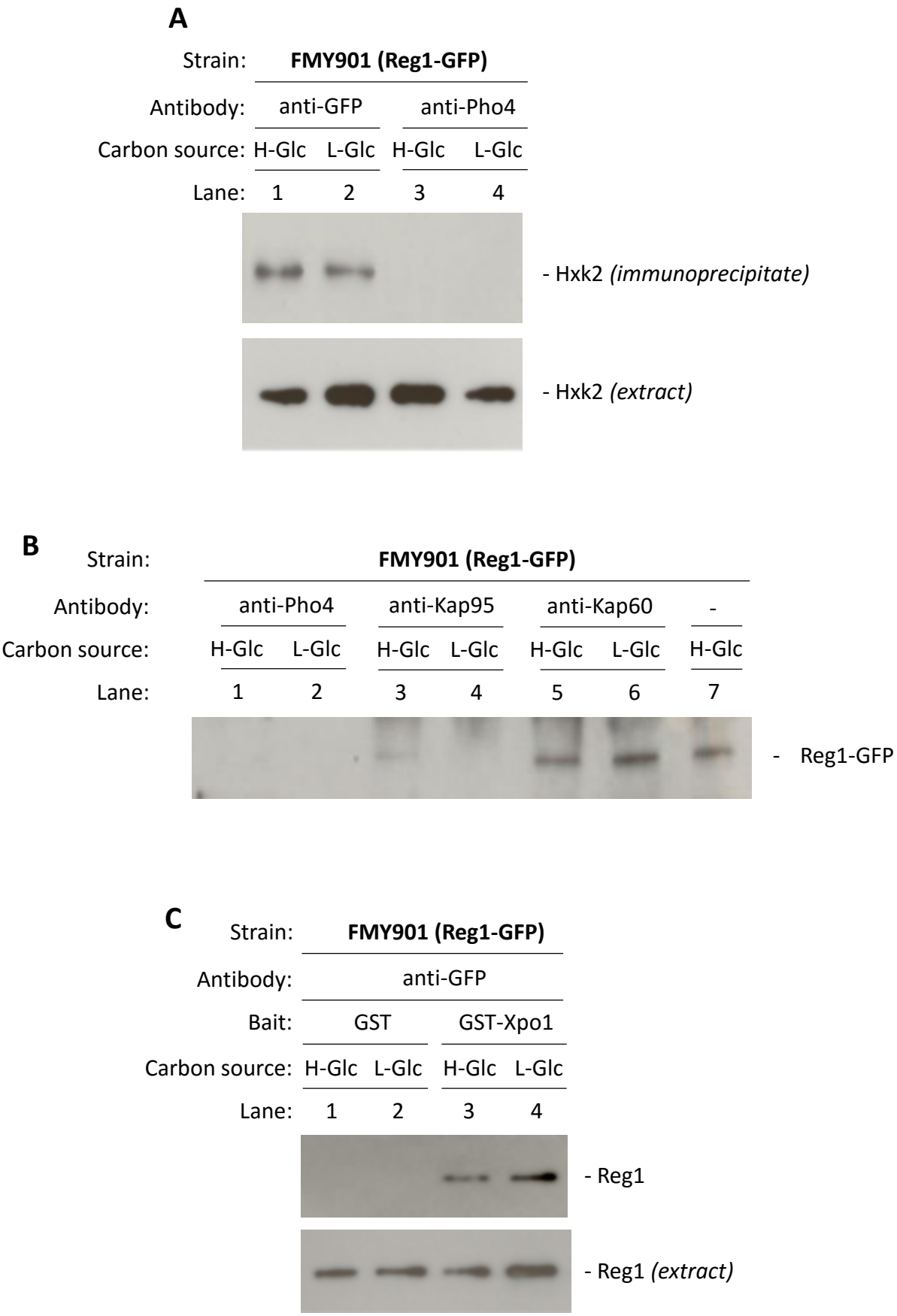
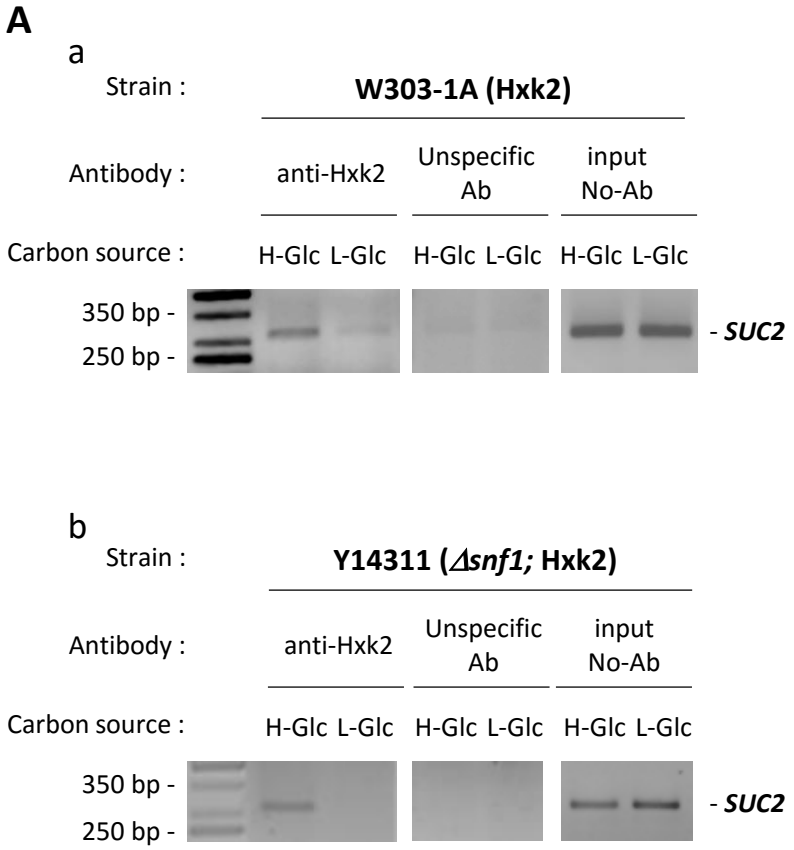

A
Strain: FMY901 (Reg1-GFP)
Antibody: anti-GFP anti-Pho4
Carbon source: H-Glc L-Glc H-Glc L-Glc
Lane: 1 2 3 4
- Hxk2 (immunoprecipitate)
- Hxk2 (extract)
B
Strain: FMY901 (Reg1-GFP)
Antibody: anti-Pho4 anti-Kap95 anti-Kap60 -
Carbon source: H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc H-Glc
Lane: 1 2 3 4 5 6 7
- Reg1-GFP
C
Strain: FMY901 (Reg1-GFP)
Antibody: anti-GFP
Bait: GST GST-Xpo1
Carbon source: H-Glc L-Glc H-Glc L-Glc
Lane: 1 2 3 4
- Reg1
- Reg1 (extract)

Figure 10


A
a
Strain :
W303-1A (Hxk2)
Antibody :
anti-Hxk2
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2
b
Strain :
Y14311 (Δsnf1; Hxk2)
Antibody :
anti-Hxk2
Unspecific Ab
input No-Ab
Carbon source :
H-Glc L-Glc H-Glc L-Glc H-Glc L-Glc
350 bp -
250 bp -
- SUC2

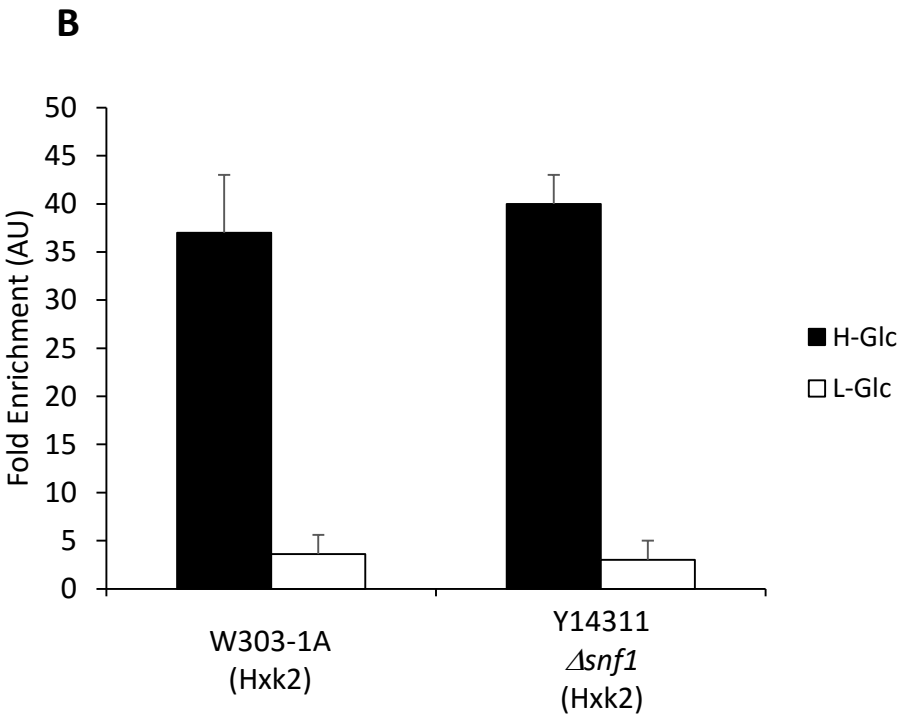
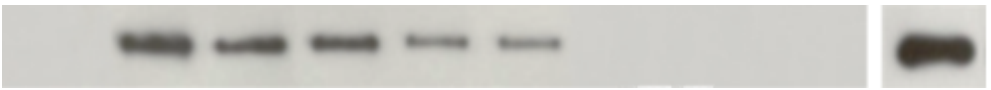

B
Fold Enrichment (AU)
50
45
40
35
30
25
20
15
10
5
0
H-Glc
L-Glc
W303-1A (Hxk2)
Y14311 Δsnf1 (Hxk2)

Figure 11

A

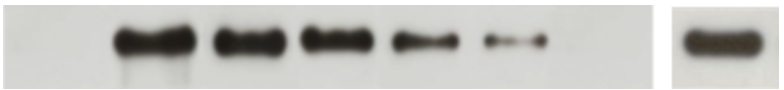
Bait:	GST	GST-Mig1								-
Antibody:	anti-Hxk2									
Hxk2:	+	+	+	+	+	+	+	+	+	+
Glucose (mM):	-	-	-	-	2	2	-	-	-	-
Xylose (mM):	-	-	-	-	-	-	4	4	4	-
ATP (mM):	-	-	1	-	1	-	1	-	-	-
AMP-PNP (mM):	-	-	-	1	-	1	-	1	-	-
Lane:	1	2	3	4	5	6	7	8	9	10



- Hxk2

B

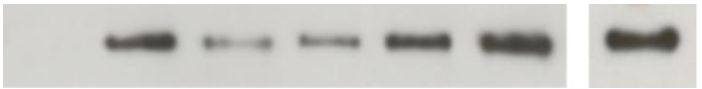
Bait:	GST	GST-Mig1						-
Antibody:	anti-Hxk2							
Hxk2:	+	+	+	+	+	+	+	+
Glucose (mM):	-	-	0,5	0.5	0.5	0.5	0.5	-
Xylose (mM):	-	-	-	0.5	1	2	4	-
Lane:	1	2	3	4	5	6	7	8



- Hxk2

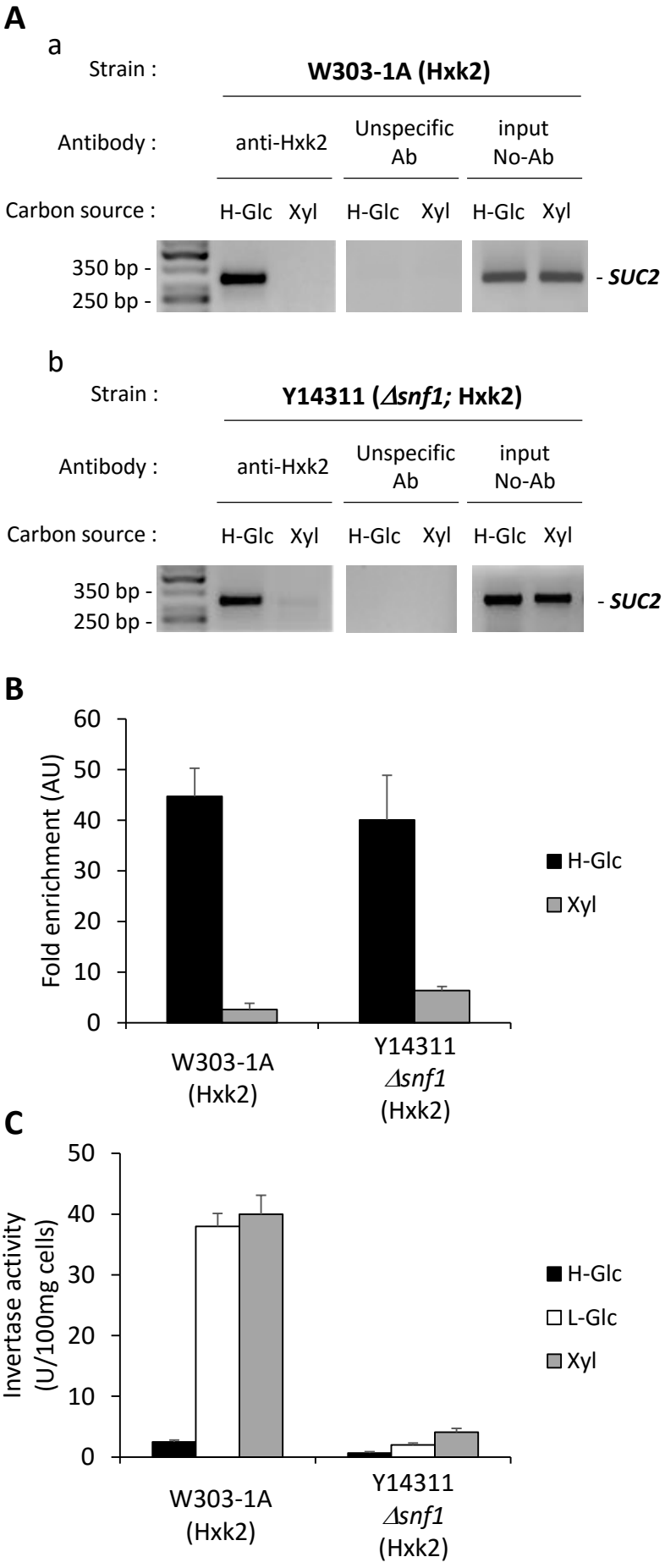
C

Bait:	GST	GST-Mig1					-
Antibody:	anti-Hxk2						
Hxk2:	+	+	+	+	+	+	+
Glucose (mM):	-	-	-	0.5	4	8	-
Xylose (mM):	-	-	2	2	2	2	-
Lane:	1	2	3	4	5	6	7



- Hxk2

Figure 12


A
a
Strain :
W303-1A (Hxk2)
Antibody :
anti-Hxk2
Unspecific Ab
input No-Ab
Carbon source :
H-Glc Xyl H-Glc Xyl H-Glc Xyl
350 bp -
250 bp -
- SUC2
b
Strain :
Y14311 (Δsnf1; Hxk2)
Antibody :
anti-Hxk2
Unspecific Ab
input No-Ab
Carbon source :
H-Glc Xyl H-Glc Xyl H-Glc Xyl
350 bp -
250 bp -
- SUC2
B
Fold enrichment (AU)
60
50
40
30
20
10
0
H-Glc
Xyl
W303-1A (Hxk2)
Y14311 Δsnf1 (Hxk2)
C
Invertase activity (U/100mg cells)
50
40
30
20
10
0
H-Glc
L-Glc
Xyl
W303-1A (Hxk2)
Y14311 Δsnf1 (Hxk2)