Universidade do Minho
Escola de Ciências

Neide Marina Vieira Pereira

The Transport of Carboxylic Acids in Yeasts: from physiology towards structural-functional characterization of permeases

Tese desenvolvida no âmbito da bolsa de doutoramento concedida pela
Fundação para Ciência e a Tecnologia (FCT- PhD) grant ref. SFRH/BD/23503/2005,
inserida no projecto de investigação Perm Yeast
(POCI/BIA-BCM/57812/2004 (Eixo 2, Medida 2.3, QCAIII – FEDER))

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

The Transport of Carboxylic Acids in Yeasts: from physiology towards structural-functional characterization of permeases
Neide Marina Vieira Pereira
UMinho | 2010

Março de 2010

Universidade do Minho
Escola de Ciências

Neide Marina Vieira Pereira

The Transport of Carboxylic Acids in Yeasts: from physiology towards structural-functional characterization of permeases

Tese de Doutoramento em Ciências
Área de Conhecimento em Biologia

Trabalho realizado sob a orientação da
Professora Doutora Sandra Paiva
Professora Doutora Margarida Casal

Março de 2010

DECLARAÇÃO

Nome: NEIDE MARINA VIEIRA PEREIRA

Endereço Electrónico: nvieira@bio.uminho.pt **Telefone:** +351 253 278 824 / +351 965 151 313

N.° do Bilhete de Identidade: 12452255

Título da Tese de Doutoramento:

The Transport of Carboxylic Acids in Yeasts: from physiology towards structural-funcional characterization of permeases

Orientadoras:

Professora Doutora Sandra Paiva
Professora Doutora Margarida Casal

Ano de conclusão: 2010

Designação do Doutoramento:

Área de Ciências
Especialidade de Biologia

Universidade do Minho, / /

Assinatura: _____

Acknowledgments / Agradecimentos

"Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada coisa a Lua toda
Brilha, porque alta vive"
Ricardo Reis (Fernando Pessoa)

Em primeiro lugar gostaria de manifestar o meu sincero agradecimento a todos os que de uma forma, ou de outra, me auxiliaram durante este sinuoso caminho, porque na realidade o trajecto do sonho à obra é tão importante como a sua concretização. Foram tantos os precalços, os receios, as dúvidas, mas também inúmeras as alegrias, as surpresas e as conquistas! Um muito obrigado a todos.

Não poderia deixar de manifestar o meu profundo agradecimento à Professora Sandra Paiva e à Professora Margarida Casal, orientadoras deste trabalho. À Professora Sandra Paiva gostaria de demonstrar a minha eterna gratidão pela admirável orientação desta tese, pela preseverança em todas as fases do trabalho, pelo estímulo intelectual constante mas acima de tudo pela amizade e pelo carinho com que me recebeu e transmitiu os seus conhecimentos. O meu mais sincero obrigada, por tudo. À Professora Margarida Casal pelo constante apoio, dedicação, determinação e rigor científico que me fortaleceram como investigadora. O meu mais profundo reconhecimento.

Gostaria também de agradecer ao Professor Björn Johansson pela constante disponibilidade e apoio no desenvolvimento dos trabalhos. Um muito obrigada!

I am also very grateful to Professor Alistair Brown for welcoming me so well in his laboratory in Aberdeen, Scotland. For providing the conditions and all the scientific inputs I ever needed for the development and conclusion of the work concerning *Candida albicans* carboxylate permeases homologs. I truly felt as a member of the Aberdeen Fungal Group and I thank Professor Al Brown and all the members in the laboratory for treating me that way. I can't express my gratitude for having the chance to work in such a highly regarded group with so high scientific standards.

I would also like to thank Professor Danièle Urban-Grimal and Professor Rosine Haguenauer-Tsapis for welcoming me in their laboratory for several times and for introducing me to the wonderful world of protein trafficking. Thank you so much for your help and advices.

The collaboration established with Professor Frederic Devaux and Xavier Darzacq was also very productive for my career, I'm extremely thankful for that opportunity.

Aos elementos que trabalham no Departamento de Biologia, nomeadamente os seus docentes, investigadores e técnicos por toda amizade e apoio disponibilizados. À Filipa minha colega de bancada por todo o apoio e amizade que demonstrou durante estes últimos anos. Por me "aturar" e encorajar nos momentos menos felizes e por celebrar verdadeiramente as minhas conquistas. Um muito obrigada do fundo do coração e o desejo que o seu percurso científico seja luminoso. Aos colegas do LGM que enriqueceram e alegraram os meus dias, sem qualquer ordem de preferência, à Sara, à Mónica, à Luísa, à Carina, à Jéssica, à Raquel, ao Ricardo, ao Nuno e ao João. O meu sincero obrigada pelos momentos de diversão que proporcionaram e pelo carinho com que me ajudaram sempre que precisei. O almoço pode ser sempre arroz de pato!

Aos colegas das antigas Micro I, Micro IV e Micro II, que agora dispersaram pelo departamento (ou por novas paragens), e a todos os amigos do Departamento de Biologia, a minha profunda gratidão pelos momentos que vivemos. Gostaria de agradecer particularmente (espero não esquecer ninguém) e desde já me desculpo se o fizer, ao Rui, à Patrícia, ao Mythos, à Gabriela, à Clara, à Júlia, à Andreia, à Susana, à Sónia, à Célia, à minha quiducha Leonor, à Isabel João, à Rita, ao Raúl, ao Huguinho, ao Paulinho e ao Paulo Bessa, ao Jorginho, à Teresa, à Nádia, ao JP, ao Cristovão, à Cristina, à Natacha e à Joana por todo o regojizo nos bons momentos, pelo forte apoio nos não tão bons e pela amizade que sempre demonstraram. A special thank to Daniel Alpern for always having my back, providing me all the papers I didn't have access to!!

Às minhas grandes manas, Marta, Irene, Carla, Cristina e Ana porque a vida seguiu rumos distintos mas as nossas almas nunca deixaram de se tocar. Obrigada por toda a alegria, pelo carinho e pela coragem, não esquecendo as importantes risadas perdidas pelas ruas desta cidade!

À minha Mãe, ao meu irmão Nuno e ao meu tio Dérito um obrigada do tamanho do mundo pela atmosfera de amor incondicional com que sempre me protegeram, por me providenciarem força, coragem e determinação nos momentos em que fraquejei e duvidei e o sonho por instantes se abafou. A vida pode e dá muitas voltas mas nada separará o amor que nos une. À minha Avózinha e Pai por todo o apoio nesta aventura e por nunca terem duvidado de mim e das minhas capacidades. Ao Telmo, o meu amor, por todos os momentos que partilhamos nestes quatro anos. Rimos, choramos, sofremos

e continuamos juntos, nos bons e maus momentos. Obrigada por termos crescido juntos e seguirmos felizes nesta roda viva da vida.

Finalmente dedico esta tese ao meu Avozinho, um muito obrigada por tudo Avô. Onde quer estejas eu sei que estás sempre a torcer por mim e estás feliz com as minhas conquistas, as conquistas da tua caracoleta. Obrigada por me teres mostrado o caminho! O horizonte reflecte o brilho dos olhos de quem o mira e os meus brilham muito Avô, emanam a intensidade dos nossos sonhos. Um beijinho.

Abstract

Plasma membrane proteins occupy a central role in cell biology. They control many important aspects on nutrient transport, intracellular signaling and homeostasis, thus they are subjected to a surprisingly tight regulation, according to different physiological conditions. In this thesis we aimed at studying the physiology, biochemical features and molecular regulation of carboxylic acids plasma membrane transporters, in different yeast species.

Homologs to Jen1 monocarboxylate transporter of *Saccharomyces cerevisiae* have been described in different microorganisms. In the first part of this work we have demonstrated that in *Candida albicans* a *CaJEN1* paralog, Ca*JEN2*, previously assigned by homology as a lactate transporter, encodes a novel dicarboxylate plasma membrane transporter, induced by succinic and malic acids. A strain deleted in both genes lost the ability to transport lactic, malic and succinic acids, by a mediated mechanism and it displayed a growth defect on these substrates. *CaJEN2* was heterologously expressed in a *S. cerevisiae jen1* strain leading to the detection of a succinate and malate permease activity. Moreover, in *in vivo* and *ex vivo* models of infection, it was found that both CaJen1 and CaJen2 are expressed in glucose-poor niches within the host, contributing to carbon metabolism during the early stages of infection.

We attempted to identify genes encoding carboxylate permeases in *Candida glabrata*, the second most prevalent yeast pathogen in humans. Acetic acid-grown cells of *C. glabrata* ATCC2001 display activity for a mediated mechanism for labelled acetic acid, at pH 5.0, with the following kinetic parameters: K_m 8,16 mM and V_{max} 8,08 nmol s^{-1} mg dry wt^{-1}. This yeast experienced a reductive evolution after the WGD, and although it has no predicted homologs of Jen1, it encodes in its genome two putative homologs of ScAdy2, *S. cerevisiae* acetate transporter. The deletion of the phylogenetically closest ScAdy2 homolog, *CAGLOMO3465g*, affected the uptake of labelled acetic acid. Nevertheless, wild-type cells and cells disrupted in this gene display similar behaviour, in the distinct growth conditions tested. These results suggest that, besides *CAGLOMO3465g*, the second ScAdy2 homolog has a putative role in acetic acid transport, in *C. glabrata*.

The heterologous characterization of two predicted ScAdy2 homologs, identified in the acetotrophic methanogen *Methanosarcina acetivorans* was performed, in an effort to validate their function as acetate transporters. The uptake of labelled

acetic acid was followed in *S. cerevisiae jen1ady2* strain, transformed with the expression vector ptYEplac181, harboring each one of the ORFS. Nevertheless, no significant differences were encountered between the tested strains.

In the second part of the work we studied the regulation of Jen1 and Ady2 expression in *S. cerevisiae*. *JEN1* and *ADY2* are subjected to glucose repression and their transcription is induced in cells grown in non-fermentable carbon sources. Formic acid is not used as sole carbon and energy source by *S. cerevisiae*, however it acts as a non-competitive substrate for Jen1 and as a competitive inhibitor of Ady2. Cells incubated in formic acid do not express the mRNAs of any of the transporter. However, a striking result was found in a strain deleted in the *DHH1* gene: the transcripts for both transporters were found in 4h formic acid-induced cells. We demonstrate that Dhh1, a DEAD-box RNA helicase, affects the half-live times of Jen1 and Ady2 mRNAs. Global transcriptional analyses performed in the wild-type and mutant *dhh1* strains suggested that other important genes, like the transcription factor encoding *CAT8* gene, encountered similar regulation.

In the third part of this work, we have studied the mechanisms underlying glucose-induced down-regulation of Jen1 monocarboxylate transporter of *S. cerevisiae*. By monitoring the Jen1-GFP subcellular localization, protein stability and permease activity, in several mutant strains, we demonstrated that glucose-downregulation of Jen1 is dependent on casein kinase 1 phosphorylation, on the HECT ubiquitin ligase Rsp5 ubiquitylation and on the presence of the arrestin-like adaptor protein, Art4. Furthermore, we have shown that Jen1 is modified at the cell surface by oligo-ubiquitylation, with ubiquitin-Lys_{63} linked chains(s), and that Jen1-Lys_{338} of Jen1 is one of the target residues. Finally, ubiquitin-Lys_{63} linked chains(s) were revealed to be directly or indirectly involved in the sorting of Jen1 into MVBs, making Jen1 one of the few examples for which it was demonstrated the requirement of ubiquitin- Lys_{63} linked chains for the correct trafficking at two stages of endocytosis.

Resumo

As proteínas de membrana plasmática ocupam um lugar central na biologia celular, sendo determinantes no controlo do transporte de nutrientes, da sinalização intracelular e da homeostasia, e por conseguinte, encontram-se sujeitas a uma regulação apertada, de acordo com as alterações fisiológicas. A presente tese teve como objectivo estudar a fisiologia, as características bioquímicas e a regulação molecular dos transportadores de ácidos carboxílicos, em diferentes espécies de leveduras.

Recentemente, foram descritos diversos homólogos do transportador de monocarboxilatos de *Saccharomyces cerevisiae* Jen1, em diferentes microrganismos. Na primeira parte do trabalho, demonstrámos que o parálogo de *CaJEN1* de *Candida albicans*, o gene *CaJEN2*, anotado por homologia como um transportador de lactato, codifica um transportador de membrana plasmática de dicarboxilatos, sujeito a indução pelos ácidos succínico e málico. Uma estirpe com delecção em ambos os genes perdeu a capacidade de transportar ácido láctico, málico e succínico, através de um mecanismo mediado, apresentando deficiências de crescimento, na presença dos mesmos substratos. A expressão heteróloga de *CaJEN2* na estirpe *S. cerevisiae jen1* conduziu à detecção da actividade de transporte mediado para succinato e malato. Em modelos *in vivo* e *ex vivo* de infecção, CaJen1 e CaJen2 são expressos em nichos pobres em glucose, no interior do hospedeiro, contribuindo dessa forma para o metabolismo de carbono, durante as fases iniciais de infecção.

A procura de transportadores de ácidos carboxílicos foi efectuada em *Candida glabrata*, a segunda levedura patogénica mais prevalente em humanos. Em células de *C. glabrata* ATCC2001, crescidas em ácido acético, foi caracterizado um sistema de transporte mediado para ácido acético marcado radioactivamente, a pH 5.0, com os seguintes parâmetros cinéticos: K_m 8,16 mM and V_{max} 8,08 nmol s^{-1} mg peso $seco^{-1}$. Esta espécie sofreu uma evolução reductora após o WGD e, embora não possua qualquer homólogo de Jen1, o seu genoma apresenta dois homólogos de ScAdy2. A delecção de *CAGLOMO3465g*, o homólogo filogeneticamente mais relacionado com ScAdy2, afectou o transporte mediado de ácido acético. Todavia, as células com delecção neste gene apresentaram um comportamento idêntico ao encontrado para a estirpe selvagem, em todas as condições de crescimento testadas. Possivelmente, para além de *CAGLOMO3465g*, haverá o envolvimento do segundo homólogo de ScAdy2 no transporte de ácido acético em *C. glabrata.*

A expressão heteróloga em *S. cerevisiae* de dois homólogos de ScAdy2, identificados em *Methanosarcina acetivorans*, foi efectuada, com o intuito de avaliar a sua função fisiológica, nesta arquea metanogénica acetotrófica. O transporte de ácido acético marcado radioactivamente foi seguido na estirpe *S. cerevisiae jen1ady2* transformada com o vector de expressão ptYEplac181, contendo cada uma das ORFs, contudo não foram encontradas diferenças significativas entre as estirpes testadas.

Na segunda parte do trabalho estudámos a regulação da expressão de Jen1 e de Ady2 em *S. cerevisiae. JEN1* e *ADY2* estão sujeitos a repressão pela glucose e a sua transcrição é induzida em células crescidas em fontes de carbono não-fermentescíveis. O ácido fórmico não é utilizado como fonte de carbono e energia por *S. cerevisiae*, actuando contudo, como um inibidor não-competitivo de Jen1 e como um inibidor competitivo de Ady2. Células induzidas em ácido fórmico não expressam os mRNAs de nenhum dos transportadores. Todavia, um resultado surpreendente foi obtido numa estirpe com delecção no gene *DHH1*: os transcritos de ambos os transportadores foram encontrados em células induzidas em ácido fórmico, durante 4h. Foi ainda demonstrado que a proteína Dhh1, uma DEAD-box RNA helicase, afecta os tempos de meia-vida dos mRNAs de Jen1 e Ady2. Análises de expressão global, efectuadas nas estirpes selvagem e mutante, sugerem que outros genes importantes, nomeadamente o *CAT8*, gene codificador de um factor de transcrição, sofrem uma regulação semelhante.

Finalmente, estudámos os mecanismos subjacentes à repressão do transportador de monocarboxilatos em *S. cerevisiae*, Jen1 por glucose. Através da monitorização da localização subcelular de Jen1-GFP, da sua estabilidade e da sua actividade, demonstrámos que a inactivação de Jen1 promovida por glucose é dependente da fosforilação, efectuada pela caseína cinase 1, da ubiquitilação promovida pela ubiquitina ligase Rsp5 e da presença do adaptador tipo-arrestina, Art4. Adicionalmente, demonstrámos que a permease Jen1 é modificada na superfície da célula por oligo-ubiquitilação com cadeia(s) de ubiquitina ligadas pela $Lis_{63,}$ e que Jen1-Lis_{338} de Jen1 é um dos resíduos alvo. Finalmente, cadeia(s) ubiquitin Lis_{63} encontram-se, directa ou indirectamente, relacionadas com o direccionamento do Jen1 para os MVBs, tornando o Jen1 um dos poucos exemplos onde se demonstrou que as cadeia(s) Ub-Lis_{63} são necessárias para o tráfego correcto em dois passos da endocitose.

SECTION III - TRAFFICKING AND TURNOVER OF Jen1

Abbreviations List

ABC – ATP-binding cassette Superfamily

ACO – Aconitase

ACS – Acetyl-CoA synthetase

AMP – Ampicilin

APC – Amino acid-Polyamine-organoCation Superfamily

APS – Ammonium persulphate

BSA – Bovine serum albumin

cDNA – Complementary deoxyribonucleic acid

CGD – *Candida albicans* genome database

CIT – Citrate synthase

CFU – Colony forming unit

D-LCR – D-lactate cytochrome c oxireductase

DMEM – Dulbecco's modified eagle's medium

DNA - Deoxyribonucleic acid

DPM – Disintegrations per minute

EC – Enzyme classification system

EDTA - Ethylenediamine tetraacetic acid

FBP – Fructose-1,6-biphosphatase

FBS – Fetal bovine serum

gDNA – Genomic DNA

GFP – Green Fluorescent Protein

ICL1 – Isocitrate lyase

ILV – Intralumenal vesicle

K_d – Difusion Constant

K_m – Affinity constant

KO – knock-out

LB – Luria-Bertani medium

L-LCR – L-lactate cytochrome c oxireductase

mCi – milliCurie

MDH – Malate dehydrogenase

MFS – Major Facilitator Superfamily

MLS1 – Malate synthase

MVB – Multivesicular bodies

mRNA – Messenger ribonucleic acid

OD – Optical density

PAGE – Polyacrylamide gel electrophoresis

PBS – Phosphate buffer saline

PCK – Phosphoenolpyruvate carboxykinase

PCR – Polymerase chain reaction

PDC1,5,6 – Pyruvate decarboxylase isoenzymes

PDH – Pyruvate dehydrogenase complex

PYC – Pyruvate carboxylase

RNA - Ribonucleic acid

R.p.m – Rotations per minute

RT-PCR – Reverse transcriptase polymerase chain reaction

qRT-PCR – Quantative Real Time polymerase chain reaction

SD – Synthetic dextrose

SDH – Succinate dehydrogenase

SDS – Sodium dodecyl sulfate

SGD – *Saccharomyces cerevisiae* genome database

TC – Transport classification

TCDB – Transport classification database

VIC – Voltage-gated ion channel Superfamily

WGD – Whole genome duplication

YE – Yeast extract

YEPD – Yeast extract, Peptone and Dextrose

Short *Curriculum Vitae*

Neide Vieira was born in Pretoria, South Africa in the 10th of January 1984. She presently lives in Braga, Portugal, and works, as a researcher, in the Molecular and Environmental Biology Centre, under the supervision of Prof. Sandra Paiva and Prof. Margarida Casal.

Her background includes a four-year graduation in Applied Biology, by the School of Sciences, University of Minho, where she graduated with the highest mark. She has just submitted her PhD thesis entitled: "The Transport of Carboxylic Acids in Yeasts: from physiology towards structural-functional characterization of permeases" to the University of Minho, which was prepared in cooperation with: the University of Aberdeen, Aberdeen, UK; the Institut Jacques Monod and École Normale Supérieure both in Paris, France.

During the last year of her graduation Neide Vieira worked in the molecular and biochemical characterization of carboxylate permeases in yeasts, in Braga, Portugal.

In 2005 Neide Vieira joined Margarida Casal's group as a researcher in the FCT (Portuguese Foundation for Science and Technology) project POCI/BIA-BCM/57812/2004 "PermYeast – Molecular biology of the Monocarboxylic Acids Permeases in Yeasts: from functional to structural analysis", coordinated by Professor Sandra Paiva. She started to work on the physiological, biochemical and molecular characterization of *S. cerevisiae* monocarboxylate transporters, Jen1 and Ady2. In 2006 she began her PhD thesis aiming at increasing the knowledge on carboxylate permeases overall, with the collaboration of three well-regarded European educational facilities. During this phase she worked for different time periods at the Department of Biology, Braga, Portugal, at the Aberdeen Fungal group in Aberdeen, Scotland and in Paris, both at the Institut Jacques Monod and in École Normale Supérieure, always under the same scope of study.

Neide Vieira has been involved in the organization of one international conference, the 26th Small Meeting on Yeast Transport and Energetics, SMYTE, held from the 6th till the 9th of September 2008 in Braga, Portugal. Additionally, she was a member of the

organization committee of the Post-graduating training course on Plasma membrane Transporters, held from the 4th till the 8th of June 2007 in Braga, Portugal.

As a result of her research work she attended several important international and national meetings in the present field of research. Moreover, she was awarded four grants to participate in EMBO and FEBS advanced practical, lecturer courses and workshops. Presently she is the author of three papers published in international peer-reviewed journals, and of 2 papers under preparation for submission in international peer-reviewed journals.

List of Publications

The work performed during this PhD thesis resulted in the following publications:

PAPERS IN REFEREED JOURNALS

Paiva, S., Vieira, N., Nondier, I., Haguenauer-Tsapis, R., Casal, M. and Urban-Grimal, D. (2009) "Glucose-Induced Ubiquitylation and Endocytosis of the Yeast Jen1 Transporter: role of Ubiquitin-K63 chains", *J Biol Chem*, **29:** 19228-36.

Vieira, N., Casal, M., Johansson, B., MacCallum D., Brown, A. and Paiva, S. (2009) "Functional specialization and differential regulation of short chain carboxylic acid transporters in the pathogen *Candida albicans*". *Molecular Microbiology* **76:**1337-54.

Vieira, N., Pereira, F., Casal, M., Brown, A., Paiva, S. and Johansson, B. (2010) "A set of plasmids for *in vivo* construction of integrative *Candida albicans* vectors in *Saccharomyces cerevisiae". YEAST.*

IN PREPARATION

Vieira, N., Devaux, F., Barbosa, S., Darzacq, X., Paiva, S. and Casal, M. "Role of the *DHH1* gene in the regulation of monocarboxylic acids transporters expression in *Saccharomyces cerevisiae*"

Vieira, N., Léon, S., Gomes, J., Cunha, C., Haguenauer-Tsapis, R., Casal, M. and Paiva, S. "Art4 arrestin-like protein mediates Rsp5 dependent ubiquitylation in glucose-induced endocytosis of the Jen1 transporter in yeast"

COMMUNICATIONS IN NATIONAL AND INTERNATIONAL MEETINGS

Paiva S., Vieira N., Barbosa S. and Casal M. "Role of the *DHH1* gene in the regulation of monocarboxylic acids transporters expression in *Saccharomyces cerevisiae*". Micro'05-Bioec'05 congress, Póvoa de Varzim, Portugal, November 2005, poster

Vieira N., Barbosa S., Paiva S., and Casal, M. "Role of the *DHH1* gene in the regulation of monocarboxylic acids transporters expression in *Saccharomyces cerevisiae*". Yeast Genetics and Molecular Biology Meeting (YGM), in Princeton, New Jersey, USA, July 2006, poster

Vieira N., Casal M., Brown A. and Paiva S. "Role of carboxylate permeases in the pathogenicity of *Candida albicans".* 25th Small Meeting on Yeast Transport and Energetics, SMYTE, Arraial d'Ajuda, Bahia, Brazil, August 2007, oral

Paiva S., Vieira N., Haguenauer-Tsapis R., Urban-Grimal D. and Casal M. "Glucose regulated ubiquitylation of the Jen1 permease of *Saccharomyces cerevisiae*". Workshop on Endocytic Systems: Mechanism and Function, in Villars-sur-Ollon, Switzerland, September 2007, poster

Vieira N., Casal M., Johansson B., Brown A. and Paiva S. "Role of carboxylate permeases in the pathogenicity of *Candida albicans".* Micro'07-Bioec'07 congress, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal, November 2007, poster

Paiva S., Vieira N., Haguenauer-Tsapis R., Urban-Grimal D. and Casal M. "Glucose-Induced Ubiquitylation and Endocytosis of the Yeast Jen1 Transporter", in the Workshop "Lipids as regulators of cell function" in Island of Spetses, Greece, June 2008, poster

Vieira N., Casal M., Johansson B., Brown A. and Paiva S. "Assessing the role of *CaJEN2* dicarboxylate permease in fungal virulence*".* Yeast Genetics and Molecular Biology Meeting (YGM), in Toronto - Ontario, Canada, July 2008, poster

Vieira N., Casal M., Johansson B., MacCallum D., Odds F., Brown A. and Paiva S. "Functional specialization and differential regulation of short chain carboxylic acid transporters in the pathogen *Candida albicans*", XVII Jornadas de Biologia das Leveduras "Professor Nicolau Van Uden", University of Minho, Braga, Portugal, June 2009, oral

Vieira N., Casal M., Johansson B., MacCallum D., Brown A. and Paiva S. "Functional specialization and differential regulation of short chain carboxylic acid transporters in the pathogen *Candida albicans*". Yeast Genetics and Molecular Biology Meeting (YGM), Manchester, UK, July 2009, poster

Chapter I

General Introduction

YEASTS AS MODEL ORGANISMS

The yeast *S. cerevisiae* was the first eukaryote organism to have its genome fully sequenced (Goffeau *et al.*, 1996), sharing a high level of complexity with higher eukaryotes. The molecular genetic tools available, its ability to be transformed and the capacity to grow in simple culture media with short generation times, makes this unicellular organism easy to manipulate in the laboratory and suitable to decipher some of the basic properties of all eukaryotic cells. Interestingly, almost 20% of the genes involved in human disease have counterparts in its genome, making it a simple model to study basic cellular processes associated to health.

Helpful global analysis tools have been developed and validated in this microorganism, such as DNA hybridisation microarrays, ChIP and RIP-chips, proteins chips, 2D electrophoresis and two-hybrid screens contributing to the elucidation of the overall cell functionality. Furthermore, the availability of a complete set of deletion mutants has further enhanced the potential use of *S. cerevisiae* as a model for unravelling the overall eukaryotic cell functionality.

Presently, genome sequences of several eukaryotic and prokaryotic organisms are becoming available, including the genomes of pathogenic fungi, namely, *Candida albicans* and *Candida glabrata*. However, due to the lack of information and of efficient molecular tools, the function of novel genes is often attributed based on sequence similarity to the *S. cerevisiae* genome, to circumvent the difficulties of carrying out genetic analyses in these fungi. However, the molecular techniques are improving and new tools are being developed to work in these non-conventional organisms. The increasing knowledge on the physiology and genetics of these other species is leading to a new research field that aims at identifying the mechanisms behind genome evolution.

Yeasts can in this manner, contribute to the elucidation of basic cellular mechanisms since they can be exploited/dissected to look at physical and functional protein relationships enabling the characterization of complexes and/or pathways, putatively conserved in higher eukaryotes.

The study of the transport of nutrients across the plasma membrane and their metabolism is essential to understand how cells adapt to different physiological conditions and how a protein interacts with their partners in larger complexes, what in turn will help to understand the mechanisms by which cellular pathways interlink. Nowadays, several molecular mechanisms of primary and secondary transporters are

still unknown and yeasts provide an exceptional model for their basic studies. In this manner, this thesis work aimed at the physiological, biochemical and molecular characterization of carboxylic acid transporters in three different yeast species: *S. cerevisiae*, *C. albicans* and *C. glabrata*, contributing to unravel their role in cell adaptation to different environments.

PHYLOGENY OF THE YEASTS STUDIED IN THIS WORK

S. cerevisiae, *C. albicans* and *C. glabrata* are Ascomycetes (Kingdom Fungi: phylum Ascomycota: subphylum Saccharomycotina: class Saccharomycetes: order Saccharomycetales). This phylum constitutes a monophyletic group, with three major groups: the Euascomycetes (molds), the Hemiascomycetes (yeasts) and the Archiascomycetes (the earliest ascomycetous lineage) (Nishida, 1994). The Hemiascomycetes comprise a large order, the Saccharomycetales established in 1960 (Kurtzman, 1998; Kirk, 2001). The order Saccharomycetales has approximately 1000 known species, that live as saprobes. Some of the species are parasites of animals, others of plants and some live in their interfaces (Suh *et al.*, 2006).

The Ascomycota together with the Basidiomycota compose the subkingdom of Dikarya, within the kingdom of Fungi (Tehler, 1988; James *et al.*, 2006; Hibbett *et al.*, 2007). The Ascomycetes differ significantly from the Basidiomycetes: the first have a cell wall composed mainly by β–glucan and the second by chitin; display a lower nuclear DNA guanine and cytosine content (less than 50%) (Kurtzman, 1998); show a holoblastic bud formation with wall layers remaining continuous in opposite to the enteroblastic formation in the Basidiomycetes; very often present more fermentative ability and a more specialized nutrition (Kurtzman, 1998); and usually have inability to stain with diazonium blue (Van Der Walt and Hopsu-Havu, 1976). The Ascomycetes are usually found in liquid carbon rich niches, which enable interactions with plants and animals, that contribute to their dispersal. In contrast, the Basidiomycetes colonize nutrient-poor and solid surfaces and rely less on the animal vectors for dispersal (Lachance, 1998).

The yeast *S. cerevisiae* is a commensal, domesticated microorganism. *S. cerevisiae* clade members are not well known in nature, but usually occur in high-sugar environments like fermenting fruits. Members of this clade have high tolerance to alcohol and over the last 8000 – 10000 years have been naturally selected as brewing

and wine yeasts (Suh *et al.*, 2006), due to high ethanol production, and as baker's yeast due to high rates of CO_2 production (Wills, 1990).

C. albicans is a diploid opportunistic commensal organism which is part of the normal gastrointestinal and urogenital microflora in healthy individuals. This species can cause frequent mucosal and cutaneous infections and in immunocompromised patients it can be responsible for life threatening systemic infections. Initially, *C. albicans* was phylogenetic placed in the imperfect fungi, the Deuteromycota, due to the lack of a sexual stage (teleomorph) (Calderone, 2002). However, several pathogenic and non pathogenic *Candida* species presented teleomorph stages, and those sexual stages are similar to the ascomycetous (Meyer *et al.*, 1997). Around the early 1990s, a molecular revolution in fungal taxonomy arose with the standardization of the PCR-amplified ribosomal RNA genes analyses (White, 1990). Different molecular techniques (Diezmann *et al.*, 2004) and sequence similarities (Gargas *et al.*, 1995) supported the localization of the *Candida* species in the phylum Ascomycota. Moreover, several differences separated *Candida* species from the Basidiomycota, namely their fermentative behaviour, the fact that they do not assimilate inositol, nor produce starch or carotene pigments, possess β–glucan in their cell wall and have homogenous cell wall septa, compared to the tripartite cell wall septa in the Basidiomycotas, among others (Calderone, 2002).

C. glabrata is a haploid asexual ascomycetous yeast recognized as the second most common cause of *Candida* infections. This fungus is often found in rotten fruits but also as a commensal in the gastrointestinal microflora of humans. In susceptible individuals, *C. glabrata* can efficiently proliferate and cause systemic and disseminated candidiasis. Currently, *C. glabrata* belongs to the *Candida* genus (Odds *et al.*, 1997) but initially it was classified as *Cryptococcus glabratus* (Anderson, 1917). In 1938 it was reclassified as *Torulopsis glabrata*, because of the inability to form true hyphae, displaying pseudohyphal growth only during nitrogen starvation (Csank and Haynes, 2000). The application of several molecular approaches ultimately placed *Toruplopsis glabrata* in the *Candida* genus despite of its inability to form true hyphae (Table 1).

Several evolutionary studies have been performed in the major clade of life, the Fungi. The scientific community intends to draw the "Fungal tree of life" and date the divergences of the different clades all through the evolutionary path. These studies are usually based on sequence similarities, nucleic acid variation, conservation and

orientation of ortholog's neighbour genes, among others (Dujon *et al.*, 2004; Kellis *et al.*, 2004; Lutzoni *et al.*, 2004; James *et al.*, 2006; Taylor and Berbee, 2006; Hibbett *et al.*, 2007) (Fig. 1).

The class of Hemiascomycetes has such a vast range of genetic diversity that is only comparable to the diversity that one can encounter in the entire phylum of chordates (Dujon *et al.*, 2004). The whole genome duplication (WGD) constituted an evolutionary trait for these microorganisms. The WGD provided the emergence of paralogous genes that through the action of mutations and natural selection acquired different functions (Ohno, 1970). Although rare, this event has played an important role in the evolution of many species, not only in the Hemiascomycetes but also in other organisms, namely in the vertebrate lineage (Ohno, 1970; Ohno, 1998), but this polyploidy state comes with a cost of severe genomic instability, till functional normal ploidy is achieved through mutations, rearrangements and massive redundant genes loss. The two duplicated copies must lose the redundancy either by subfunctionalization (partitioning of the ancestral function) (Force *et al.*, 1999; Lynch and Force, 2000) or by neofunctionalization (where one of the redundant genes acquires a new function) (Ohno, 1970).

Table 1 – Summary of *S. cerevisiae*, *C. albicans* and *C. glabrata* major attributes.

	S. cerevisiae	***C. albicans***	***C. glabrata***
Polyploidy	Haploid/Diploid	Diploid	Haploid
Chromosomes	16	8	13
Genome size	≈ 12 Mb	≈ 15.6 Mb (haploid)	≈ 12.3Mb
Genes	≈ 6200	≈ 6500	≈ 6500
Pathogenicity	Non-Pathogenic	Pathogenic (≈55%)	Pathogenic (≈20%)
Matting genes	Present	Present	Present
Sexual cycle	Complete	Parasexual	Unknown
Phenotypic switching	Absent	Present	Present
Filamentation	No pseudo nor true hyphae	Pseudo and true hyphae	Pseudohyphae
Biofilm formation	Present	Present	Present
Azole resistance	Susceptible	Susceptible	Innate resistance
Codon	Cg compatible codons	Altered codons	Sc compatible codons

There are indications that *S. cerevisiae* and *C. albicans* shared a common ancestor around 800 million years ago (Heckman *et al.*, 2001), before the WGD. After that divergence between the *Kluyveromyces* and *Saccharomyces* speciation occurred the WGD (Byrne and Wolfe, 2006).

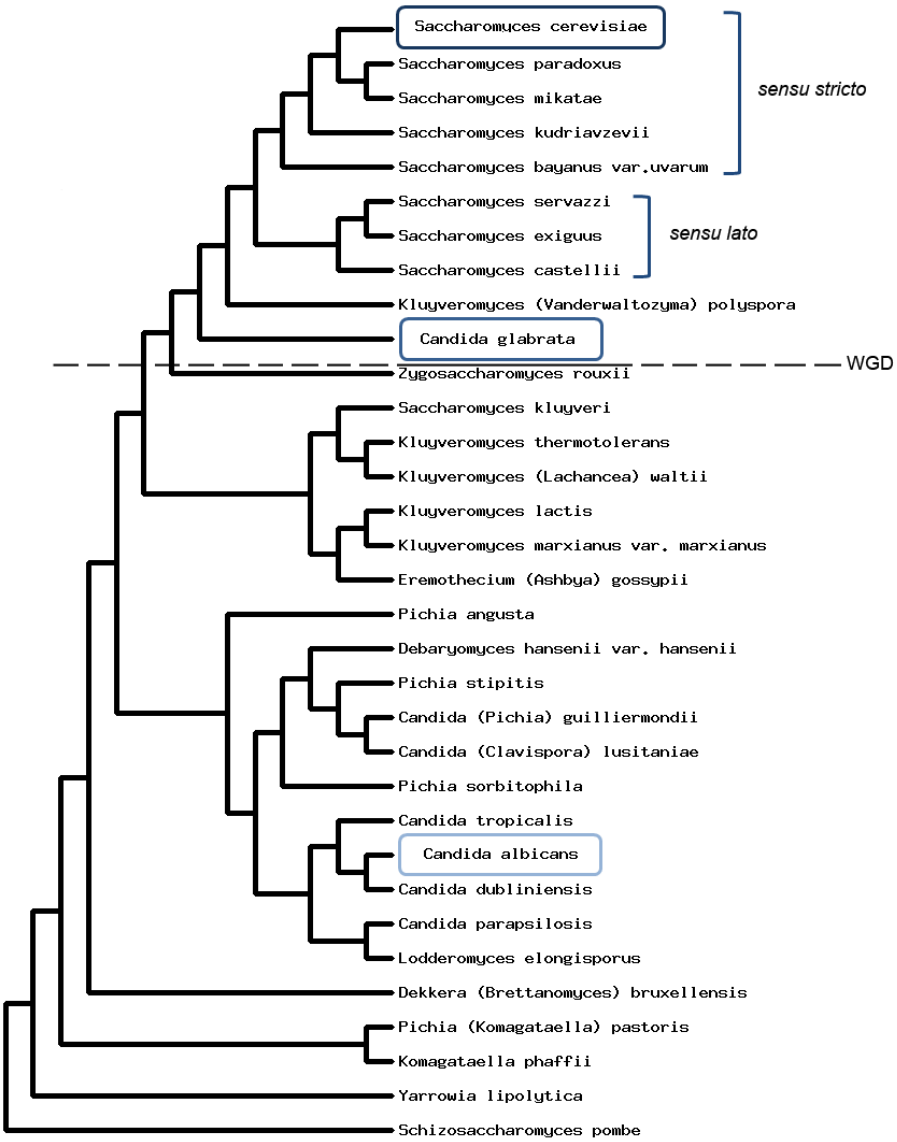

Figure 1 – Indicative cladogram of sequenced yeast genomes, adapted from http://www.genolevures.org/yeastgenomes.html#. The strains used in this work are indicated with a box.

Many have doubted the occurrence of the WGD in *S. cerevisiae*, since it was based on the analyses of a small percentage of yeast genes. This idea was supported by the presence of ancestral duplication blocks in the paralogs genomic locations, by independent local duplication events (Mewes *et al.*, 1997; Llorente *et al.*, 2000a;

Llorente *et al.*, 2000b; Koszul *et al.*, 2004). However, Kellis and co-workers have shown that indeed *S. cerevisiae* has undergone a complete duplication of the eight ancestral chromosomes, followed by an accentuated loss of approximately 90% of the redundant genes. Moreover, there are indications that the relative yeast species, *Kluyveromyces waltii* and *Ashbya gossypii*, originated over 100 million years ago from a common ancestor, and that *S. cerevisiae* arose from the whole genome duplication of that common ancestor. In this manner, the closely related species dated the WGD after the emergence of *K. waltii* (Kellis *et al.*, 2004), *A. gossypii* (Dietrich *et al.*, 2004) and *K. lactis* (150 million years ago) (Dujon *et al.*, 2004), but before all *sensus stricto* and *sensus lato Saccharomyces* species (Cliften *et al.*, 2003; Kellis *et al.*, 2003) and *C. glabrata* (Dujon *et al.*, 2004). Recent phylogenetic analyses indicated that although *S. cerevisiae* lineage went through the WGD to acquire genetic novelty, several other mechanisms have occurred during the evolution of the *Hemiascomycetes*, like tandem repeats and block or segmental duplications (Dujon *et al.*, 2004). The study of ancient genomes like *C. albicans* genome (Jones *et al.*, 2004) and an Euascomycete *Neurospora crassa* genome (Galagan *et al.*, 2003), supports this view.

Although *C. glabrata* belongs to the *Candida* genus and shares with *C. albicans* its pathogenic behaviour, this yeast is phylogenetically placed in the *Saccharomyces* clade (Diezmann *et al.*, 2004; Dujon *et al.*, 2004; Butler *et al.*, 2009). In fact, *S. cerevisiae* and *C. glabrata* are very closely related and share thousands of genes, which are located in more than 500 syntenic clusters (Ochman *et al.*, 2005). Both species went through the WGD but present different levels of redundancy. *C. glabrata*, which split from the *Saccharomyces* lineage after this event, presents a higher loss of duplicated genes. This loss was severe and led to a reductive evolution, with a degree of redundancy similar to prior-WGD yeasts, like *Kluyveromyces lactis*, or even lower than in *Yarrowia lipolytica* (Dujon *et al.*, 2004). There are no logical explanations for this functional reduction but probably it was related to the adaptation of this yeast to being a human pathogen.

The majority of phylogenetic studies involving Hemiascomycetes was performed on non-membrane proteins or RNA. Nevertheless, De Hertogh and co-workers dedicated their efforts to study the phylogeny of membrane transporters, which account for 10% of the coding genes in Hemiascomycetes (De Hertogh *et al.*, 2006). The evolution pattern of membrane permeases was followed through the analyses of the

complete genomes of S. *cerevisiae*, *C. glabrata*, *K. lactis*, *Debaryomyces hansenii* and *Y. lipolytica*. This study allowed the identification of ubiquitous families, present in all species, and of the emergence and/or loss of specie-specific transporters. The gain and loss of specific transporters, throughout evolution, stimulates the differentiation of several large metabolic pathways, in the diverse yeasts. In summary, *Y. lipolytica* and *D. hansenii* emerged with a significant gain of transporters compared to the gain detected in the emergence of *K. lactis*, *C. glabrata* and *S. cerevisiae*, probably due to substrate availability that was imposed by natural selection and constricted the transporters evolution. In *C. glabrata* there is a specific loss of several transporter subfamilies in comparison with the other species. In fact, amongst the five, *C. glabrata* is the one that contains in its genome the lowest number of putative and established transporters (398 in comparison to≈500 in the other species) (De Hertogh *et al.*, 2006).

TRANSPORT CLASSIFICATION SYSTEM

Integral membrane proteins mediate almost all the transmembrane transport processes, functioning both with extracytoplasmic receptors or receptors domains and with cytoplasmic proteins or protein domains. These complexes can be referred as transport systems, transporters, porters, permease systems or permeases and always catalyze a vectorial reaction (Saier, 2000). Transmembrane proteins are encoded by 5 to 15% of the genome of all organisms (Tanner and Caspari, 1996; Diallinas, 2008; Conde *et al.*, 2009). Phylogenetic tools have been used since 1993 to predict functional features of transporters and to characterize their evolution (Marger and Saier, 1993). With the improved DNA sequencing technologies the primary structures of many genes became available, facilitating the phylogenetic characterization of transporter families. This was based on the fact that, highly homologous proteins usually have very similar three dimensional structures, and that the degree of tertiary structural similarities correlates reasonably well with primary structure similarity. A nonambigous transport classification system (TC) was then elaborated combining mechanistic, functional and phylogenetic analyses (Saier, 2000). This system was slightly similar to the already implemented enzyme classification system (EC) and it was approved by a panel of experts of the International Biochemistry and Molecular Biology (IUBMB) (http://www-biology.ucsd.edu/~msaier /transport/) (Busch and Saier, 2004).

The Transporter Classification Database (TCDB - http://www.tcdb.org/) is freely available and provides all sort of information regarding the transport systems of a wide variety of living organisms (Saier and Ren, 2006). The classification system groups the transporters according to five criteria and each criteria corresponds to one of the five digits within the TC number of a specific transporter (V, W, X, Y and Z). The first component, V, corresponds to the transporter class (1-channel and pores, 2-electrochemical potential-driven transporters, 3-primary active transporters, 4-group translocators, 8-accessory factors involved in transport, 9-incompletely characterized transport systems). The second level of classification, W, corresponds to the subclass and classifies permeases according to the type of transport and energy coupling processes. In this manner, the two first digits identify the global transport mechanism. The third digit, X, is related to the phylogenetic family (or superfamily) to which a transporter is assigned to. The fourth number, Y represents a phylogenetic cluster within a family or superfamily (a subfamily in a family or a family in a superfamily). Finally Z identifies the substrate or the substrates transported and the polarity of the transport system (Saier, 2000). In this manner, transporters that share the same subfamily and that transport the same substrate or range of substrates, using the same type of transport and energy coupling mechanisms have an equal TC number, irrespective whether they are orthologs or paralogs. Currently, this system identifies more than five hundred and fifty families of transporters and several were grouped in large superfamilies, like the Voltage-gated ion channel Superfamily, VIC (TC 1.A.1), the Major Facilitator Superfamily, MFS (TC 2.A.1), the Amino acid-Polyamine-organocation Superfamily, APC (TC 2.A.3), the ATP-binding cassette Superfamily, ABC (TC 3.A.1), among others (Saier, 2000). The MFS and the ABC superfamilies are present in all living organisms. In microorganisms these superfamilies account approximately to 50%, of the solute transporters encoded within their genomes.

The MFS is a very old superfamily, currently constituted by 65 families and including more than 10000 sequenced members. Typically, this superfamily consists of transporters with two structural units, of six transmembrane-spanning α–helical segments, connected by a cytoplasmic loop (Marger and Saier, 1993). These transporters are single-polypeptide secondary carriers that only transport small solutes, according to a chemiosmotic gradient. Originally, they were set to transport primordially sugars, nevertheless, sequential studies pointed to their far more diverse

functions. They exhibit specificity for sugars, polyols, drugs, neurotransmitters, Krebs cycle metabolites, phosphorylated glycolytic intermediates and amino acids, among others.

MCP PORTER FAMILY

Several carboxylates like lactate and pyruvate, the branched-chain oxo acids derived from leucine, valine and isoleucine, as well as the ketone body compounds acetoacetate, β-hydroxybutyrate and acetate, have important roles in the metabolism of mammalian cells (Poole and Halestrap, 1993). In mammals, the final product of pyruvate fermentation, produced by the glycolytic pathway, is lactic acid. This acid acquires significant importance due to the high quantities accumulated, produced and utilized by different cells (Price *et al.*, 1998). The glycolytic pathway represents the major source of ATP synthesis in cells like the white skeletal muscle, erythrocytes, tumoral and hypoxic cells, among others (Halestrap and Price, 1999). In these cases, the produced lactic acid must be readily exported from the cell, in order to maintain a high glycolysis rate, since its accumulation leads to a cytosolic pH decrease and consequently to the inhibition of the phosphofructokinase and hence, of glycolysis. The cells that have a glycolytic metabolism, export lactate which is then captured by the liver and reconverted to glucose, that can in turn be metabolized again by these cells, by the glycolytic pathway - Cori cycle (Voet, 1990). On the other hand, in the case of cells that metabolize lactic acid (brain, heart, red skeletal muscle cells), its intake must be accomplished in a rapid and efficient way.

Initially, these acids were believed to cross the plasma membrane by simple diffusion. However, in mammals the transport of lactate in erythrocytes was shown to be specifically inhibited by a cyano-4-hydroxycinnamate and organomercurials. In 1982, Jennings and Adam-Lackey evidenced that the transport of lactate obeyed to a Michaelis-Menten kinetics and was associated with the presence of a membrane protein present in rabbit erythrocytes (Jennings and Adams-Lackey, 1982). The unequivocal identification of a transporter was achieved by Poole and Halestrap in 1992, after solubilization of the protein and reconstitution of the transport activity in proteoliposomes (Poole and Halestrap, 1992). The protein was further characterized and named MCT1 (monocarboxylate transporter 1), being found that it had an N-terminal identical to a putative transporter, previously cloned from Chinese-hamster ovary cells

(Kim *et al.*, 1992). MCT2 was the second transporter cloned, isolated from hamster liver and sharing 60% of identity with MCT1 (Garcia *et al.*, 1995). MCT3 was then isolated from chicken retinal pigment epithelium and heterologously expressed in a thyroid epithelial cell line (Yoon, 1997). Sequentially, with the aid of the Expression Sequence Tags (EST) database, additional human MCT-related sequences were annotated and cloned from a human cDNA library (Price *et al.*, 1998), MCT3-MCT6. Concomitantly, several searches were performed in diverse protein databases leading to the annotation of several MCT homologs in other organisms, namely in *S. cerevisiae*, *Caenorhabditis elegans* and *Sulfolobus solfataricus*. The existence of non-mammalian MCTs allowed the elaboration of a new family of transporters, with eukaryotic and prokaryotic members (Price *et al.*, 1998), the proton-linked monocarboxylate transporters family. In this manner, the MCTs have been included in the MFS superfamily, and according to the TC system belong to the Monocarboxylate Porter family (MCP), with the TC number, 2.A.1.13.

MCT9 was the next monocarboxylate porter to be identified, based on a human EST database search (Halestrap and Price, 1999). An amino acid transporter, with homology to the MCT family, was then annotated as a MCT family member, *TAT1*. However, Tat1 transported amino acids, not lactate or pyruvate, by a facilitated diffusion mechanism, in contrast to the proton-linked uptake mechanism typical of the MCTs (Kim *et al.*, 2001). Another gene with homology to *TAT1* was found by Kim and co-workers, MCT8. This protein was involved in the transport of thyroid hormones T4, and T3 and not in the uptake of amino acids, or lactate. Latter, MCT11, MCT12, MCT13 and MCT14 were discovered by additional sequence homology searches in diverse databases (Halestrap and Meredith, 2004).

The MCT family comprises members with a predicted topology of 10-12 transmembrane domains (TMD), with a cytoplasmic loop between TM segments 6-7 and with both C and N-terminals located in the cytoplasm. Topological studies were performed only in MCT1, and confirmed the previous predicted topology (Poole, 1996). The sequence conservation between different family members is higher in the TM segments and lower in the intervening hydrophilic regions of the sequences. It has been proposed that the two halves of the molecule (TM helices 1-6 and 7-12) have different functional roles. The N-terminal domains may be more important for energy coupling (e.g. via H^+ or Na^+ co-transport), membrane insertion, and/or correct structure

maintenance, whereas the C-terminal domains may be more important for the determination of substrate specificity (Halestrap and Price, 1999).

Several expression studies, of all the different MCT isoforms, have been performed at the mRNA level (Lin *et al.*, 1998; Price *et al.*, 1998) and at the protein level (Garcia *et al.*, 1994; Garcia *et al.*, 1995; Gerhart *et al.*, 1997; Jackson *et al.*, 1997; Gerhart *et al.*, 1998; Wilson *et al.*, 1998), in mammalian tissues. These studies indicated that the MCT genes have a very different tissue distribution, in mammals. For instance, MCT1 was found to be present in almost all tissues, in many cases with specific locations within each tissue. In contrast, MCT2 is expressed in fewer tissues, and is absent or barely detectable in skeletal muscle. It is found together with MCT1 in several tissues such as liver, kidney and brain but its exact location within each tissue differs from that of MCT1, suggesting a distinct functional role. These facts can explain the need for the existence of several homologues genes and probably their different specificities besides the involvement in monocarboxylate transport (Halestrap and Meredith, 2004). A recent study showed that in mice placenta, after 11.5 gestational days, a strong mRNA expression of MCT1, MCT4 and MCT9 was detected. MCTs could be identified at the plasma membrane, but also at the subcellular level and presented a completely opposite localization, in comparison to the human placenta. Probably, related to differences between human and mice in the origin of lactate and its respective utilization by the fetuses (Nagai *et al.*, 2009).

MCTs were indeed, primarily identified in mammals, but with the advents of sequencing technologies, several homologs have been annotated in distinct organisms. In *S. cerevisiae* five homologs were identified, in *Drosophila melanogaster* six members were discovered in the X chromosome, six sequences in *C. elegans* genome (Halestrap and Price, 1999) and more recently three members were found in *A. nidulans* (Semighini *et al.*, 2004). Some MCT1 homologs have also been described in bacterial genomes like in *Escherichia coli* and in *Bacillus subtilus* (Halestrap and Price, 1999).

The first homologs in *S. cerevisiae* were identified by searching the 186 proteins identified as possible MFS members, divided in 23 families, present in this baker's yeast entire genome. One of the families was designated by the Monocarboxylate Permease Homologs (MCH) and included four members, *YOR306c*, *YOL119c*, *YKL221w* and *YNL125c* (Nelissen *et al.*, 1997). The fifth homolog, encoded by *YDL054*c, was identified by Bruno André and published in the Yeast Transport Protein

database (YTPdb) (http://alize.ulb.ac.be/YTPdb/). This homolog exhibited striking similarities to the other four putative permease genes encoded in *S. cerevisiae* genome (homologs of mammalian MCTs), the *MCH* genes (Fig. 2) (Andre, 1995). Another membrane protein classification study also included these five *S. cerevisiae* proteins in the MCP family (De Hertogh *et al.*, 2002).

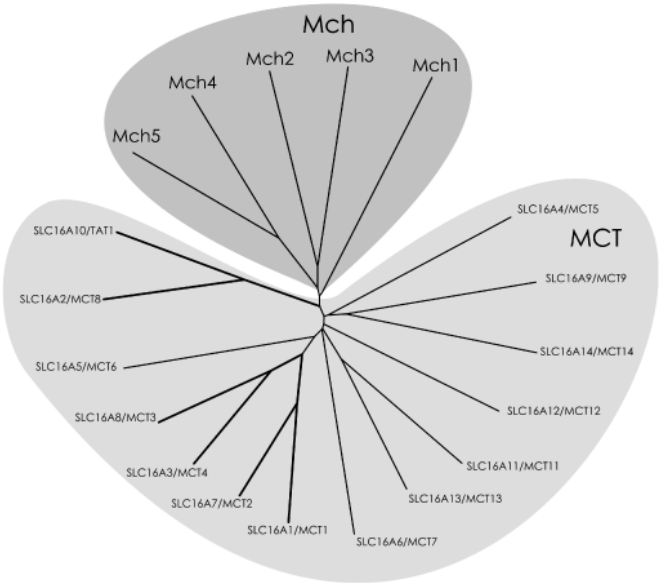

Figure 2 – Phylogenetic tree elaborated with the known human MCTs and *S. cerevisiae* Mchs. Sequences were obtained by SGD and by homology searches at NCBI. Copied from (Casal *et al.*, 2008).

The functional analyses of these MCH genes were carried out by Makuc and co--workers (Makuc *et al.*, 2001). The simultaneous disruption of the five homologs had no effect on the uptake of monocarboxylates, proving that these homologs do not share substrate specificity with the human MCTs. Additionally, they were also not involved in the secretion of monocarboxylates, as some mammalian MCT members are (Makuc *et al.*, 2001). None of the five MCH genes was glucose repressed nor required the presence of lactate to be induced, in contrast to the monocarboxylate transport system of *S. cerevisiae* (Cassio *et al.*, 1987; Casal *et al.*, 1996). The expression pattern in medium containing lactate, ethanol or pyruvate was vey similar and their subcellular localization was generally dubious, with exception of Mch4-GFP that localized to the vacuole membrane. Overall, none of the five homologs was significantly induced by monocarboxylates nor localized to the plasma membrane (Makuc *et al.*, 2001) and their functions remain unknown so far, except for Mch5 (*YOR306*c). Reihl and co-workers provided experimental evidences that Mch5 encodes a transporter that facilitates the diffusion of riboflavin (vitamin B2), when riboflavin concentrations in the cytoplasm

are lower than the external concentrations (Reihl and Stolz, 2005). The heterologous expression of this transporter in *Schizosaccharomyces pombe*, that naturally doesn't transport riboflavin, confirmed its function (Reihl and Stolz, 2005). It is clear that the Mch proteins have distinct roles than their mammalian counterparts. Although sharing a common ancestor, both families must have diverged in what concerns the substrate and transport mechanism specificities (Fig. 2).

YEAST CARBOXYLATE PERMEASES

Yeasts are capable of utilising different organic acids, as sole carbon and energy sources (Barnett, 1976). Carboxylic acids are organic acids that dissociate partially in aqueous solutions, according to their pKa and the pH of the solution. Their utilization by yeasts, as sole carbon and energy sources, is dependent primordially on two factors: their transport across the plasma membrane, and the correct function of the pathways and enzymes involved in their metabolism. The undissociated form of the acid, being lipid-soluble, can cross the plasma membrane by simple diffusion (Casal *et al.*, 1995), an energy-independent (passive) transport mechanism. However, the dissociated form of the acid requires a specific transport system (Cassio *et al.*, 1987; Casal *et al.*, 1996). In this form, carboxylic acids can be transported by a secondary transport mechanism, energy-dependent (active), characterized by the movement of the dissociated form of the acid against its electrochemical gradient, and by the simultaneous movement of ions H^+ in favor of their concentration gradient and the electric potential of the membrane.

In yeast, the existence of a mediated transport system for carboxylic acids was described for the first time in *K. lactis* and involved the active transport of L-malic acid (Zmijewski and MacQuillan, 1975). Twenty years after, the *MAE1* gene was the first gene to be functional characterized as a carboxylate permease, in the yeast *S. pombe* (Grobler *et al.*, 1995). In *S. cerevisiae* only two carboxylate permeases have been described so far, Jen1 (Casal *et al.*, 1999) and Ady2 (Paiva *et al.*, 2004). Comparing to *K. lactis*, which encodes in its genome two transporters of the sialate:H^+ symport family (SHS), *S. cerevisiae* only has one member of that family, Jen1, even though both species encode similar number of porters in their genomes (206 for *K. lactis* and 218 for *S. cerevisiae*) (De Hertogh *et al.*, 2006). It seems like, throughout evolution, yeasts were selectively loosing porters, probably due to substrate availability that shaped the loss of irrelevant porters.

Jen1 is a lactate-pyruvate-acetate-propionate transporter induced by non-fermentable carbon sources, mainly in lactate and pyruvate (Cassio *et al.*, 1987) being glucose repressed (Andrade and Casal, 2001; Paiva *et al.*, 2002). An efficient lactate-proton symporter activity is dependent on some internal metabolite, derived from the acid metabolism (Casal *et al.*, 1995). In the Transport classification database Jen1 is classified as a lactate/pyruvate:H^+- symporter, 2.A.1.12.2, that belongs to the Sialate:H^+ Symporter (SHS) Family included in the Major Facilitator Superfamily. It has no homology to the MCP family members. The gene *JEN1* has 12 putative transmembrane domains and is regulated negatively by both DNA-binding repressor proteins Mig1 and Mig2, in the presence of glucose, and is fully derepressed by the transcriptional activator Cat8, in non-fermentative growth conditions (Bojunga and Entian, 1999). Besides transcriptional repression, glucose regulation also acts at the posttranscriptional level. Glucose addition, to *JEN1* expressing cells, promotes mRNA degradation through the action of a second *JEN1* transcript (starting at position +391), that acts as a glucose sensor, stimulating mRNA degradation, even in the presence of low glucose concentrations (Andrade and Casal, 2001; Andrade *et al.*, 2005). In addition to Mig1/2 and Cat8, other proteins are involved in the transcriptional regulation of *JEN1*. Lodi and co-workers identified the involvement of the Hap2/3/4/5 complex in the derepression of *JEN1*, in lactic acid-grown cells (Lodi *et al.*, 2002). Additionally, Snf1, a protein kinase that enables the release of glucose repressible genes from glucose catabolite repression (Carlson, 1999), is implicated in the control of *JEN1* transcription (Lodi *et al.*, 2002). Moreover, glucose also exerts a repression effect at the protein level promoting an efficient ubiquitylation of the permease and its subsequent endocytosis and sorting to the vacuole, for degradation (Paiva *et al.*, 2002; Paiva *et al.*, 2009).

ADY2/ATO1 encodes an acetate-proprionate-formate transporter present in non-fermentable carbon sources-grown cells (Casal *et al.*, 1996; Paiva *et al.*, 2004). This permease displays 6 putative transmembrane spanning domains and was previously classified in the TC system, as a possible or putative membrane transporter, belonging to the Gpr1/Fun34/YaaH membrane protein family (TC, 9.B.33). Additional roles have been attributed to this protein, especially its involvement in ammonia export (Palkova *et al.*, 2002) and in *asci* formation (Rabitsch *et al.*, 2001), which could have contributed to a dubious classification of this permease. *S. cerevisiae* has two homologs of *ADY2* encoded by *ATO2* and *ATO3*, presumably also involved in the export of ammonia

(Palkova *et al.*, 2002). These genes together with *ADY2* are homologs of the *GPR1* gene of *Y. lipolytica*, whose disruption affects acetic acid utilization (Tzschoppe *et al.*, 1999). Moreover, the AcpA, another homolog of Ady2, was recently described as an acetate transporter present in the hyphal fungus *Aspergillus nidullans*, induced by ethanol and ethyl acetate (Robellet *et al.*, 2008). Furthermore, two homologs of ScAdy2 have been identified in the archaea domain, in the acetate consuming *Methanosarcina acetivorans*. Together, these data reinforce the role of Ady2 as an acetate transporter in *S. cerevisiae*. Very recently, the previous TCDB annotation was updated and now Ady2 is classified as an acetate permease required for normal sporulation included in the YaaH family (TC, 2.A.96.1.4).

In a vast range of global expression analyses, *JEN1* and *ADY2* show a similar expression profile (Kal *et al.*, 1999; Boer *et al.*, 2003; Vachova *et al.*, 2004), which is also detected at the regulatory level. Similarly to *JEN1*, *ADY2* is repressed by glucose and is under the control of the transcription factor *CAT8*, which regulates catabolite derepression (Haurie *et al.*, 2001). The Adr1p, which is known to be involved in the metabolism of non-fermentable carbon sources, binds to the promoter of both genes (Young *et al.*, 2003) and *ADY2* also requires the presence of Snf1 to be fully derepressed (Tachibana *et al.*, 2005).

Homologs of Jen1 were encountered in several Hemiascomycetes genomes, namely, in *K. lactis* and in *C. albicans* (Lodi *et al.*, 2007). Interestingly, no Jen1 homolog is present in the genome of *C. glabrata.* This yeast lacks several porters (TC, 2.A) that can be found on other Hemiascomycetes genomes, probably due to its reductive evolution. *C. glabrata* encodes only 162 porters in comparison to the 218 porters found in *S. cerevisiae* or the 316 present in *Y. lipolytica* genome (De Hertogh *et al.*, 2006). In *K. lactis*, a Krebs positive yeast (Barnett and Kornberg, 1960) that can use malic acid and other Krebs cycle intermediates, as sole carbon and energy sources, two homologs of *S. cerevisiae JEN1* were identified, *KlJEN1* and *KlJEN2* (Lodi *et al.*, 2004). *KlJEN1* encodes a monocarboxylate permease, with 12 transmembrane domains, and its disruption impaired the mediated uptake of lactate, in lactic acid *K. lactis* derepressed cells. In turn, *KlJEN2* encodes a dicarboxylate permease shared by malic and succinic acids (Queiros *et al.*, 2007). In *C. albicans*, two homologs of *JEN1* were also functionally characterized (Soares-Silva *et al.*, 2004; Vieira *et al.*, 2009). *CaJEN1* encodes a lactate-pyruvate-proprionate proton symporter, Cat8 activated (Soares-Silva

et al., 2004) and its paralog *CaJEN2* encodes a malate-succinate transporter (Vieira *et al.*, 2009). Both permeases display 10 transmembrane domains (predicted by the TMHMM program) and are subjected to a tight glucose repression (≥0.1% of glucose). Expression profile studies, at the transcriptional and translational level, indicated that both genes are expressed in mono and dicarboxylic acids containing media, although the expression of *CaJEN1* tends to be higher in the presence of monocarboxylic acids, and *CaJEN2* expression increased in the presence of dicarboxylic acids. Additionally, both permeases are expressed after phagocytosis of *C. albicans* cells by human neutrophils and murine macrophages (Vieira *et al.*, 2009).

Jen1 homologs have transmembrane structures typical of secondary transporters with 9 to 12 predicted transmembrane spanning helices (TMS), indicating a conservation of this protein family throughout evolution (Lodi *et al.*, 2007). A phylogenetic tree containing Jen1 orthologs and paralogs, found in specific yeast species of biotechnological/biomedical significance, was drawn with the information resultant from the complete sequencing of those various yeast genomes (Fig. 3). Two main clusters can be identified, Jen1 and Jen2. All the proteins functionally characterized as monocarboxylate transporters are grouped in the Jen1 cluster, whereas the two dicarboxylate transporters, described so far, are represented in cluster Jen2. This approach enables the prediction of the functional roles of uncharacterized transporters. Nevertheless, all *Y. lipolytica* homologs constitute a separated group, of totally unknown function.

Regarding the *S. cerevisiae* acetate permease Ady2, eight homologs are encoded in *C. albicans* genome (*AFF1*, *AFF2*, *AFF3*, *AFF4*, *AFF5*, *AFF7*, *AFF10* and *AFF11*). Global analyses of the transcriptional response of *C. albicans* cells, infecting a murine macrophage cell line, indicated that these genes are overall significantly upregulated in this model of infection (Lorenz *et al.*, 2004). However, none of these genes was functionally characterized, till date. The studies performed on *C. albicans* Jen homologs indicated that they are not involved in the mediated transport of acetate in this pathogen. Both permeases were, in fact, induced in acetate containing media, but their disruption had no effect on growth rates of the mutant strains, nor in the active uptake of radioactive labelled acetate (Vieira, N. unpublished results).

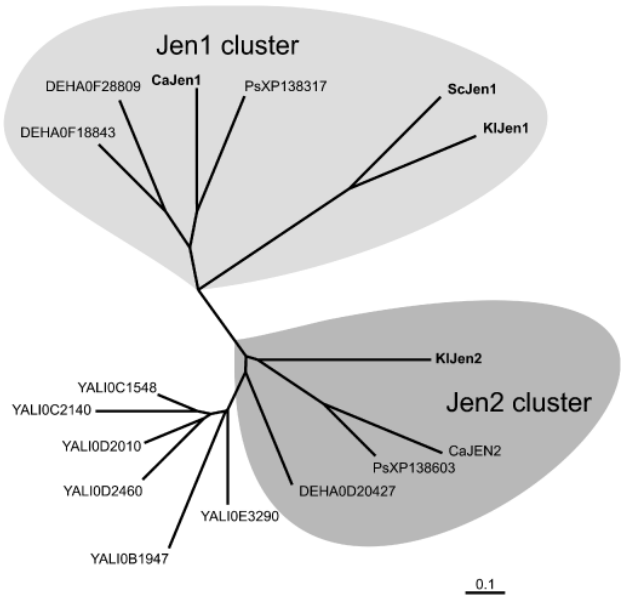

Figure 3 – Phylogenetic tree drawn with ScJen1 homologs. The sequences were obtained from SGD, CGD and Génolevures, except *Pichia stipitis* homologs which were obtained by homology search with BLASTP (http://www.ncbi.nml.nih.gov/blast/Blast.cgi). The multiple alignments were carried out at MUSCLE – Multiple Sequence Comparison by Log-Expectation (http://www.ebi.ac.uk/Tools/muscle/index.html). Tree construction was performed by Phylip suite (Felsenstein, 1989). Evolutionary distances estimations were elaborated by PROTDIST and the clustering was achieved by NEIGHBOUR programs. Finally TREEVIEW was used for the unrooted tree draw.

In *C. glabrata* there are two homologs of *S. cerevisiae ADY2* and one of *ATO3*. In this thesis work we aimed at functionally characterizing these homologs.

Using the information resultant from the complete sequence of different yeast and bacteria genomes, a phylogenetic tree is represented in figure 4. This tree presents the phylogenetic distances between some of the YaaH family members in: *S. cerevisiae*, *A. nidulans* and in *E. coli*; as well as the uncharacterized putative ScAdy2 homologs in *C. albicans* and in *C. glabrata*.

FPS1 A CHANNEL FOR CARBOXYLIC ACIDS

The first channel for carboxylic acids was identified in yeast in 2007. *S. cerevisiae FPS1* encoded a plasma membrane aquaglyceroporin involved in the facilitated diffusion of the undissociated form of acetic acid, to the interior of the cell (Mollapour and Piper, 2007). Initially, Fps1, member of the intrinsic protein family, MIP, (TC, 1.A.8.5.1), was described as a glycerol channel (Tamas *et al.*, 1999) involved in its efflux/influx in response to turgor changes (Tamas *et al.*, 2000), or even as a mediator for the influx of arsenite and antimonite (Wysocki *et al.*, 2001). However, a *S.*

cerevisiae strain lacking *FPS1*, presented a more resistant behaviour upon an acetic acid stress accompanied by a lower retention of this acid, in the interior of the cell, in comparison to the wild-type strain (Mollapour and Piper, 2007).

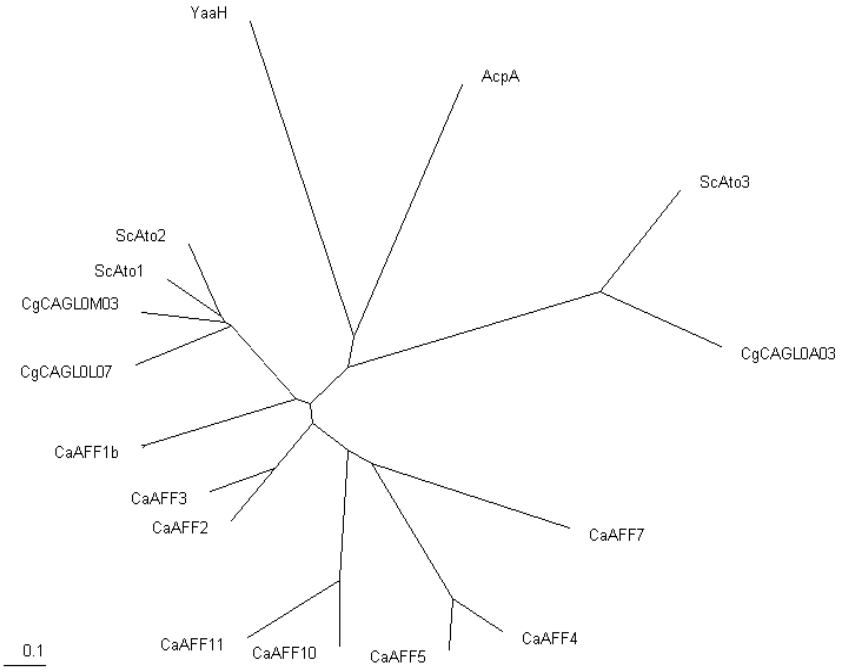

Figure 4 – Phylogenetic tree elaborated with YaaH family members and predicted ScAdy2 homologs in *C. albicans* and in *C. glabrata*. The tree construction followed the methodology described in figure 3.

Hog1 is a kinase that belongs to the HOG mitogen-activated protein (MAP) kinase cascade. In *S. cerevisiae* this pathway responds to a limited range of stress conditions, namely, high osmolarity stress, heat stress (Winkler *et al.*, 2002), intracellular methylglyoxal accumulation (Aguilera *et al.*, 2005; Maeta *et al.*, 2005), citric and acetic acid stress (Lawrence *et al.*, 2004; Mollapour and Piper, 2006) and oxidative stress (Bilsland *et al.*, 2004). The response to acetic acid seems to involve only Hog1 and not Slt2 (another stress activated MAP kinase in yeast) and to require the functionality of the Sln1/Ssk1 branch of the upstream signaling to the Pbs2, the MAPKK activator of Hog1, and not of the Sho1 branch (Mollapour and Piper, 2006). When cells are in contact with 80 or 100 mM of acetic acid, at pH 4.5, Hog1 is activated, conducting the Fps1 removal from the plasma membrane through Hog1-dependent phosphorylation and ubiquitylation of Fps1 and its consequent endocytosis and degradation in the vacuole (Mollapour and Piper, 2007). It seems that acetic acid may have to enter the cell in order to generate the Hog1 activatory signal, since at pH 6.8 the acetate activation is significantly slower (Mollapour and Piper, 2006).

C. glabrata encodes in its genome two putative homologs of the Sc*FPS1* gene, but their function remains unknown. On the other hand, *C. albicans* has no close homolog of *S. cerevisiae FPS1*. While performing a search for homologies in NCBI no hits were encountered in *C. albicans* genome. However, in the Candida Genome Database (http://www.candidagenome.org/) the best hit encoded an aquaporin, *AQY1*. Other predicted homologs were encountered in *Y. lipolytica*, *K. lactis* and *K. marxianus.*

The role of orthologs of the *S. cerevisiae* HOG MAPK pathway have been studied in the pathogenic yeasts *C. albicans* and *C. glabrata* (Smith *et al.*, 2004; Gregori *et al.*, 2007). Probably due to evolutionary impositions, CaHog1 seems to play more general roles in the yeast stress response, in comparison with ScHog1 (Smith *et al.*, 2004), since its disruption attenuates *C. albicans* virulence (Alonso-Monge *et al.*, 1999). CaSho1 also seems to have different roles from its osmosensing function in *S. cerevisiae* (Roman *et al.*, 2005). Moreover, *C. albicans* and *C. glabrata* seem relatively more resistant to the environmental osmotic stress than *S. cerevisiae* (Nikolaou *et al.*, 2009). As referred previously, *C. glabrata* is closely related to *S. cerevisiae* and supposedly most of the adaptive functions of this stress pathway should be conserved. However, the genome sequencing of the ATCC2001 strain indicated that this strain carries a truncated non-functional Cg*ssk2-1* allele, with no kinase domain, and that the *SSK22* homolog was absent in this strain genome. These alterations implied the debilitation of the Sln1 branch in the HOG MAPK pathway and consequently an enhanced sensitivity to acetate and methylglyoxal. Nevertheless, all the other *C. glabrata* backgrounds, and clinical isolates, display normal *SSK2* alleles and the Sln1 branch is completely functional. Overall, this stress pathway is very similar in *C. glabrata* and in *S. cerevisiae,* although it seems to vary in what concerns activation. For instance, in *S. cerevisiae* only acetic acid seems to trigger Hog1 activation and not other weak organic acids. In contrast, *C. glabrata* double mutants in the CgSsk2 and CgSho1 branch are extremely sensitive to weak organic acids, with a medium chain length, a phenotype not visualized in *S. cerevisiae*. The authors suggest that probably *S. cerevisiae* has an alternative mechanism that compensates the mutant phenotype regarding acid sensitivity (Gregori *et al.*, 2007).

Elucidation of the role of ScFps1, and of its putative orthologs will help to understand the overall carboxylic acid metabolism in yeasts and to clarify the differences encountered in the HOG MAPK pathway in these *Hemiascomycetes*.

METABOLISM OF MONO AND DICARBOXYLIC ACIDS IN YEASTS

Yeast species are able to utilize non-fermentable substrates as the sole carbon and energy sources, under certain physiological conditions (Barnett, 1976). The utilization of these alternative carbon sources, namely, glycerol, ethanol, and short-chain carboxylic acids, depends on the transport of these nutrients into the cell, as described above, and on the existence of adequate metabolic processes for degradation. Some of these substrates require the presence of specific transport systems while others can freely diffuse through the plasma membrane, like ethanol. For instance, the extracellular ethanol produced after glucose fermentation can cross the plasma membrane and be consumed in a secondary respiratory growth phase (Beck and von Meyenburg, 1968).

Monosaccharides, such as glucose and fructose, are among the preferred carbon sources in yeasts. In the presence of these monosaccharides the metabolic pathways involved in the degradation of alternative carbon sources are repressed (Gancedo, 1998; Carlson, 1999). *S. cerevisiae* usually proliferates in glucose rich niches in contrast to the human pathogens, *C. albicans* and *C. glabrata* that colonize low sugar environments, such as the gastrointestinal tract and mucous membranes. The glucose sensing pathways seems to be quite similar between *S. cerevisiae* and *C. albicans*, differing, however, on some aspects, such as the range of glucose concentration to which both yeast respond (Sexton *et al.*, 2007). Several studies have been performed in *S. cerevisiae* and in *C. albicans* to unravel the pathways controlling alternative carbon metabolism, but little is known about *C. glabrata*. Considering that *C. glabrata* is so closely related to *S. cerevisiae* and that the more distant yeast, *C. albicans*, shares similar patterns of behaviour with both yeasts, one can assume that *C. glabrata* behaviour shouldn't be much different. We will further discuss some aspects on the metabolic pathways involved in the metabolism of alternative carbon sources, mainly in *S. cerevisiae* and *C. albicans*.

The metabolism of non-fermentable carbon sources, namely of carboxylic acids, relies on the operation of the Krebs cycle (or tricarboxylic cycle–TCA cycle), gluconeogenesis and respiratory chain, requiring in these manner aerobic conditions (Gancedo, 1989). It is also dependent on anaplerotic pathways, such as the glyoxylate cycle and pyruvate carboxylase (Gancedo, 1998) that replenish the biosynthetic intermediaries of the Krebs cycle. The di- and tricarboxylic acids that belong to the

Krebs cycle can enter directly into this pathway, while monocarboxylates need to be previously converted to acetyl-coenzyme A (acetyl-coA). New carbon units entry this pathway through acetyl-CoA, that can derive either from pyruvate, through glycolysis, or from the oxidation of fatty acids. Since in eukaryotic cells glycolysis occurs in the cytoplasm, and the Krebs cycle, and all the subsequent reactions, in the mitochondria, pyruvate must first be transported into this organelle. One of the first mitochondrial transporters to be studied was the pyruvate transporter. Its existence was primarily demonstrated in mammals, by the specific inhibition of pyruvate uptake by a cyano-4-hydroxycinnamate (Halestrap, 1976). In yeast, the search for the pyruvate transporter has been extensive and till date, unfruitful. Its existence was first demonstrated in isolated mitochondria by Briquet (Briquet, 1977). A sequential analysis of strains mutated in all the mitochondrial carriers, with unknown function, identified the *YIL006w* gene as a putative pyruvate transporter. A mutant deleted in this gene presented no mitochondrial pyruvate uptake and was not sensitive to cyano-4-hydroxycinnamate, which suggested its role as a pyruvate transporter (Hildyard and Halestrap, 2003). Ultimately, the reconstitution of the purified recombinant protein into phospholipid vesicles indicated that this gene does not code for a pyruvate transporter (Todisco *et al.*, 2006). In fact, it was shown to transport NAD^+, as a uniport, as well as other nucleotides in exchange for NAD^+ (Todisco *et al.*, 2006).

The connective link, between glycolysis and the Krebs cycle, is established by the pyruvate dehydrogenase complex (PDH) that catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA. In *S. cerevisiae* this conversion may also proceed indirectly via the so-called PDH bypass, which requires the sequential action of pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-coenzyme A synthetase (Van den Berg and Steensma, 1995). The first step of the Krebs cycle (or tricarboxylic acid cycle – TCA cycle) is accomplished by the condensation of acetyl-CoA with oxaloacetate to form a six carbon compound, citrate. Several subsequent reactions will oxidize citrate and the respective products of each reaction, until oxaloacetate (four carbons) is regenerated. Two successive decarboxylations occur in each turn of this cycle, as well as the production of three NADH molecules, one of GTP and one of $FADH_2$. In this manner, the production of biosynthetic intermediates by the Krebs cycle can not be supported by acetate-based growth, since for each two carbon acetate group that enters the cycle, two CO_2 are produced, and there is no net

assimilation of carbon for the production of biosynthetic intermediates. Therefore, growth on acetic acid or fatty-acids requires the operation of alternative reactions to provide the cell with C4 and C3 intermediates, essential for the biosynthesis of cellular components. This is accomplished by the glyoxylate cycle, a modification of the Krebs cycle that bypasses the CO_2-producing reactions, diverting part of the flux of carbon molecules at isocitrate. This cycle is present in plants, fungi and bacteria and involves two oxidative steps, associated to electron transport, providing some energy. However, the core of cellular energy is derived from the concomitant oxidation of other acetate molecules by the Krebs cycle (Cozzone, 1998).

The glyoxylate cycle functions, in this way, as an anaplerotic pathway, allowing the reposition of Krebs cycle intermediaries with four carbons (like oxaloacetate, C4), from C2 compounds (like acetate) (Voet, 2004). The glyoxylate cycle consists of five enzymatic activities, two are unique to the cycle: isocitrate lyase (ICL1, EC 4.1.3.1) and malate synthase (MLS1, EC 2.3.3.9); and the other three: citrate synthase (CIT, EC 2.3.3.1), aconitase (ACO, EC 4.2.1.3) and malate dehydrogenase (MDH, EC 1.1.1.37) are shared with the Krebs cycle (tricarboxylic acid cycle –TCA), and are often carried out by isoenzymes. Whenever glucose becomes available in the culture medium, the glyoxylate bypass is turned off due to catabolite repression (Voet, 2004).

In the *S. cerevisiae* genome two genes code for isocitrate lyase (*YER065c–ICL1* and *YPR006c–ICL2*, a mitochondrial methylisocitrate lyase) (Fernandez *et al.*, 1992; Luttik *et al.*, 2000), for malate synthase (*YNL117w–MLS1* and *YIR031c–DAL7*, a malate synthase involved in allantoin degradation) (Hartig *et al.*, 1992; Fernandez *et al.*, 1993) and for aconitase (*YLR304c–ACO1* and *YJL200c–ACO2* both mitochondrial) (Gangloff *et al.*, 1990; van den Berg *et al.*, 1998). There are three genes coding for citrate synthases (*YNR001c–CIT1*, cytoplasmic, *YCR005c–CIT2*, a peroxisomal isoenzyme and *YPR001w–CIT3*, a mitochondrial isoenzyme) (Kim *et al.*, 1986; Jia *et al.*, 1997), and for malate dehydrogenase (*YKL085w–MDH1*, mitochondrial, *YOL126c–MDH2*, cytoplasmic and *YDL078c–MDH3*, peroxisomal) (McAlister-Henn and Thompson, 1987; Minard and McAlister-Henn, 1991; Steffan and McAlister-Henn, 1992).

In *C. albicans*, only one gene encodes Icl1 (*orf19.6844*), Mls1 (*orf19.4833*) (Lorenz and Fink, 2001) and citrate synthase (*orf19.4393*) (Doedt *et al.*, 2004). Three genes code for malate dehydrogenase: *MDH1* (*orf19.7481*) that encodes the mitochondrial isoform, *MDH1-1* (*orf19.4602*, uncharacterized), and *MDH1-3*

(*orf19.5223,* uncharacterized) (Murad *et al.*, 2001; Lorenz *et al.*, 2004) and two for aconitase (*orf19.6385–ACO1* and *orf19.6632–ACO2*, uncharacterized) (Pitarch *et al.*, 2001; Yin *et al.*, 2004).

In *C. glabrata* two homologs of isocitrate lyase can be identified (*CAGL0J03058g–ICL1* and *CAGL0L09273g–ICL2*), one of malate synthase (*CAGL0L03982g–MLS1*), three homologs of citrate synthase (*CAGL0H03993g–CIT1*, *CAGL0B03663g–CIT1* and *CAGL0L09086g–CIT3*), three homologs of malate dehydrogenase (*CAGL0L05236g–MDH1*, *CAGL0E01705g–MDH2* and *CAGL0L06798g–MDH3*) and of aconitase (*CAGL0D06424g–ACO1*, *CAGL0F02431g–ACO2* and *CAGL0H00484g–IBA57*).

Several studies have been performed in different fungi indicating that the key enzymes of the glyoxylate cycle, Icl1 and Mls1, are usually compartmentalized in the peroxisomes, and the enzymes common to both Krebs and glyoxylate cycle exist as isoenzymes, with distinct spatial localization, either cytosolic or mitochondrial (Hikida *et al.*, 1991; Tanaka, 1993; Valenciano *et al.*, 1996; Titorenko *et al.*, 1998; Maeting *et al.*, 1999). Nevertheless, in *S. cerevisiae*, Icl1 is a cytosolic enzyme (McCammon *et al.*, 1990) and Mls1 is localized either to the peroxisome or to the cytosol, according to the growth conditions (Taylor *et al.*, 1996). For instance, during growth on oleic acid, Mls1 localizes to the peroxisome (Kunze *et al.*, 2002).

The glyoxylate cycle enzymes seem to play a crucial role in the virulence of several microorganisms, namely in: *C. albicans*; *Mycobacterium tuberculosis,* a bacterial pathogen of mammals (McKinney *et al.*, 2000); *Leptoshaeria maculans*, *Magnaporthe grisae* and *Stagonospora nodorum*, plant-pathogenic fungi (Idnurm and Howlett, 2002; Wang *et al.*, 2003; Solomon *et al.*, 2004); and *Rhosococcus fascians*, a bacterial plant-pathogen(Vereecke *et al.*, 2002). Moreover, it has been suggested that *C. albicans* relies on acetyl-CoA derived from lipid catabolism and from other simple carbon sources such as acetate and lactate, for survival in its human host (Lorenz and Fink, 2002; Boshoff and Barry, 2005; Piekarska *et al.*, 2006; Vieira *et al.*, 2009). In this manner, the glyoxylate cycle is extremely important for the survival of animal and plant pathogens, within its hosts, where carbon sources, such as glucose, are often scarce. In agreement with this, the ability to efficiently utilize non-fermentable carbon sources was reported as a virulence attribute, supported by the very similar transcriptional profile of

C. albicans upon phagocytosis or in carbon starvation conditions (Ramirez and Lorenz, 2007).

Another pathway extremely important for the survival of fungi, while growing in the presence of non-fermentable carbon sources, is gluconeogenesis. This pathway allows the formation of monophosphate hexoses, essential for biosynthetic processes, enabling some fermentative yeasts to utilize the principal end-product of glucose fermentation, ethanol, and to respire organic acids, other end products of fermentation (Gancedo, 1989). In gluconeogenesis, glucose is synthesized, ATP is consumed and $NADH^+$ oxidized. The majority of the enzymes involved in this pathway are shared with glycolysis, with the exception of: pyruvate carboxylase (PYC, EC 6.4.1.1) that converts pyruvate to oxaloacetate, phosphoenolpyruvate carboxykinase (PCK, EC 4.1.1.49) that converts oxaloacetate to phosphoenolpyruvate; and fructose-1,6-biphosphatase (FBP, EC 3.1.3.11) that catalyzes the hydrolysis of fructose-1,6-biphosphate to fructose-6-phosphate. Gluconeogenesis and glycolysis are tightly regulated, so that when glycolysis is turned on, gluconeogenesis is turned off and vice versa (Voet, 2004).

In *S. cerevisiae*, pyruvate carboxylase is localized to the cytoplasm, instead of being mitochondrial, like in many higher eukaryotes (Haarasilta and Taskinen, 1977; van Urk *et al.*, 1989; Rohde *et al.*, 1991). *S. cerevisiae* has two genes encoding for pyruvate carboxylase (*YGL062w–PYC1* and *YBR218c–PYC2*) (Walker *et al.*, 1991), and each one encodes an apoenzyme activated by biotin; one gene codes for phosphoenolpyruvate carboxykinase (YKR097w–*PCK1*) (Valdes-Hevia *et al.*, 1989), also cytosolic, and one gene encodes fructose-1,6-biphosphatase (YLR377c–*FBP1*) (Entian *et al.*, 1988).

In. *C. albicans* only one gene is identified as a putative pyruvate carboxylase (*orf19.789–PYC2*) (Chauhan *et al.*, 2003), as a phosphoenolpyruvate carboxykinase (*orf19.7514–PCK1*) (Leuker *et al.*, 1997) and as a fructose-1,6-biphosphatase (*orf19.6178–FBP1*) (De la Rosa *et al.*, 2000).

In *C. glabrata* genome two genes are encoding pyruvate carboxylase putative homologs (*CAGL0F06941g–PYC1* and *CAGL0K06787g–PYC2*), one encoding a phosphoenolpyruvate carboxykinase homolog (*CAGL0H06633g–PCK1*) and two coding for fructose-1,6-biphosphatase homologs (*CAGL0H04939g* and *CAGL0I04048g*).

In *C. albicans* gluconeogenesis, the glyoxylate cycle and fatty acid β-oxidation pathways are required for its full virulence. Several studies on carbon metabolism, pointed to the existence of a more integrated metabolic behaviour for carbon use in this pathogen. Deletions in several genes involved either in gluconeogenesis, fatty acid β-oxidation or in the glyoxylate cycle have wider repercussions than the deletion of their orthologs in *S. cerevisiae*. For instance, a strain disrupted in a key component of fatty acid β-oxidation, *FOX2*, displayed inability to utilize several fatty acids, as in *S. cerevisiae*, but also of other carbon sources whose assimilation is independent of β-oxidation, namely ethanol, and presented poor growth on citric acid and glycerol. A mutant lacking the *ICL1* also presented more severe carbon utilization phenotypes. It was unable to grow on fatty acids, ethanol and oleate as predicted, but also on citrate and glycerol. Furthermore, a mutant strain disrupted in the gluconeogenesis gene *FBP1*, failed to utilize any of the non-fermentable carbon sources, as expected (Ramirez and Lorenz, 2007).

Alternative carbon utilization regulatory networks were also examined, and indicated divergences between the regulatory proteins in *S. cerevisiae* and *C. albicans*. A homolog of *MIG1*, a transcriptional regulator involved in glucose repression (Carlson, 1999; Schuller, 2003) was identified in *C. albicans* and exerts a similar function (Murad *et al.*, 2001). However, some genes seem to be differentially regulated in *S. cerevisiae* and *C. albicans*, namely *FBP1*, where no significant differences are found in expression based on carbon sources (M. Lorenz unpublished data). Both regulators, *CAT8* and *ADR1* involved in the derepression of genes involved in non-fermentable carbon sources utilization, namely in gluconeogenesis and in the glyoxylate cycle (*CAT8*) or ethanol and acetate (*ADR1*), also have homologs in *C. albicans*. One homolog of *CAT8* was identified (Soares-Silva *et al.*, 2004) and two homologs of *ADR1* (Ramirez and Lorenz, 2007). Nevertheless, *C albicans* mutant strains disrupted in *CAT8* or *ADR1* have no obvious phenotypes, indicating that these genes are not regulating key enzymes involved in these pathways. Additionally, in *C. albicans*, there are no homologs of the regulators *OAF1* and *PIP2*, involved in the derepression of genes related to fatty acids and peroxisome biogenesis, only one homolog of A. nidulans Far proteins, *CTF1* (Ramirez and Lorenz, 2009).

In this manner, *C. albicans* must display a more connected network, where a small defect could extrapolate to the downregulation of the whole system (Ramirez and

Lorenz, 2007). Probably, *C. albicans* developed a highly integrated metabolic network, to meet the nutritional needs of a pathogen, enabling the concomitant assimilation of different carbon sources during infection. In *S. cerevisiae* this is not favoured, there is a switch from fermentative metabolism to respiratory metabolism, according to the nutrient availability, and that switch is tightly regulated. It is possible that a pathogen like *C. albicans* cannot afford the period of metabolic inactivity between this switch, while facing the host immunological pressure (Ramirez and Lorenz, 2007), and that it needs to assimilate whichever carbon sources it encounters, within the different body sites it is colonizing.

A BLASTP homology search performed in *C. glabrata* genome (www.genolevures.org) identified several genes homologs of the previously described carbon metabolism regulators: Mig1 (*CAGL0A01628g*), Cat8 (*CAGL0M03025g*), Adr1 (*CAGL0E04884g*) and two homologs of Oaf1 and Pip2 (*CAGL0M12298g* and *CAGL0J07150g*). Homologs were also identified for the key component of fatty acid β-oxidation, Fox2 (*CAGL0L02167g*) and, as referred previously, for Icl1, (*CAGL0J03058g–ICL1* and *CAGL0L09273g–ICL2*). Overall, the key components of carbon metabolism are conserved in these *Hemiascomycetes*, as expected. Nevertheless, differences are always found, for instance, in respect to sugar metabolism, it is known that the majority of *C. glabrata* strains can only assimilate glucose and trehalose, not being able to ferment or assimilate glucose-repressible sugars, namely sucrose, maltose or galactose, such as *S. cerevisiae* and *C. albicans* (Kreger-vav Rij, 1984; Hazen, 1995; Barnett, 2000). Moreover, *C. glabrata* contains in its genome genes encoding Oaf1 and Pip2 that are lacking in *C. albicans* genome. Further studies should be developed in order to clarify the level of divergence between carbon metabolism pathways in *C. glabrata*, in *C. albicans* and in *S. cerevisiae.* If *C. glabrata* presents a more integrated metabolic network, as a human pathogen, or if it relies on the metabolic switching such as *S. cerevisiae*, is yet to be unraveled.

Lactic acid metabolism in yeasts

Lactic acid is a monocarboxylic acid that when present in the culture medium in the undissociated form, at low pH, can cross the plasma membrane by diffusion. However, at pH above 5, lactic acid is predominantly present in its anionic form and its

assimilations depends on transporter-mediated uptake by ScJen1, in *S. cerevisiae,* or by CaJen1, in *C. albicans*.

The metabolism of lactic acid involves its oxidization to pyruvate. *S. cerevisiae* cells possess a D-lactate cytochrome c oxireductase (D-LCR; EC 1.1.2.4) and a L-lactate cytochrome c oxireductase, or the cytochrome b2 (L-LCR; EC 1.1.2.3) for D or L- lactate, respectively (Labeyrie and Slonimski, 1964) (Fig. 5). Both enzymes are localized in the mitochondria. D-lactate cytochrome c oxireductase is a flavoprotein localized in the internal mitochondria membrane (Nygaard, 1961), whereas L-lactate cytochrome c oxireductase is a soluble protein located in the mitochondrial intermembrane space (Daum *et al.*, 1982). In *S. cerevisiae DLD1*, *DLD2* and *DLD3* genes encode the D-LCR enzymes. The first codes for the D-lactate dehydrogenase located in the inner mitochondrial membrane, whereas the second codes for the D-lactate dehydrogenase that localizes to the mitochondrial matrix and the third for a D-lactate dehydrogenase, whose expression is stimulated by mitochondrial damage (Lodi and Ferrero, 1993; Chelstowska *et al.*, 1999). *CYB2* encodes the L-LCR enzyme, Cytochrome b2 (L-lactate cytochrome-c oxidoreductase) (Guiard, 1985) that is the only active enzyme in the conversion of external lactate (L-lactate) into pyruvate. Expression of both *DLD1* and *CYB2* genes is transcriptionally repressed by glucose, by anaerobic conditions and induced by non-fermentable substrates, such as lactate (Guiard, 1985). Homologs of *DLD1/2* and *CYB2* have been identified in *C. albicans*: two homologs of *DLD1* (*orf19.5805* and *orf19.6043*, uncharacterized) (Tsong *et al.*, 2003; Maglott *et al.*, 2007); *DLD2* (*orf19.6755*, uncharacterized) (Maglott *et al.*, 2007); *CYB2* (*orf19.5000*, uncharacterized) (Lan *et al.*, 2004); and in *C. glabrata*: *DLD1* (*CAGL0I05148g*); *DLD2* (*CAGL0J06314g*); and *CYB2* (*CAGL0K10736g*).

The pyruvate is then decarboxylated to acetyl-CoA by the action of the pyruvate dehydrogenase (PDH) complex. In *S. cerevisiae* and in *C. albicans* the PDH complex is localized inside the mitochondria. This complex includes multiple copies of subunits possessing three catalytic activities: pyruvate dehydrogenase (E1), which includes subunits E1α and E1β, dihydrolipoamide S-acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) (Kresze and Ronft, 1981; Reed, 1987). These three enzymatic subunits catalyze the conversion of pyruvate to acetyl-CoA (Pyruvate + CoA + $NAD^+ \rightarrow$ acetyl-CoA + NADH + H^+). One last component was identified, the component X, which plays a structural role in this complex, binding the E3 component

to the E2 component (De Marcucci and Lindsay, 1985). The different genes encoding the three subunits of this complex have been identified, both in *S. cerevisiae* and in *C. albicans: ScPDA1* and *CaPDA1* that code for the E1α component of pyruvate dehydrogenase (Behal *et al.*, 1989; Steensma *et al.*, 1990; Copping *et al.*, 2005); *ScPDB1* and *CaPDB1* that code for the E1β subunit (Miran *et al.*, 1993; Copping *et al.*, 2005); *ScLAT1* and *CaLAT1* encode the E2, dihydrolipoamide S-acetyltransferase (Niu *et al.*, 1988; Chibana *et al.*, 2005); *ScLPD1* and *CaLPD1* code for the dihydrolipoamide dehydrogenase, E3 (Douglas and Dawes, 1987; Ross *et al.*, 1988; Cheng *et al.*, 2003); and finally, *ScPDX1* and *CaPDX1* that code for the X component (Behal *et al.*, 1989; Lawson *et al.*, 1991; Vellucci *et al.*, 2007).

In the genome of *C. glabrata* homologs of all the genes indicated above were identified: *PDA1* (*CAGL0L12078g*); *PDB1* (*CAGL0K06831g*); *LAT1* (*CAGL0J10186g*); *LPD1* (*CAGL0F01947g*); and *PDX1* (*CAGL0G09361g*). As mentioned before, *C. glabrata* has no homolog of the *S. cerevisiae* monocarboxylate transporter, Jen1. Transport studies with radioactive labelled lactic acid, were performed on the ATCC2001 strain, and confirmed the lack of a saturable (second order mechanism) transport system for the uptake of this acid in *C. glabrata* (Vieira, N. unpublished results). Additionally, physiological studies indicated that this strain grows extremely slow in the presence of lactic acid, as the sole carbon and energy source. Probably the limiting step is, in fact, the lack of efficient mechanisms of transport for the acid into the intracellular space, since *C. glabrata* seems to encode in its genome all the components involved in the metabolism of this substrate.

Acetic acid metabolism in yeasts

When acetate is present, as the sole carbon and energy source, it is primarily converted to acetyl-CoA by the enzyme acetyl-CoA synthetase (ACS, EC 6.2.1.1). Acetyl-CoA will then enter the glyoxylate cycle bypassing the CO_2 loss reactions of the Krebs cycle, and in this manner, becoming usable for replenishing intermediates of the Krebs cycle (Fig. 5). There are two isoforms of acetyl-CoA synthetase in *S. cerevisiae* (Satyanarayana *et al.*, 1974), Acs1 (*YAL054C*) and Acs2 (*YLR153C*). The aerobic form is encoded by the gene *ACS1* (De Virgilio *et al.*, 1992), and the non-aerobic form is encoded by the gene *ACS2* (Van den Berg and Steensma, 1995), presenting lower affinity for acetate. In this yeast, *ACS1* seems to be only required for growth on acetate,

but not on ethanol or glucose (De Virgilio *et al.*, 1992). In contrast, *acs2* mutants grow well in the presence of acetate and ethanol, but are unviable in the presence of glucose (Van den Berg and Steensma, 1995).

In *C. albicans* two homologs have been identified, Acs1 (*orf19.1743*, uncharacterized) and Acs2 (*orf19.1064*). Carman and co-workers demonstrated that *ACS2* is essential for growth, not only on glucose, but also on several other non-fermentable carbon sources, such as acetate and ethanol. In this manner, the *acs2* mutants present more severe growth defects, being only able to metabolize fatty acids and glycerol, confirming the divergence between these yeasts metabolic pathways (Piekarska *et al.*, 2006; Ramirez and Lorenz, 2007; Zhou and Lorenz, 2008). Conversely, the *ACS1* gene doesn't seem to play an essential role in the metabolism of alternative carbon courses in *C. albicans*, since *acs1* mutant presents only mild growth defects. However, *ACS1* is strongly induced in the presence of fatty acids (Ramirez and Lorenz, 2007).

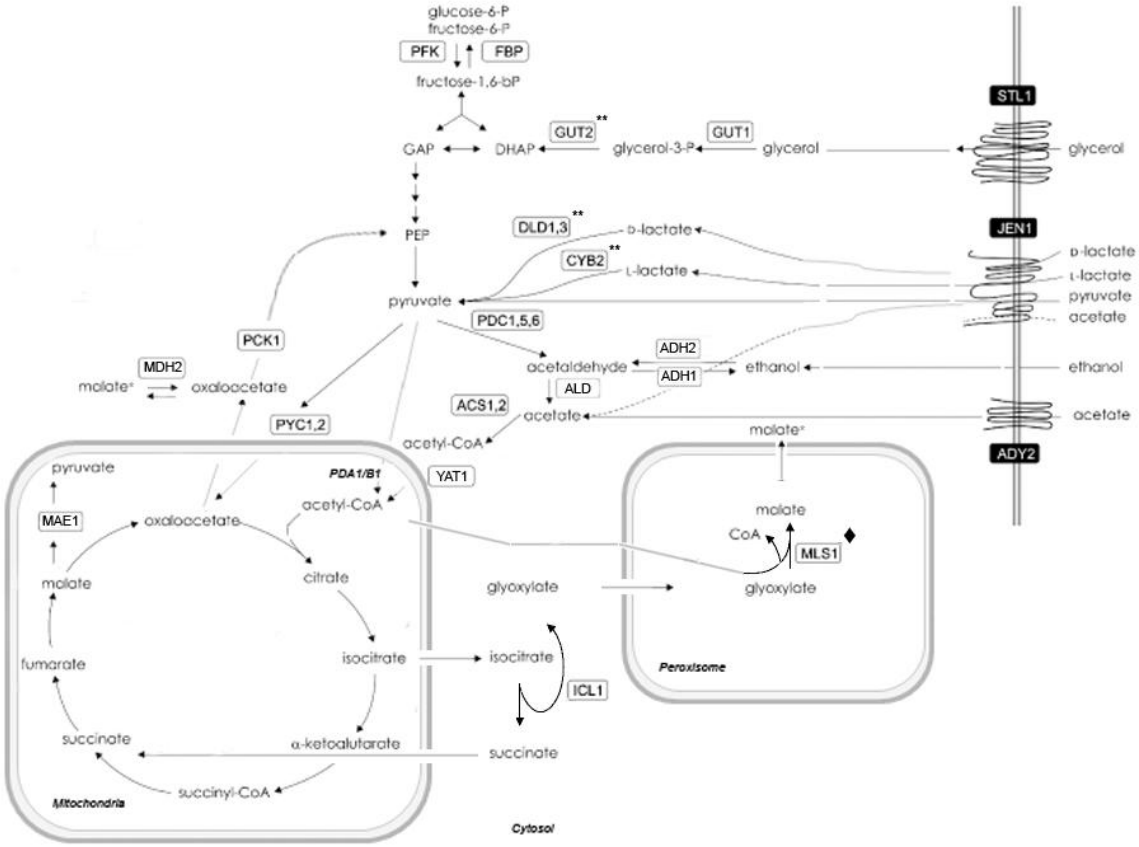

Figure 5 – Schematic drawing of metabolic routes representing the oxidative and fermentable carbon metabolism in *S. cerevisiae*. The figure shows the Glycerol proton transporter, Stl1 (Glycerol enters the glycolytic pathway at the level of triose phosphate (dihydroxyacetone phosphate; DHAP), which is converted into pyruvate to some degree but also allows production

of glucose-6-phosphate), the lactate transporter, Jen1 and the acetate transporter, Ady2. Some genes involved in the utilization of non-fermentable carbon sources are represented: *GUT1* – Glycerol kinase; *GUT2* – Mitochondrial glycerol-3-phosphate dehydrogenase; *FBP1* – Fructose-1,6-bisphosphatase; *PFK1,2* – phosphofructokinase; *PCK1* – Phosphoenolpyruvate carboxykinase; *PYC1/2* – Pyruvate carboxylase; *DLD1,3* – D-lactate dehydrogenase; *CYB2* – L-lactate cytochrome-c oxidoreductase; *ADH1,2* – Alcohol dehydrogenase; *ALD* – Acetaldehyde dehydrogenase; *PDC1,5,6* – Pyruvate decarboxylase; *PDA/B* – Pyruvate dehydrogenase complex; *ACS1/ACS2* – Acetyl-coA synthetase; *YAT1* – Carnitine acetyltransferase; *ICL1* – Isocitrate lyase; *MLS1* – Malate synthase; *MDH2* – Cytoplasmic Malate dehydrogenase. ** Mitochondrial localization; ♦ Malate synthase (MLS1) can be both peroxisomal or cytosolic, according to the growth conditions. Adapted from (Casal *et al.*, 2008).

C. glabrata is able to grow in minimal media, supplemented with acetic acid, as the sole carbon and energy source. Studies with radioactive labelled acetic acid demonstrated that *C. glabrata* presents a mediated transport system for the uptake of this acid, probably encoded by one, or more, of the two homologs of ScAdy2 (Vieira, N., unpublished). In this pathogenic yeast genome two predicted homologs of acetyl-CoA synthetases have also been annotated: Acs1 (*CAGL0L00649g*), Acs2 (*CAGL0B02717g*).

Succinic acid metabolism in yeasts

Succinic acid is a dicarboxylic acid that can enter directly into the Krebs cycle, where it originates fumarate by the action of succinate dehydrogenase (SDH, EC 1.3.5.1). Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate with concomitant reduction of FAD to $FADH_2$. Succinate dehydrogenase is an integral membrane protein, tightly associated with the inner mitochondrial membrane that is actually part of the succinate-coenzyme Q reductase (complex II), of the electron transport chain. This enzymatic complex is constituted by two catalytic and two structural subunits, all encoded by nuclear genes (Lemire and Oyedotun, 2002).

In *S. cerevisiae*, all the genes coding for the components of this enzymatic complex have been identified and characterized. The flavoprotein subunit responsible for the oxidation of succinate to fumarate is encoded by the paralog genes *SDH1* (*YKL148C*) and *SDH1b* (*YJL045W*), but only *SDH1* is essential for growth in respiratory substrates (Chapman *et al.*, 1992; Robinson and Lemire, 1992). *SDH2* (*YLL041C*) gene codes for the iron-sulphur protein subunit, which contains three Fe-S centers, and that together with the Sdh1 constitute the catalytic center of this complex, guiding the electron flow from $FADH_2$ to the Fe-S centers and ultimately to ubiquinone

(Gould *et al.*, 1989; Lombardo *et al.*, 1990). *SDH3* (*YKL141W*) encodes the cytochrome b560 subunit of the respiratory complex II that anchors the protein complex to the inner mitochondrial membrane. Cytochrome b is a mono-haem transmembrane protein with three transmembrane domains, that presents conserved histidines involved in the binding of the haem group (Abraham *et al.*, 1994; Daignan-Fornier *et al.*, 1994; Oyedotun and Lemire, 1999). There is also one uncharacterized non essential ORF in *S. cerevisiae* genome (*YMR118c*) that codes for a protein of unknown function with similarity to the cytochrome b subunit (Giaever *et al.*, 2002; Balakrishnan *et al.*, 2005). *SDH4* (*YDR178W*) codes for the second small hydrophobic peptide involved in the anchoring of the protein complex to the internal mitochondrial membrane (Bullis and Lemire, 1994; Oyedotun and Lemire, 1997). An additional ORF (*YLR164W*) is present in *S. cerevisiae* genome coding for a protein with similarities to *SDH4* (Kerscher *et al.*, 2000). Deletion of *SDH3* leads to more severe growth defects than *SDH4* deletion, indicating a more important role for *SDH3* in the respiratory growth (Daignan-Fornier *et al.*, 1994). Recently, *sdh3* mutant strain was reported to present growth defects, both in aerobic and anaerobic conditions, indicating an additional role for *SDH3* besides its involvement in respiration (Cimini *et al.*, 2009). Finally *SDH5* or *EMI5 (YOL071W)* is a subunit required for the attachment of the FAD cofactor to Sdh1 (Deutschbauer *et al.*, 2002; Hao *et al.*, 2009). All the above mentioned genes are repressed by glucose and derepressed in the presence of respiratory substrates. Loss of function of this complex abolishes cell growth in the presence of any carbon source (Saliola *et al.*, 2004).

In *C. albicans* genome two homologs of *SDH1* were annotated: *SDH1* (*orf19.440*, uncharacterized) that codes for a protein described as a mitochondrial succinate dehydrogenase, induced in macrophages and in pseudohyphae (Singh *et al.*, 2005) and *SDH12* (*orf19.2871*) encoding a protein with similarity to the flavoprotein subunit of the Sdh complex that is soluble in hyphae, downregulated by Efg1 (transcriptional repressor) and after contact with macrophages (Keller *et al.*, 1997; Doedt *et al.*, 2004; Hernandez *et al.*, 2004; Fernandez-Arenas *et al.*, 2007). An *SDH2* (*orf19.637*) homolog was also identified and codes for a Fe-S subunit of succinate dehydrogenase, that localizes to the surface of yeast cells, but not on hyphae (Urban *et al.*, 2003; Lan *et al.*, 2004). Two homologs of *SDH4* have been identified in the Candida Genome Database: one is classified as *SDH4* (*orf19.4022*, uncharacterized), coding for a putative membrane subunit of the succinate dehydrogenase complex,

transcriptionally regulated by iron (Lan *et al.*, 2004). The other one is only referred as a predicted ortholog of *S. cerevisiae SDH4* (*orf19.4468*). Another ORF (*orf19.1480*) was identified in *C. albicans* genome as a putative ortholog of the *S. cerevisiae* gene (*YMR118c*), with similarities to cytochrome b (*SDH3*),downregulated by Efg1 (Doedt *et al.*, 2004; Hromatka *et al.*, 2005; Maglott *et al.*, 2007). Finally, one homolog of *SDH5/EMI5* was identified in *C. albicans* genome (*orf19.4727*), but remains so far uncharacterized (Maglott *et al.*, 2007).

In *C. glabrata*, all the components of this enzymatic complex have predicted homologs. Through evolution this yeast must have lost one of the *SDH1/b* paralogs since only one gene (*CAGL0J00847g)* is homologous to both. There are two homologs of the iron-sulphur subunit *SDH2* (*CAGL0C03223g* and *CAGL0E03850g)*, and one homolog of *SDH3* and *YMR118c*, (*CAGL0D01958g)*. One of the anchor proteins encoded by *SDH4*, and its paralog *YLR164w*, are represented by *CAGL0F05863g*, and finally one homolog of *SDH5* is annotated, *CAGL0I08085g*. This yeast has the ability to grow in the presence of succinic acid, as the sole carbon and energy source, but lacks homologs to the Jen family members, more specifically of the dicarboxylate permease Jen2.

Regarding the synthesis of succinic acid in *S. cerevisiae*, it can occur through two pathways: the Krebs cycle and the glyoxylate cycle. In both cases, acetyl-CoA is primarily converted to citrate by the action of citrate synthase. The tertiary alcohol of citrate is then isomerised to a secondary alcohol, originating isocitrate, by the action of aconitase, by a two step process. The isocitrate formed, can then follow the cyclic reactions of the Krebs cycle, culminating in the synthesis of succinate by the hydrolysis of succinyl-CoA, trough the action of succinyl-CoA synthetase (EC, 6.2.1.4), accompanied by GTP production. If the isocitrate is conducted through the glyoxylate cycle, the isocitrate lyase will catalyze an aldol cleavage of the substrate, originating succinate and glyoxylate (Voet, 2004).

Malic acid metabolism in yeasts

Malic acid metabolism has been essentially studied due to its significance in the wine industry. The concentration of this organic acid is determinant for the quality of the wine. The yeast *S. cerevisiae* is only able to utilize small amounts of malic acid during fermentation, and never consumes it totally (Usseglio-Tomasset, 1989). This is

probably due to the lack of a mediated transport system for the uptake of this substrate, and to the low efficiency of its intracellular metabolism, in *S. cerevisiae* (Kuczynski and Radler, 1982; Salmon, 1987; Radler, 1993). The assimilation of malic acid varies according to the yeast strain, the initial substrate concentration and the pH of the culture media (Delcourt *et al.*, 1995).

In contrast to *S. cerevisiae,* where the undissociated form of malic acid enters the cells by simple diffusion, both *Zygossacharomyces bailii* and *S. pombe* present a mediated transport system for this substrate, which requires the presence of an assimilable carbon source, such as glucose or glycerol to be active. In *S. pombe* malic acid is transported in the anionic form, by a glucose-induced proton symport mechanism, inhibited by ethanol and acetic acid, whereas in *Z. bailii* it is transported in the undissociated form by a facilitated diffusion mechanism, induced by glucose and repressed by fructose (Baranowski and Radler, 1984; Osothsilp and Subden, 1986; Sousa *et al.*, 1992; Grobler *et al.*, 1995).

In *S. cerevisiae*, Z. *bailii* and in *S. pombe,* the malo-alcoholic fermentation conducts the degradation of malate into ethanol (Rodriguez and Thornton, 1990; Radler, 1993). Firstly, the malic enzyme (EM, EC 1.1.1.38) promotes the oxidative decarboxylation of malic acid, in a NAD^+ dependent manner, to pyruvate. Pyruvate is then decarboxylated by pyruvate decarboxylase (PDC, EC 4.1.1.1), originating acetaldehyde. Most of the produced acetaldehyde is subsequently reduced to ethanol by the action of alcohol dehydrogenase (ADH, EC 1.1.1.1), but some is required for cytosolic acetyl-CoA production for biosynthetic pathways. In this pathway, for each malate molecule consumed, one molecule of ethanol is produced, with concomitant production of two CO_2 molecules. Another malate degradation pathway can occur through fumarate. Malate is primarily dehydrated to fumarate, by fumarase (FUM, EC 4.2.1.2). Fumarate is then reduced to succinate by fumarate reductase (FRDS, EC 1.3.1.6) (Kuczynski and Radler, 1982) (Fig. 6). In *S. cerevisiae*, *MAE1* encodes the malic enzyme (Boles *et al.*, 1998); the pyruvate decarboxylase is encoded by *PDC1*, the major isoenzyme (Kellermann *et al.*, 1986), *PDC5* (Hohmann and Cederberg, 1990) and *PDC6* (Hohmann, 1991), both minor isoforms; alcohol dehydrogenase is encoded by *ADH1* (Bennetzen and Hall, 1982); Fumarase by *FUM1* (Przybyla-Zawislak *et al.*, 1999) and, finally, fumarate reductase by two isoforms, *FRDS1* and *FRDS2* (Enomoto *et al.*, 1996; Arikawa *et al.*, 1998; Muratsubaki and Enomoto, 1998).

Although the biochemical mechanism underlying the utilization of this acid is similar in these yeasts, the efficiency of the process differs greatly. In *S. cerevisiae*, the absence of a mediated transport system and the higher K_m value of the mitochondrial malic enzyme, *MAE1* (K_m =50mM), in comparison to what is found for other yeasts, lowers significantly the efficiency of malic acid utilization in this yeast (Kuczynski and Radler, 1982; Viljoen *et al.*, 1994; Boles *et al.*, 1998).

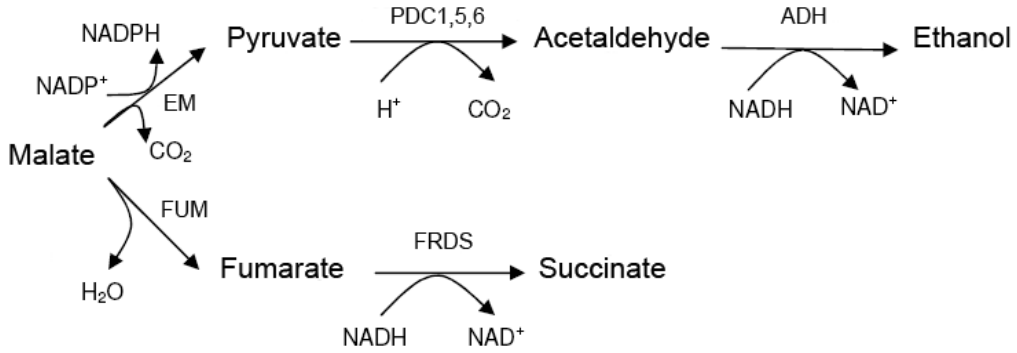

Figure 6 –Malic acid degradation pathways: Malo-alcoholic fermentation and fumarate pathway. *EM* – Malic enzyme; *PDC1,5,6* – Pyruvate decarboxylase; *ADH* – Alcohol dehydrogenase; *FUM* – Fumarase and *FRDS* – Fumarate reductase. Adapted from (Redzepovic *et al.*, 2003) in Pereira L. Master thesis, 2005.

Malic acid can be utilized by Krebs negative yeasts, as the ones referred previously, but also by Krebs positive yeasts, such as, *Candida sphaerica*, *Hansenula anomala*, *Candida utilis* and *K. marxianus*. In these Krebs positive species malic acid degradation is induced by the substrate itself, and the respective metabolic pathways are subjected to glucose repression (Côrte-Real, 1989; Corte-Real and Leao, 1990; Cassio and Leao, 1993; Queiros *et al.*, 1998).

In *C. albicans*, it is not clear how malic acid is metabolized. According to the Candida Genome Database, *orf19.1867*, which displays homology to *S. pombe MAE1* (Grobler *et al.*, 1995), encodes a putative malate permease induced during phagocytosis by macrophages (Prigneau *et al.*, 2003). Also, *orf19.3419* encodes a putative mitochondrial malic enzyme (Murad *et al.*, 2001), with homology to *S. cerevisiae MAE1* (Boles *et al.*, 1998). This has resulted in a nomenclature conflict, since both *orf19.1867* and *orf19.3419* are referred to *MAE1* in *C. albicans*. In *S. pombe* the malic enzyme is encoded by *MAE2*, and it is a cytoplasmatic protein that, unlike the mitochondrial ScMae1, has a high affinity for its substrate (Viljoen *et al.*, 1994). The disruption of *CaJEN2* impaired the uptake of malic and succinic acids (Vieira *et al.*, 2009), and thus, one can assume that the ORF with homology to *S. pombe MAE1* is not

involved in the uptake of dicarboxylic acids, across the plasma membrane, at least in the conditions tested. However, it is possible that *C. albicans* expresses other malic acid transporters, at alternative cellular locations, such as the peroxisome (Tournu *et al.*, 2005; Piekarska *et al.*, 2008), or that another malate permeases exist with a distinct pattern of regulation. A search in the Candida genome database identified *CaSSU12*, a predicted homolog of *S. cerevisiae* plasma membrane sulphit pump, which belongs to the MFS superfamily (*ScSSU1*), as a putative malate permease induced during macrophage infection. Its role as a putative malate transporter in *C. albicans* should be further studied.

Homologs of pyruvate decarboxylase have also been annotated in *C. albicans* genome: *PDC11* (*orf19.2877*) and *PDC12*, a fungal specific putative pyruvate decarboxylase (*orf19.4608*, uncharacterized), both similar to *ScPDC1* (Urban *et al.*, 2003; Braun *et al.*, 2005). Several putative alcohol dehydrogenases are also annotated: *ADH1* (*orf19.3997*) (Shen *et al.*, 1991); *IEF2* (*orf19.5288*, uncharacterized) (Harcus *et al.*, 2004), *ADH2* (*orf19.5113*) and *ADH5* (*orf19.2608*) (Lan *et al.*, 2002), among others. Regarding fumarase, two ORFs are annotated as putative fumarate hydratases, *FUM11* (*orf19.543*, uncharacterized) and *FUM12* (*orf19.6724*, uncharacterized), (Doedt *et al.*, 2004; Lan *et al.*, 2004). Finally, only one mitochondrial fumarate reductase is identified, *OSM2* (*orf19.5005*) (Garcia-Sanchez *et al.*, 2005).

C. glabrata is able to grow in the presence of malic acid, as the only carbon and energy source (Vieira, N. unpublished results), nevertheless, no transport studies with radioactive labelled malic acid have been performed in this yeast yet. A Blastp search was performed on Ncbi (http://www.ncbi.nlm.nih.gov/) to search for putative homologs of *S. pombe* Mae1, a malate permease, in *C. glabrata* genome. No homologs for *S. pombe* Mae1 or for the *C. albicans* dicarboxylate permease Jen2 were identified. However, this yeast presents homologs that putatively encode all the enzymes involved in the degradation of malic acid: one homolog of *MAE1* (*CAGL0L02035g*); two homologs of *PDC1,5,6* (*CAGL0M07920g* and *CAGL0G02937g*); one homolog of *ADH1* and *ADH5* (*CAGL0I07843g*); and of the *ADH3* isoenzyme (*CAGL0J01441g*); one of fumarase, *FUM1* (*CAGL0A01045g*); and finally two homologs of fumarate reductase, *FRDS1* (*CAGL0L01177g*) and *FRDS2* (*CAGL0I01320g*).

Some yeast strains are capable of synthesizing and accumulating malic acid (Fatichenti, 1984; Schwartz, 1988). The synthesis of this acid is favoured in the

presence of high sugar concentrations and requires CO_2, limiting nitrogen concentrations and pH values around 5.0 (Radler, 1993). In *S. cerevisiae* malic acid is produced through the action of pyruvate carboxylase that conducts the carboxylation of pyruvate to oxaloacetate. The formed oxaloacetate is further reduced to malate by malate dehydrogenase (MDH, EC 1.1.1.37) (Pines *et al.*, 1996). However, this synthesis is very low, when compared to the quantities obtained by filamentous fungi, in aerobic conditions (Peleg *et al.*, 1990).

Inhibition studies in *S. cerevisiae,* supported that malic acid synthesis occurs through the described pathway, since pyruvate carboxylase inhibitors, such as the slow-binding inhibitor avidin, abolished the synthesis of malic acid, whereas inhibitors of malic acid synthesis through the Krebs cycle did not (Schwartz, 1988). There are also evidences suggesting the involvement of fumarase in the cytosolic synthesis of malic acid, since the overexpression of this enzyme led to an accumulation of malic acid. However, that accumulation was proven to be due to the concomitant rise of malate dehydrogenase levels, and hence of malic acid synthesis from oxaloacetate (Pines *et al.*, 1996; Pines *et al.*, 1997).

In *S. cerevisiae* malate dehydrogenase is encoded by three isoenzymes, as referred previously. The *MDH1* codes for the mitochondrial isoform whereas *MDH2* codes for the cytoplasmic isoform and finally *MDH3* encodes the peroxisome isoform (Fig. 5). *C. albicans* and *C. glabrata* both present three homologs, as described previously in the text. Overexpression studies performed by Pines and co-workers established the direct connection between Mdh2 concentration and malic acid production, indicating that most probably Mdh2 constitutes the limiting step in malic acid synthesis. Additionally, overexpression of this cytosolic enzyme led to an accumulation of other organic acids, namely of fumaric acid, probably due to an increase of intracellular malic acid, and hence of the fumarase enzyme (Pines *et al.*, 1997).

Regulation and carboxylic acids permease turnover in S. cerevisiae

Plasma membrane proteins, such as transporters and receptors, have a determinant role in the regulation of the overall cellular metabolism, contributing to the sensing, adhesion, signaling and nutrient uptake, allowing the cell to adapt and respond

to distinct environmental cues. In this manner, the protein composition of the plasma membrane will directly influence the cellular metabolic context.

Considering the complexity of plasma membrane proteins at the cell surface, the need for a permanent homeostatic regulation and turnover of damage/misfolded plasma membrane proteins, and the demand to frequently remodel the plasma membrane protein composition, in response to environmental changes, it is not surprising that cells have developed several mechanisms to control their composition. In yeast, there may be hundreds of different plasma membrane proteins at any given time, so it is essential to have a highly specific system to selectively remove them, under certain conditions. The downregulation of membrane proteins is achieved by means of a selective endocytosis, where target proteins are internalized into endosomes, and either sorted to Multivesicular Bodies (MVBs) and delivered to the vacuole/lysosome for degradation, or recycled back to the plasma membrane (Miranda and Sorkin, 2007). Ubiquitylation is a post-translational modification that has been shown to trigger downregulation of several membrane proteins, from mammal to yeast organisms, and has emerged as a crucial endocytic regulatory mechanism (Weissman, 2001). Many of the original observations of ubiquitylation of the endocytic cargo and regulation of endocytosis by ubiquitylation were made in the yeast *S. cerevisiae* (Kolling and Hollenberg, 1994; Hicke and Riezman, 1996), a very favorable model organism that continues to be in the frontline of this area of research. Strikingly, the mechanisms controlling plasma membrane transporters trafficking are essentially conserved from fungi to mammals.

The first link between ubiquitylation and endocytosis resulted from the study on the endocytosis of the Ste6p, a member of the ABC transporter family in *S. cerevisiae* (Kolling and Hollenberg, 1994). After that, similar studies have shown the ubiquitylation of several proteins of the plasma membrane, including Jen1. Indeed, the *S. cerevisiae* monocarboxylate transporter Jen1, has proven to be an excellent system for genetically dissecting mechanisms that regulate trafficking of an eukaryote plasma membrane protein, according to physiological constrains. In lactic acid-grown cells, Jen1 is fairly stable at the plasma membrane, however the addition of glucose triggers, within minutes, its ubiquitin dependent internalization and sorting to the vacuole for irreversible degradation (Paiva *et al.*, 2002; Paiva *et al.*, 2009) (Fig. 7).

Modifications of Proteins by ubiquitin

Ubiquitylation is a post-translational modification that mediates the covalent conjugation of ubiquitin to protein substrates and intimately regulates many processes in the cell, besides promoting the degradation of target proteins, it is also involved in gene transcription, DNA repair, signaling, intracellular trafficking, virus budding, among others (Haglund and Dikic, 2005). Ubiquitin is 76 amino-acid globular protein highly conserved among eukaryotes containing a di-glycine motif, at the C-terminal, allowing the last residue, Gly_{76} to be conjugated to the ε-amino group of a lysine residue in a target protein. Classically, ubiquitylation is related to the target of proteins for degradation in the proteasome, an ATP dependent protease (Kloetzel, 2001). Nowadays, the view of the involvement of ubiquitylation as a signal for degradation of membrane proteins in vacuoles and/or lysosomes is common. In this manner, the attachment of a single ubiquitin or the attachment of ubiquitin chains to one or several residues can target proteins to different proteolytic structures. This plasticity relies on several factors, namely, the intermediate enzymes that recognize the ubiquitylable substrate, the type of ubiquitin conjugate (mono or poly-ubiquitylation), the lysine residues involved in the formation of ubiquitin chains and the intracellular localization of the post-translational modification, among others.

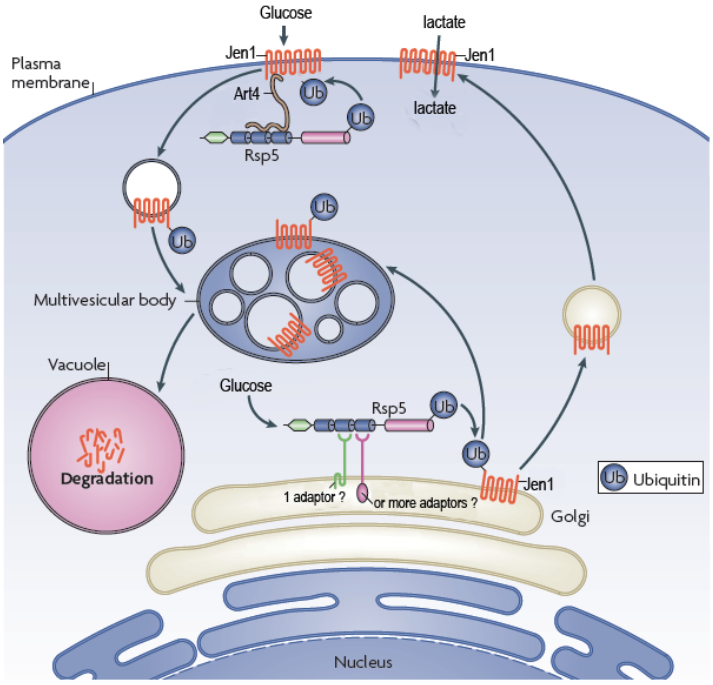

Figure 7 – Schematic representation of Jen1 trafficking and turnover in *S. cerevisiae* cells. When *S. cerevisiae* cells are grown in lactic acid, Jen1 is delivered to the plasma membrane in a Sec6 dependent manner (Paiva *et al.*, 2002). Upon a glucose pulse to the culture media, the

HECT ligase, Rsp5, promotes Jen1 ubiquitylation, at the cell surface and its subsequent sorting to MVBs for degradation in the vacuole (Paiva *et al.*, 2009). Jen1 exiting the Golgi apparatus after the glucose pulse might eventually be sorted to the vacuole for degradation. Nevertheless, the adaptor(s) protein(s) involved in the VPS pathway between the Golgi and the vacuole is/are yet to be determined, as are the ones required for the MVB sorting. Adapted from (Rotin and Kumar, 2009).

Different ubiquitin-like (UBLs) and ubiquitin-domain proteins (UDPs) have also been annotated. The first share with ubiquitin the ability to covalently modify substrate proteins, whereas the second have homologous domains to ubiquitin and a broad action in cellular functions, for instance, regulating ubiquitin-mediated processes. As examples, UDPs can be ubiquitylable substrates, enzymes related to ubiquitin addition and removal from the target proteins, among others (Weissman, 2001).

Another post-translational modification involved in endocytosis is phosphorylation. The phosphorylation of enzymes that catalyze the addition of ubiquitin or of the target protein can augment or inhibit ubiquitylation (Davies *et al.*, 2009b) and sometimes be required to drive the subsequent ubiquitylation and internalization of distinct substrates (Hicke *et al.*, 1998; Marchal *et al.*, 1998; Marchal *et al.*, 2000; Paiva *et al.*, 2009).

E1, E2 and E3 enzymes

Ubiquitylation of target substrates requires the action of several enzymes. Firstly, in an ATP-dependent manner, E1 ubiquitin-activating enzymes form a thiol-ester bond between its catalytic cystein and the C-terminal $Glycine_{76}$ of ubiquitin. Secondly, an E2 ubiquitin-conjugating enzyme accepts the activated ubiquitin molecule by a *trans*-thiolation reaction, also between its cystein and ubiquitin. Finally, ubiquitin is transferred to the ε-amino group of a lysine residue in the target protein, and this process is catalyzed by an E3 ubiquitin-ligase. The diversity of E3 ligases increases the target recognition specificity in the ubiquitin-ligase process (Ma, 2007).

The E2 ubiquitin-conjugating enzymes have a 14 kDa core that is 35% conserved among the family members, presenting different amino or carboxyterminal extensions, which in turn increase the specificity of the interaction with distinct E3 ligases. In this manner there is a potential combinatorial diversity in terms of binding of E2-E3 to the target protein, relying on the E3 domain and in the E2 polypeptides that can establish a stable isopeptide bond between ubiquitin and the acceptor protein (Rotin and Kumar, 2009). Two major E3 families have been identified to date, the RING

(defined structurally by two interleaved metal-coordinating sites) or HECT (homologous to E6-AP carboxylic terminus) domain-containing enzymes. Most of the E3 ubiquitin-ligases are RING-finger containing proteins. In this protein family there is a direct transfer of the activated ubiquitin from E2 to the E3-bound substrate. The RING E3 acts as scaffolds that bring the E2 near to the substrate facilitating the direct transfer, and can be a single chain or part of multimeric complexes. On the other hand, the HECT E3 ligases contain a conserved cystein residue that allows the formation of an intermediate thioester bond with the ubiquitin C-terminus, prior to catalyzing target ubiquitylation. The HECT E3 ligases can act independently or in association with adaptor proteins (Rotin and Kumar, 2009). Many of the HECT E3 ligases have also a shared WW domain, involved in targeting substrates for ubiquitylation. These motifs are tryptophan based and can establish a hydrophobic pocket for proline-rich sequences (PPXY or related proline-containing sequences, the PY elements) and some phosphoserine and phosphotreonine sequences. The HECT domains have been found in sequences of more than one hundred species. The human forms can be divided in three major groups according to the N-terminal domain: the Nedd4 family (9 members), the HERC family (6 members) and other HECTS (13 members) (Rotin and Kumar, 2009). The Nedd4 family members contain a N-terminal C2 domain, 2-4 WW domains and a characteristic C-terminal HECT domain. The C2 lipid interaction domain mediates intracellular targeting to the plasma membrane, endosomes and multi vesicular bodies (MVBs) (Plant *et al.*, 2000; Dunn *et al.*, 2004).

Rsp5 is the single member of the HECT ubiquitin ligases of the Nedd4 family, in *S. cerevisiae*. This ubiquitin ligase contributes to several cellular functions, namely, chromatin remodeling, regulation of transcription, regulation of endocytosis and the sorting of membrane proteins, transporters and receptors (Huibregtse *et al.*, 1997; Hicke and Dunn, 2003; Rotin and Kumar, 2009). Rsp5, is in this manner, involved in the ubiquitylation of distinct proteins, in different cellular compartments, and is known to be involved in the endocytosis of most, if not all the endocytosed proteins (Horak, 2003). However, most of the plasma membrane transporters or receptors lack the typical PY motifs, implying the need for adaptor molecules, that will recognize the substrate and bind simultaneously, through the PY elements, to the Rsp5 ligase (Shearwin-Whyatt *et al.*, 2006).

Ubiquitin as sorting signal

A substrate can be modified at a single Lys residue (monoubiquitylation), or at several Lys residues (multimonoubiquitylation) or even by the attachment of short-chains of two to three ubiquitins at one or more target Lys residues (oligo-ubiquitylation), since the ubiquitin molecule has lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63), covalently linked ubiquitin can modify ubiquitin itself, giving rise to polyubiquitin chains (Fig. 8). The type of ubiquitylation and of the formed ubiquitin chains will, ultimately, determine the fate of the protein substrate.

In yeast, it was previously shown that all these residues could be involved in the formation of ubiquitin chains (Peng *et al.*, 2003), although Lys48 and Lys63 are most commonly used (Fig. 8). Substrates bearing polyubiquitin chains linked at Lys48 are predominately associated with proteasomal degradation, which is a ordinary destiny for misfolded membrane proteins or soluble proteins recognized by the ERAD pathway (Finley *et al.*, 1994; Ward *et al.*, 1995; Jarosch *et al.*, 2002; Xu *et al.*, 2009). In contrast, the Lys63-linked ubiquitin chains are associated with trafficking and signaling (Galan and Haguenauer-Tsapis, 1997; Pickart and Fushman, 2004), inflammatory response, protein translation and DNA repair (Pickart and Fushman, 2004). More recently, by mass spectroscopy analysis, there were indications that a single ubiquitin molecule can be modified at several residues, originating different topology chains, but the involvement of these modifications with cellular trafficking is still unclear (Kim *et al.*, 2007).

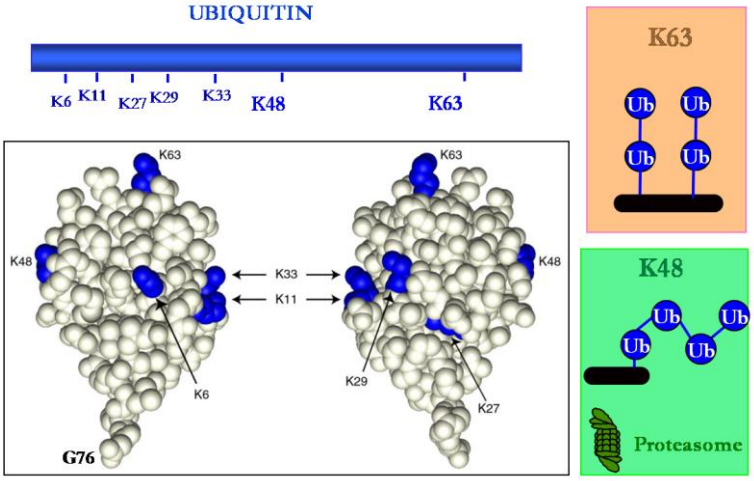

Figure 8 – Localization of the lysine residues present in a space filling model of ubiquitin. Schematic representation of the most common ubiquitin chains, K48 and K63. Adapted from (Staub and Rotin, 2006).

In yeast, Rsp5 preferentially catalyzes K63 linked polyubiquitylation of substrates (Kee *et al.*, 2005; Kee *et al.*, 2006). Recent findings by Kim and co-workers indicated that the specificity of the chain type is related uniquely to the Rsp5 HECT domain and not to the interactions between the E3 ligase and the cooperative E2 protein, and that the last 60 amino acids of the C lobe of the HECT domain dictate this chain specificity (Kim and Huibregtse, 2009). Nevertheless, the mechanisms involved in polyubiquitylation remain intriguing. The E4 enzymes bearing an U-box motif, have also been assigned to this process (Koegl *et al.*, 1999; Henry *et al.*, 2003; Levy *et al.*, 2005).

Deubiquitylation enzymes (DUBs)

Ubiquitylation is a reversible process and, in this way, there are tight controls for the removal of ubiquitin. The isopeptidases involved in this removal are called deubiquitylation enzymes (DUBs) (Millard and Wood, 2006). There are approximately 19 annotated yeast DUBs and significantly more in mammals. DUBs, thiol proteases, can be divided in UCHs (ubiquitin carboxy-terminal hydrolases) and UBPs (ubiquitin-specific processing enzymes). These enzymes can cleave ubiquitin from target proteins, from the residual proteasome-associated peptides and also dismantle the multi-ubiquitin chains. Adittionally, the UCHs, can process immature ubiquitin, encoded on multiple genes and translated as fusion proteins (with other ubiquitins or with two small ribosomal subunits), originating mature ubiquitin (Finley *et al.*, 1989; Wilkinson, 2000).

Overall, DUBs play crucial cellular roles, especially in the maintenance of the cellular homeostasis, and are important for the regulation of ubiquitin-mediated effects (Weissman, 2001).

Sorting of protein targets to Multi Vesicular Bodies

Transmembranar proteins are cotranslationally translocated to the endoplasmic reticulum (ER), postranslationally modified in the ER-Golgi membrane systems and then sorted to specific sites of residence within the cell. A tight regulation of these mechanisms lies on the correct delivering and removal of the proteins to the cell surface. Endosomes play a crucial role in this process coordinating the vesicular transport between the Trans-Golgi network (TGN), the plasma membrane and the lysosome/vacuole for degradation (Morvan *et al.*, 2004). Many transporters and

receptors in the membrane can be internalized to endosomes and either recycled back to the membrane or degradated in the lysome/vacuole. In yeast, recent reports also suggest that, according to nutritional conditions, cell surface transporters can be sorted for premature degradation in the vacuole, directly from the Golgi apparatus to the endosomal system (vacuolar protein sorting pathway-VPS) (Blondel *et al.*, 2004; Stimpson *et al.*, 2006). Endosomes can be divided in early or late endosomes depending on the kinetics with which the endosomes are loaded with endocytosed material and on their morphological aspect. The early endosomes tend to be more tubular and stay in the cell periphery in opposition to the late endosomes that are localized near the nucleus and that present a more spherical appearance (Gruenberg, 2001). Additionally, some of these late endosomes present a multivesicular appearance and are denominated MVB-multi vesicular bodies, resulting from the invagination and budding of the endosomal membrane towards the lumen.

The delivery of endocytic membrane proteins to the lysome/vacuole requires a prior distribution of these molecules to the MVBs, which ultimately fuse with these organelles (Futter *et al.*, 1996). MVBs provide in this manner an important cellular mechanism for terminating the function of integral membrane proteins tagged for degradation (Tanaka *et al.*, 2008). Nevertheless, the proteins that do not enter this pathway can be removed by standard vesicular transport (Morvan *et al.*, 2004). The MVB sorting machinery is highly conserved among eukaryotes, from yeast to humans, and its study in *S. cerevisiae* has proven to be essential for the discovery of the molecular components involved in this pathway.

The entry of several proteins into MVBs requires their prior ubiquitylation, indicating a role for ubiquitin in the MVB sorting (Katzmann *et al.*, 2001; Urbanowski and Piper, 2001). Rsp5 mediated ubiquitylation has been proven to regulate the sorting of the cargo into MVBs from the endocytic vesicles, and from the Golgi apparatus to the endosomes (Belgareh-Touze *et al.*, 2008) for subsequent vacuolar degradation (VPS pathway) (Beck *et al.*, 1999; Helliwell *et al.*, 2001; Soetens *et al.*, 2001; Blondel *et al.*, 2004). Additionally, several ubiquitin-binding domains (UBDs) were identified within components of the MVB sorting machinery, which suggest a mechanism by which these ubiquitylated cargoes are detected. However, the optimal ubiquitylation pattern for the promotion of MVB sorting is yet to be determined (Urbanowski and Piper, 2001; Mosesson *et al.*, 2003; Katzmann *et al.*, 2004; Morvan *et al.*, 2004; Umebayashi *et al.*,

2008). The type of ubiquitin chain(s) involved in MVB sorting is still unclear, both K63 and K48-linked chains promote the lysosomal delivery of the cargoes, but K63 seems to have a more prominent role in this process.

More recently, the need for a prior deubiquitylation at the MVB followed by re-ubiquitylation of the cargo for correct sorting into the MVBs has been mentioned, based on the fact that Ubp2 deubiquitylation enzyme was suggested to be involved in the regulation of Fur4 Rsp5-mediated MVB sorting (Lam *et al.*, 2009). Nevertheless, these assumptions are merely speculative.

Class E vacuolar protein sorting proteins (VPS) are also essential for cargo sorting into the MVB pathway. Some of them are organized in three distinct endosomal sorting complexes required for transport: ESCRT-I, II and III. These protein complexes are fundamental for MVB biogenesis and for the sorting of ubiquitylated targets to MVBs (Fig. 9) (Katzmann *et al.*, 2002). Ubiquitylated MVB cargo (endocytosed material) recognition is stablished by the Vps27-Hse1 (ESCRT-0) complex that recruits ESCRT-I (Vps23/Tsg101, Vps28, Vps37, and Mvb12) (Bilodeau *et al.*, 2002; Shih *et al.*, 2002; Katzmann *et al.*, 2003). ESCRT-II (Vps22, Vps25, Vps36) appears to function downstream, or simultaneously to the ESCRT-I, and is believed to interact with the MVB cargo, being involved in the concentration and sorting of this cargo (Alam *et al.*, 2006; Im and Hurley, 2008). Finally ESCRT-II also helps in the recruitment and assembly of the ESCRT-III subunits (Vps20/CHM6, Snf7/CHMP4, Vps2/CHMP2, Vps24/CHMP3, Did2/CHMP1, Vps60/CHMP5) (Teo *et al.*, 2004; Yorikawa *et al.*, 2005).

The ubiquitin isopeptidase Doa4 is then recruited by the ESCRT-III complex to release the ubiquitin molecules from ubiquitin bond MVB cargoes (after the initial ubiquitin dependent phase for the correct MVB sorting, is completed) (Morvan *et al.*, 2004). Ultimately, the ESCRT-III interacts with Vps4-Vta1 complex promoting the disassembly of ESCRT-III itself and probably the conclusion of the formation of intralumenal vesicles (ILV) (Obita *et al.*, 2007; Stuchell-Brereton *et al.*, 2007; Azmi *et al.*, 2008). The MVB cargoes will then be delivered into the ILVs for degradation in the lysosome/vacuole (Fig. 9). It's interesting that while lysosomal/vacuolar degradation effectively abolishes the function of the transmembrane proteins, the sorting into the MVB allows the silencing of their function, prior to its degradation, since the active domains are sequestered into the endosomal lumen (Davies *et al.*, 2009a).

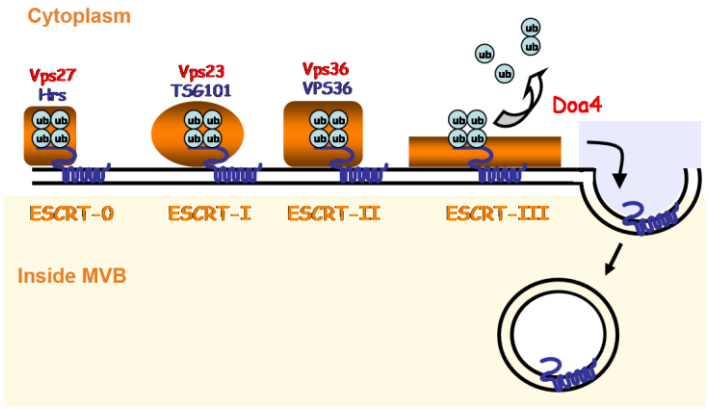

Figure 9 – Schematic representation of the ESCRTs complexes (yeast and mammalian members) that have been demonstrated to execute critical functions during MVB cargo sorting. The ESCRT complexes are involved in the recognition of ubiquitylated cargoes and in their sorting to the internal vesicles of MVBs. Rsp5 ubiquitin ligase and the adaptor proteins play a critical role in cargo selection through covalent modification of cargoes. The yeast Doa4 was the first DUB to be implicated in the MVB cargo sorting involved in the regulation of ubiquitin-mediated cargo recognition and contributing to ubiquitin recycling.

OUTLINE OF THE THESIS

Carboxylic acids can be used as carbon and energy sources and are subproducts of carbon metabolism in yeasts, contributing to the overall cell functionality. For decades, intensive biochemical and physiological studies have been developed, aiming at the characterization of the metabolism and transport of these acids across the plasma membrane, in different organisms.

The global aim of this thesis work was to increase the scientific knowledge on yeast carboxylate permeases, overall. We dedicated our efforts to unravel the molecular mechanisms underlying Jen1 expression and regulation in *S. cerevisiae*, and concomitantly, to identify and characterize carboxylate permeases homologs, in different pathogenic fungi and in an archaeon.

In the first chapter of this thesis, a general introduction is presented, reviewing the main concepts underlying the topics of the work performed

The thesis is then organized in three main sections. The first section is divided in four chapters and embraces the characterization of carboxylate transporters homologs, in different organisms. As mentioned before, the sequencing of different eukaryotic and prokaryotic genomes allowed the identification of several homologs in different organisms. In this manner, in chapter two the functional characterization of *C. albicans* Jen homologs is described. The role of these permeases in *C. albicans* virulence is ultimately assessed in *in vitro* and *in vivo* models of infection. Chapter three explains

the followed methodologies to integrate genes in *C. albicans* that cannot be propagated in *E. coli*. This work resulted from the difficulty of cloning *C. albicans* Jen2 in the integrative plasmid, CIp20, in the scope of the work described in chapter two and resulted in the construction of a collection of *C. albicans* integrative vectors that permit gap repair in *S. cerevisiae*. Finally, chapters four and five describe the studies developed, aiming at the characterization of predicted Ady2 homologs, in *C. glabrata*, another human pathogenic yeast, and in the methanogen *M. acetivorans*, respectively.

The second part of the thesis, chapter 6, describes the work developed on *JEN1* mRNA expression and decay, in different carbon and energy sources. During the search for the acetate transporter encoding gene, in *S. cerevisiae*, *DHH1* was isolated (Paiva, S. PhD thesis). However, this gene encodes a DEAD-box RNA helicase with pleiotropic roles within the yeast cell and it is not involved in the transport of acetic acid across the plasma membrane. In fact, it seems to affect the stability of *JEN1* mRNA in some carbon and energy sources.

The last section of this thesis is divided in two chapters and is related to the glucose-induced turnover of Jen1 in the yeast *S. cerevisiae*. The addition of glucose to lactic acid-grown cells triggers a rapid loss of Jen1 activity with concomitant repression of *JEN1* expression and induction of its mRNA degradation (Andrade and Casal, 2001; Andrade *et al.*, 2005). Moreover, previous findings indicated that loss of Jen1-GFP activity upon a glucose pulse resulted from its endocytosis and vacuolar sorting degradation (Paiva *et al.*, 2002). In this manner, in chapter seven we set up to unravel mechanisms underlying Jen1 downregulation. In chapter eight we aimed at the identification of the adaptor protein involved in the Rsp5 binding to Jen1. As mentioned previously, Rsp5 possesses three WW domains probably related to the recognition of PPXY/PY motifs on specific target proteins. However, plasma membrane cargoes, like Jen1, lack these proline rich sequences, and hence, require the action of adaptor proteins (Nikko *et al.*, 2008; Leon and Haguenauer-Tsapis, 2009).

Finally, in chapter 9, a general discussion of the work performed is presented, in order to highlight the principle findings and some implications of the results provided in this thesis. Additionally, future research goals are mentioned.

References

Abraham, P. R., Mulder, A., van 't Riet, J. and Raue, H. A. 1994. Characterization of the *Saccharomyces cerevisiae* nuclear gene *CYB3* encoding a cytochrome b polypeptide of respiratory complex II. *Mol Gen Genet.* **242**: 708-716.

Aguilera, J., Rodriguez-Vargas, S. and Prieto, J. A. 2005. The HOG MAP kinase pathway is required for the induction of methylglyoxal-responsive genes and determines methylglyoxal resistance in *Saccharomyces cerevisiae*. *Mol Microbiol.* **56**: 228-239.

Alam, S. L., Langelier, C., Whitby, F. G., Koirala, S., Robinson, H., Hill, C. P. and Sundquist, W. I. 2006. Structural basis for ubiquitin recognition by the human ESCRT-II EAP45 GLUE domain. *Nat Struct Mol Biol.* **13**: 1029-1030.

Alonso-Monge, R., Navarro-Garcia, F., Molero, G., Diez-Orejas, R., Gustin, M., Pla, J., Sanchez, M. and Nombela, C. 1999. Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J Bacteriol.* **181**: 3058-3068.

Andrade, R. P. and Casal, M. 2001. Expression of the lactate permease gene *JEN1* from the yeast *Saccharomyces cerevisiae*. *Fungal Genet Biol.* **32**: 105-111.

Andrade, R. P., Kotter, P., Entian, K. D. and Casal, M. 2005. Multiple transcripts regulate glucose-triggered mRNA decay of the lactate transporter *JEN1* from *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun.* **332**: 254-262.

Andre, B. 1995. An overview of membrane transport proteins in *Saccharomyces cerevisiae*. *Yeast.* **11**: 1575-1611.

Arikawa, Y., Enomoto, K., Muratsubaki, H. and Okazaki, M. 1998. Soluble fumarate reductase isoenzymes from *Saccharomyces cerevisiae* are required for anaerobic growth. *FEMS Microbiol Lett.* **165**: 111-116.

Azmi, I. F., Davies, B. A., Xiao, J., Babst, M., Xu, Z. and Katzmann, D. J. 2008. ESCRT-III family members stimulate Vps4 ATPase activity directly or via Vta1. *Dev Cell.* **14**: 50-61.

Balakrishnan, R., Christie, K. R., Costanzo, M. C., Dolinski, K., Dwight, S. S., Engel, S. R., Fisk, D. G., Hirschman, J. E., Hong, E. L., Nash, R., *et al.* 2005. Fungal BLAST and Model Organism BLASTP Best Hits: new comparison resources at the *Saccharomyces Genome* Database (SGD). *Nucleic Acids Res.* **33**: D374-377.

Baranowski, K. and Radler, F. 1984. The glucose-dependent transport of L-malate in *Zygosaccharomyces bailii*. *Antonie Van Leeuwenhoek.* **50**: 329-340.

Barnett, J. A. and Kornberg, H. L. 1960. The utilization by yeasts of acids of the tricarboxylic acid cycle. *J Gen Microbiol.* **23**: 65-82.

Barnett, J. A., Payne, R. W. and Yarrow, D. 2000. Yeast, Characteristics and Identification. Third edition. Cambridge University Press.

Beck, C. and von Meyenburg, H. K. 1968. Enzyme pattern and aerobic growth of *Saccharomyces cerevisiae* under various degrees of glucose limitation. *J Bacteriol.* **96**: 479-486.

Beck, T., Schmidt, A. and Hall, M. N. 1999. Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. *J Cell Biol.* **146**: 1227-1238.

Behal, R. H., Browning, K. S., Hall, T. B. and Reed, L. J. 1989. Cloning and nucleotide sequence of the gene for protein X from *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A.* **86**: 8732-8736.

Belgareh-Touze, N., Leon, S., Erpapazoglou, Z., Stawiecka-Mirota, M., Urban-Grimal, D. and Haguenauer-Tsapis, R. 2008. Versatile role of the yeast ubiquitin ligase Rsp5p in intracellular trafficking. *Biochem Soc Trans.* **36**: 791-796.

Bennetzen, J. L. and Hall, B. D. 1982. The primary structure of the *Saccharomyces cerevisiae* gene for alcohol dehydrogenase. *J Biol Chem.* **257**: 3018-3025.

Bilodeau, P. S., Urbanowski, J. L., Winistorfer, S. C. and Piper, R. C. 2002. The Vps27p Hse1p complex binds ubiquitin and mediates endosomal protein sorting. *Nat Cell Biol.* **4**: 534-539.

Bilsland, E., Molin, C., Swaminathan, S., Ramne, A. and Sunnerhagen, P. 2004. Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance. *Mol Microbiol.* **53**: 1743-1756.

Blondel, M. O., Morvan, J., Dupre, S., Urban-Grimal, D., Haguenauer-Tsapis, R. and Volland, C. 2004. Direct sorting of the yeast uracil permease to the endosomal system is controlled by uracil binding and Rsp5p-dependent ubiquitylation. *Mol Biol Cell.* **15**: 883-895.

Boer, V. M., de Winde, J. H., Pronk, J. T. and Piper, M. D. 2003. The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. *J Biol Chem.* **278**: 3265-3274.

Bojunga, N. and Entian, K. D. 1999. Cat8p, the activator of gluconeogenic genes in *Saccharomyces cerevisiae*, regulates carbon source-dependent expression of NADP-dependent cytosolic isocitrate dehydrogenase (Idp2p) and lactate permease (Jen1p). *Mol Gen Genet.* **262**: 869-875.

Boles, E., de Jong-Gubbels, P. and Pronk, J. T. 1998. Identification and characterization of *MAE1*, the *Saccharomyces cerevisiae* structural gene encoding mitochondrial malic enzyme. *J Bacteriol.* **180**: 2875-2882.

Boshoff, H. I. and Barry, C. E. 2005. A low-carb diet for a high-octane pathogen. *Nat Med.* **11**: 599-600.

Braun, B. R., van Het Hoog, M., d'Enfert, C., Martchenko, M., Dungan, J., Kuo, A., Inglis, D. O., Uhl, M. A., Hogues, H., Berriman, M., *et al.* 2005. A human-curated annotation of the *Candida albicans* genome. *PLoS Genet.* **1**: 36-57.

Briquet, M. 1977. Transport of pyruvate and lactate in yeast mitochondria. *Biochim Biophys Acta.* **459**: 290-299.

Bullis, B. L. and Lemire, B. D. 1994. Isolation and characterization of the *Saccharomyces cerevisiae SDH4* gene encoding a membrane anchor subunit of succinate dehydrogenase. *J Biol Chem.* **269**: 6543-6549.

Busch, W. and Saier, M. H., Jr. 2004. The IUBMB-endorsed transporter classification system. *Mol Biotechnol.* **27**: 253-262.

Butler, G., Rasmussen, M. D., Lin, M. F., Santos, M. A., Sakthikumar, S., Munro, C. A., Rheinbay, E., Grabherr, M., Forche, A., Reedy, J. L., *et al.* 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature.* **459**: 657-662.

Byrne, K. P. and Wolfe, K. H. 2006. Visualizing syntenic relationships among the hemiascomycetes with the Yeast Gene Order Browser. *Nucleic Acids Res.* **34**: D452-455.

Calderone, R. A. 2002. *Candida and Candidiasis*. Washington, DC: American Society for Micobiology Press.

Carlson, M. 1999. Glucose repression in yeast. *Curr Opin Microbiol.* **2**: 202-207.

Casal, M., Blazquez, M. A., Gamo, F. J., Gancedo, C. and Leao, C. 1995. Lack of lactate-proton symport activity in pck1 mutants of *Saccharomyces cerevisiae*. *FEMS Microbiol Lett.* **128**: 279-282.

Casal, M., Cardoso, H. and Leao, C. 1996. Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. *Microbiology.* **142**: 1385-1390.

Casal, M., Paiva, S., Andrade, R. P., Gancedo, C. and Leao, C. 1999. The lactate-proton symport of *Saccharomyces cerevisiae* is encoded by *JEN1*. *J Bacteriol.* **181**: 2620-2623.

Casal, M., Paiva, S., Queiros, O. and Soares-Silva, I. 2008. Transport of carboxylic acids in yeasts. *FEMS Microbiol Rev.* **32**: 974-994.

Cassio, F. and Leao, C. 1993. A comparative study on the transport of L(-)malic acid and other short-chain carboxylic acids in the yeast *Candida utilis*: evidence for a general organic acid permease. *Yeast.* **9**: 743-752.

Cassio, F., Leao, C. and van Uden, N. 1987. Transport of lactate and other short-chain monocarboxylates in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* **53**: 509-513.

Chapman, K. B., Solomon, S. D. and Boeke, J. D. 1992. *SDH1*, the gene encoding the succinate dehydrogenase flavoprotein subunit from *Saccharomyces cerevisiae*. *Gene.* **118**: 131-136.

Chauhan, N., Inglis, D., Roman, E., Pla, J., Li, D., Calera, J. A. and Calderone, R. 2003. *Candida albicans* response regulator gene *SSK1* regulates a subset of genes whose functions are associated with cell wall biosynthesis and adaptation to oxidative stress. *Eukaryot Cell.* **2**: 1018-1024.

Chelstowska, A., Liu, Z., Jia, Y., Amberg, D. and Butow, R. A. 1999. Signalling between mitochondria and the nucleus regulates the expression of a new D-lactate dehydrogenase activity in yeast. *Yeast.* **15**: 1377-1391.

Cheng, S., Clancy, C. J., Checkley, M. A., Handfield, M., Hillman, J. D., Progulske-Fox, A., Lewin, A. S., Fidel, P. L. and Nguyen, M. H. 2003. Identification of *Candida albicans* genes induced during thrush offers insight into pathogenesis. *Mol Microbiol.* **48**: 1275-1288.

Chibana, H., Oka, N., Nakayama, H., Aoyama, T., Magee, B. B., Magee, P. T. and Mikami, Y. 2005. Sequence finishing and gene mapping for *Candida albicans* chromosome 7 and syntenic analysis against the *Saccharomyces cerevisiae* genome. *Genetics.* **170**: 1525-1537.

Cimini, D., Patil, K. R., Schiraldi, C. and Nielsen, J. 2009. Global transcriptional response of *Saccharomyces cerevisiae* to the deletion of *SDH3*. *BMC Syst Biol.* **3**: 17.

Cliften, P., Sudarsanam, P., Desikan, A., Fulton, L., Fulton, B., Majors, J., Waterston, R., Cohen, B. A. and Johnston, M. 2003. Finding functional features in *Saccharomyces* genomes by phylogenetic footprinting. *Science.* **301**: 71-76.

Copping, V. M., Barelle, C. J., Hube, B., Gow, N. A., Brown, A. J. and Odds, F. C. 2005. Exposure of *Candida albicans* to antifungal agents affects expression of *SAP2* and *SAP9* secreted proteinase genes. *J Antimicrob Chemother.* **55**: 645-654.

Corte-Real, M. and Leao, C. 1990. Transport of malic acid and other dicarboxylic acids in the yeast *Hansenula anomala*. *Appl Environ Microbiol.* **56**: 1109-1113.

Côrte-Real, M., Leão, C., and van Uden, N. 1989. Transport of L(-) malic acid and other dicarboxylic acids in the yeast *Candida sphaerica*. *Appl Microbiol Biotechnol.* **31**: 551-555.

Cozzone, A. J. 1998. Regulation of acetate metabolism by protein phosphorylation in enteric bacteria. *Annu Rev Microbiol.* **52**: 127-164.

Csank, C. and Haynes, K. 2000. *Candida glabrata* displays pseudohyphal growth. *FEMS Microbiol Lett.* **189**: 115-120.

Daignan-Fornier, B., Valens, M., Lemire, B. D. and Bolotin-Fukuhara, M. 1994. Structure and regulation of *SDH3*, the yeast gene encoding the cytochrome b560 subunit of respiratory complex II. *J Biol Chem.* **269**: 15469-15472.

Daum, G., Gasser, S. M. and Schatz, G. 1982. Import of proteins into mitochondria. Energy-dependent, two-step processing of the intermembrane space enzyme cytochrome b2 by isolated yeast mitochondria. *J Biol Chem.* **257**: 13075-13080.

Davies, B. A., Azmi, I. F. and Katzmann, D. J. 2009a. Regulation of Vps4 ATPase activity by ESCRT-III. *Biochem Soc Trans.* **37**: 143-145.

Davies, B. A., Lee, J. R., Oestreich, A. J. and Katzmann, D. J. 2009b. Membrane protein targeting to the MVB/lysosome. *Chem Rev.* **109**: 1575-1586.

De Hertogh, B., Carvajal, E., Talla, E., Dujon, B., Baret, P. and Goffeau, A. 2002. Phylogenetic classification of transporters and other membrane proteins from *Saccharomyces cerevisiae*. *Funct Integr Genomics.* **2**: 154-170.

De Hertogh, B., Hancy, F., Goffeau, A. and Baret, P. V. 2006. Emergence of species-specific transporters during evolution of the hemiascomycete phylum. *Genetics.* **172**: 771-781.

De la Rosa, J. M., Ruiz, T. and Rodriguez, L. 2000. Cloning and characterization of a *Candida albicans* gene homologous to fructose-1,6-bisphosphatase genes. *Curr Microbiol.* **41**: 384-387.

De Marcucci, O. and Lindsay, J. G. 1985. Component X. An immunologically distinct polypeptide associated with mammalian pyruvate dehydrogenase multi-enzyme complex. *Eur J Biochem.* **149**: 641-648.

De Virgilio, C., Burckert, N., Barth, G., Neuhaus, J. M., Boller, T. and Wiemken, A. 1992. Cloning and disruption of a gene required for growth on acetate but not on ethanol: the acetyl-coenzyme A synthetase gene of *Saccharomyces cerevisiae*. *Yeast.* **8**: 1043-1051.

Delcourt, F., Taillandier, P., Vidal, F. and Strehaiano, P. 1995. Influence of pH, malic acid and glucose concentrations on malic acid consumption by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol.* **43**: 321-324.

Deutschbauer, A. M., Williams, R. M., Chu, A. M. and Davis, R. W. 2002. Parallel phenotypic analysis of sporulation and postgermination growth in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A.* **99**: 15530-15535.

Diallinas, G. 2008. Biochemistry. An almost-complete movie. *Science.* **322**: 1644-1645.

Dietrich, F. S., Voegeli, S., Brachat, S., Lerch, A., Gates, K., Steiner, S., Mohr, C., Pohlmann, R., Luedi, P., Choi, S., *et al.*. 2004. The *Ashbya gossypii* genome as a tool for mapping the ancient *Saccharomyces cerevisiae* genome. *Science.* **304**: 304-307.

Diezmann, S., Cox, C. J., Schonian, G., Vilgalys, R. J. and Mitchell, T. G. 2004. Phylogeny and evolution of medical species of *Candida* and related taxa: a multigenic analysis. *J Clin Microbiol.* **42**: 5624-5635.

Doedt, T., Krishnamurthy, S., Bockmuhl, D. P., Tebarth, B., Stempel, C., Russell, C. L., Brown, A. J. and Ernst, J. F. 2004. APSES proteins regulate morphogenesis and metabolism in *Candida albicans*. *Mol Biol Cell.* **15**: 3167-3180.

Douglas, J. R. and Dawes, I. W. 1987. Cloning and characterization of the gene encoding lipoamide dehydrogenase in *Saccharomyces cerevisiae*. *J Gen Microbiol.* **133**: 925-933.

Dujon, B., Sherman, D., Fischer, G., Durrens, P., Casaregola, S., Lafontaine, I., De Montigny, J., Marck, C., Neuveglise, C., Talla, E., *et al.* 2004. Genome evolution in yeasts. *Nature.* **430**: 35-44.

Dunn, R., Klos, D. A., Adler, A. S. and Hicke, L. 2004. The C2 domain of the Rsp5 ubiquitin ligase binds membrane phosphoinositides and directs ubiquitination of endosomal cargo. *J Cell Biol.* **165**: 135-144.

Enomoto, K., Ohki, R. and Muratsubaki, H. 1996. Cloning and sequencing of the gene encoding the soluble fumarate reductase from *Saccharomyces cerevisiae*. *DNA Res.* **3**: 263-267.

Entian, K. D., Vogel, R. F., Rose, M., Hofmann, L. and Mecke, D. 1988. Isolation and primary structure of the gene encoding fructose-1,6-bisphosphatase from *Saccharomyces cerevisiae*. *FEBS Lett.* **236**: 195-200.

Fatichenti, F., Farris, G. A., Deiana, P., and Ceccarelli, S. 1984. Malic acid production and consumption by selected *Saccharomyces cerevisiae* under anaerobic and aerobic conditions *Appl Microbiol Biotechnol.* **19**: 427-429.

Felsenstein, J. 1989. PHYLIP – Phylogeny Inference Package (Version 3.2). *Cladistics* **5**: 5: 164–166.

Fernandez-Arenas, E., Cabezon, V., Bermejo, C., Arroyo, J., Nombela, C., Diez-Orejas, R. and Gil, C. 2007. Integrated proteomics and genomics strategies bring new insight into *Candida albicans* response upon macrophage interaction. *Mol Cell Proteomics.* **6**: 460-478.

Fernandez, E., Fernandez, M. and Rodicio, R. 1993. Two structural genes are encoding malate synthase isoenzymes in *Saccharomyces cerevisiae*. *FEBS Lett.* **320**: 271-275.

Fernandez, E., Moreno, F. and Rodicio, R. 1992. The *ICL1* gene from *Saccharomyces cerevisiae*. *Eur J Biochem.* **204**: 983-990.

Finley, D., Bartel, B. and Varshavsky, A. 1989. The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature.* **338**: 394-401.

Finley, D., Sadis, S., Monia, B. P., Boucher, P., Ecker, D. J., Crooke, S. T. and Chau, V. 1994. Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol Cell Biol.* **14**: 5501-5509.

Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y. L. and Postlethwait, J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics.* **151**: 1531-1545.

Futter, C. E., Pearse, A., Hewlett, L. J. and Hopkins, C. R. 1996. Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. *J Cell Biol.* **132**: 1011-1023.

Galagan, J. E., Calvo, S. E., Borkovich, K. A., Selker, E. U., Read, N. D., Jaffe, D., FitzHugh, W., Ma, L. J., Smirnov, S., Purcell, *et al.* 2003. The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature.* **422**: 859-868.

Galan, J. M. and Haguenauer-Tsapis, R. 1997. Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. *EMBO J.* **16**: 5847-5854.

Gancedo, C., and Serrano, R. 1989. Energy yelding metabolism. In: The yeasts, vol. 3 A. H. Rose and J. S. Harrison (Eds). Academic Press Limited, London. pp 205-251.

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

Gangloff, S. P., Marguet, D. and Lauquin, G. J. 1990. Molecular cloning of the yeast mitochondrial aconitase gene (*ACO1*) and evidence of a synergistic regulation of expression by glucose plus glutamate. *Mol Cell Biol.* **10**: 3551-3561.

Garcia-Sanchez, S., Mavor, A. L., Russell, C. L., Argimon, S., Dennison, P., Enjalbert, B. and Brown, A. J. 2005. Global roles of Ssn6 in Tup1- and Nrg1-dependent gene regulation in the fungal pathogen, *Candida albicans*. *Mol Biol Cell.* **16**: 2913-2925.

Garcia, C. K., Brown, M. S., Pathak, R. K. and Goldstein, J. L. 1995. cDNA cloning of MCT2, a second monocarboxylate transporter expressed in different cells than MCT1. *J Biol Chem.* **270**: 1843-1849.

Garcia, C. K., Goldstein, J. L., Pathak, R. K., Anderson, R. G. and Brown, M. S. 1994. Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. *Cell.* **76**: 865-873.

Gargas, A., DePriest, P. T., Grube, M. and Tehler, A. 1995. Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science.* **268**: 1492-1495.

Gerhart, D. Z., Enerson, B. E., Zhdankina, O. Y., Leino, R. L. and Drewes, L. R. 1997. Expression of monocarboxylate transporter MCT1 by brain endothelium and glia in adult and suckling rats. *Am J Physiol.* **273**: E207-213.

Gerhart, D. Z., Enerson, B. E., Zhdankina, O. Y., Leino, R. L. and Drewes, L. R. 1998. Expression of the monocarboxylate transporter MCT2 by rat brain glia. *Glia.* **22**: 272-281.

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., *et al.* 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature.* **418**: 387-391.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H. and Oliver, S. G. 1996. Life with 6000 genes. *Science.* **274**: 546, 563-547.

Gould, S. J., Subramani, S. and Scheffler, I. E. 1989. Use of the DNA polymerase chain reaction for homology probing: isolation of partial cDNA or genomic clones encoding the iron-sulfur protein of succinate dehydrogenase from several species. *Proc Natl Acad Sci U S A.* **86**: 1934-1938.

Gregori, C., Schuller, C., Roetzer, A., Schwarzmuller, T., Ammerer, G. and Kuchler, K. 2007. The high-osmolarity glycerol response pathway in the human fungal pathogen *Candida glabrata* strain ATCC2001 lacks a signaling branch that operates in baker's yeast. *Eukaryot Cell.* **6**: 1635-1645.

Grobler, J., Bauer, F., Subden, R. E. and Van Vuuren, H. J. 1995. The *mae1* gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C4 dicarboxylic acids. *Yeast.* **11**: 1485-1491.

Gruenberg, J. 2001. The endocytic pathway: a mosaic of domains. *Nat Rev Mol Cell Biol.* **2**: 721-730.

Guiard, B. 1985. Structure, expression and regulation of a nuclear gene encoding a mitochondrial protein: the yeast L(+)-lactate cytochrome c oxidoreductase (cytochrome b2). *EMBO J.* **4**: 3265-3272.

Haarasilta, S. and Taskinen, L. 1977. Location of three key enzymes of gluconeogenesis in baker's yeast. *Arch Microbiol.* **113**: 159-161.

Haglund, K. and Dikic, I. 2005. Ubiquitylation and cell signaling. *EMBO J.* **24**: 3353-3359.

Halestrap, A. P. 1976. Mechanism of inhibition of mitochondrial pyruvate transporter by alpha-cyanocinnamate derivatives. *Biochem J* **156**: 181–183.

Halestrap, A. P. and Meredith, D. 2004. The *SLC16* gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch.* **447**: 619-628.

Halestrap, A. P. and Price, N. T. 1999. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J.* **343**: 281-299.

Hao, H. X., Khalimonchuk, O., Schraders, M., Dephoure, N., Bayley, J. P., Kunst, H., Devilee, P., Cremers, C. W., Schiffman, J. D., Bentz, B. *et al.* 2009. *SDH5*, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma. *Science.* **325**: 1139-1142.

Harcus, D., Nantel, A., Marcil, A., Rigby, T. and Whiteway, M. 2004. Transcription profiling of cyclic AMP signaling in *Candida albicans*. *Mol Biol Cell.* **15**: 4490-4499.

Hartig, A., Simon, M. M., Schuster, T., Daugherty, J. R., Yoo, H. S. and Cooper, T. G. 1992. Differentially regulated malate synthase genes participate in carbon and nitrogen metabolism of *S. cerevisiae*. *Nucleic Acids Res.* **20**: 5677-5686.

Haurie, V., Perrot, M., Mini, T., Jeno, P., Sagliocco, F. and Boucherie, H. 2001. The transcriptional activator Cat8p provides a major contribution to the reprogramming of carbon metabolism during the diauxic shift in *Saccharomyces cerevisiae*. *J Biol Chem.* **276**: 76-85.

Hazen, K. C. 1995. New and emerging yeast pathogens. *Clin Microbiol Rev.* **8**: 462-478.

Heckman, D. S., Geiser, D. M., Eidell, B. R., Stauffer, R. L., Kardos, N. L. and Hedges, S. B. 2001. Molecular evidence for the early colonization of land by fungi and plants. *Science.* **293**: 1129-1133.

Helliwell, S. B., Losko, S. and Kaiser, C. A. 2001. Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. *J Cell Biol.* **153**: 649-662.

Henry, P. C., Kanelis, V., O'Brien, M. C., Kim, B., Gautschi, I., Forman-Kay, J., Schild, L. and Rotin, D. 2003. Affinity and specificity of interactions between Nedd4 isoforms and the epithelial Na^+ channel. *J Biol Chem.* **278**: 20019-20028.

Hernandez, R., Nombela, C., Diez-Orejas, R. and Gil, C. 2004. Two-dimensional reference map of *Candida albicans* hyphal forms. *Proteomics.* **4**: 374-382.

Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, M., Cannon, P. F., Eriksson, O. E., Huhndorf, S., James, T., Kirk, P. M., Lucking, R., *et al.* 2007. A higher-level phylogenetic classification of the Fungi. *Mycol Res.* **111**: 509-547.

Hicke, L. and Dunn, R. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol.* **19**: 141-172.

Hicke, L. and Riezman, H. 1996. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell.* **84**: 277-287.

Hicke, L., Zanolari, B. and Riezman, H. 1998. Cytoplasmic tail phosphorylation of the alpha-factor receptor is required for its ubiquitination and internalization. *J Cell Biol.* **141**: 349-358.

Hikida, M., Atomi, H., Fukuda, Y., Aoki, A., Hishida, T., Teranishi, Y., Ueda, M. and Tanaka, A. 1991. Presence of two transcribed malate synthase genes in an n-alkane-utilizing yeast, *Candida tropicalis*. *J Biochem.* **110**: 909-914.

Hildyard, J. C. and Halestrap, A. P. 2003. Identification of the mitochondrial pyruvate carrier in *Saccharomyces cerevisiae*. *Biochem J.* **374**: 607-611.

Hohmann, S. 1991. Characterization of *PDC6*, a third structural gene for pyruvate decarboxylase in *Saccharomyces cerevisiae*. *J Bacteriol.* **173**: 7963-7969.

Hohmann, S. and Cederberg, H. 1990. Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. *Eur J Biochem.* **188**: 615-621.

Horak, J. 2003. The role of ubiquitin in down-regulation and intracellular sorting of membrane proteins: insights from yeast. *Biochim Biophys Acta.* **1614**: 139-155.

Hromatka, B. S., Noble, S. M. and Johnson, A. D. 2005. Transcriptional response of *Candida albicans* to nitric oxide and the role of the *YHB1* gene in nitrosative stress and virulence. *Mol Biol Cell.* **16**: 4814-4826.

Huibregtse, J. M., Yang, J. C. and Beaudenon, S. L. 1997. The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. *Proc Natl Acad Sci U S A.* **94**: 3656-3661.

Idnurm, A. and Howlett, B. J. 2002. Isocitrate lyase is essential for pathogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). *Eukaryot Cell.* **1**: 719-724.

Im, Y. J. and Hurley, J. H. 2008. Integrated structural model and membrane targeting mechanism of the human ESCRT-II complex. *Dev Cell.* **14**: 902-913.

Jackson, V. N., Price, N. T., Carpenter, L. and Halestrap, A. P. 1997. Cloning of the monocarboxylate transporter isoform MCT2 from rat testis provides evidence that expression in tissues is species-specific and may involve post-transcriptional regulation. *Biochem J.* **324**: 447-453.

James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., *et al.* 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature.* **443**: 818-822.

Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H. and Sommer, T. 2002. Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat Cell Biol.* **4**: 134-139.

Jennings, M. L. and Adams-Lackey, M. 1982. A rabbit erythrocyte membrane protein associated with L-lactate transport. *J Biol Chem.* **257**: 12866-12871.

Jia, Y. K., Becam, A. M. and Herbert, C. J. 1997. The *CIT3* gene of *Saccharomyces cerevisiae* encodes a second mitochondrial isoform of citrate synthase. *Mol Microbiol.* **24**: 53-59.

Jones, T., Federspiel, N. A., Chibana, H., Dungan, J., Kalman, S., Magee, B. B., Newport, G., Thorstenson, Y. R., Agabian, N., Magee, P. T., Davis, R. W. and Scherer, S. 2004. The diploid genome sequence of *Candida albicans*. *Proc Natl Acad Sci U S A.* **101**: 7329-7334.

Kal, A. J., van Zonneveld, A. J., Benes, V., van den Berg, M., Koerkamp, M. G., Albermann, K., Strack, N., Ruijter, J. M., Richter, A., Dujon, B., Ansorge, W. and Tabak, H. F. 1999. Dynamics of gene expression revealed by comparison of serial analysis of gene expression transcript profiles from yeast grown on two different carbon sources. *Mol Biol Cell.* **10**: 1859-1872.

Katzmann, D. J., Babst, M. and Emr, S. D. 2001. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell.* **106**: 145-155.

Katzmann, D. J., Odorizzi, G. and Emr, S. D. 2002. Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol.* **3**: 893-905.

Katzmann, D. J., Sarkar, S., Chu, T., Audhya, A. and Emr, S. D. 2004. Multivesicular body sorting: ubiquitin ligase Rsp5 is required for the modification and sorting of carboxypeptidase S. *Mol Biol Cell.* **15**: 468-480.

Katzmann, D. J., Stefan, C. J., Babst, M. and Emr, S. D. 2003. Vps27 recruits ESCRT machinery to endosomes during MVB sorting. *J Cell Biol.* **162**: 413-423.

Kee, Y., Lyon, N. and Huibregtse, J. M. 2005. The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. *EMBO J.* **24**: 2414-2424.

Kee, Y., Munoz, W., Lyon, N. and Huibregtse, J. M. 2006. The deubiquitinating enzyme Ubp2 modulates Rsp5-dependent Lys63-linked polyubiquitin conjugates in *Saccharomyces cerevisiae*. *J Biol Chem.* **281**: 36724-36731.

Keller, B. A., Patel, S. and Fisher, L. M. 1997. Molecular cloning and expression of the *Candida albicans TOP2* gene allows study of fungal DNA topoisomerase II inhibitors in yeast. *Biochem J.* **324**: 329-339.

Kellermann, E., Seeboth, P. G. and Hollenberg, C. P. 1986. Analysis of the primary structure and promoter function of a pyruvate decarboxylase gene (*PDC1*) from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **14**: 8963-8977.

Kellis, M., Birren, B. W. and Lander, E. S. 2004. Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. *Nature.* **428**: 617-624.

Kellis, M., Patterson, N., Endrizzi, M., Birren, B. and Lander, E. S. 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature.* **423**: 241-254.

Kerscher, O., Sepuri, N. B. and Jensen, R. E. 2000. Tim18p is a new component of the Tim54p-Tim22p translocon in the mitochondrial inner membrane. *Mol Biol Cell.* **11**: 103-116.

Kim, C. M., Goldstein, J. L. and Brown, M. S. 1992. cDNA cloning of *MEV*, a mutant protein that facilitates cellular uptake of mevalonate, and identification of the point mutation responsible for its gain of function. *J Biol Chem.* **267**: 23113-23121.

Kim, D. K., Kanai, Y., Chairoungdua, A., Matsuo, H., Cha, S. H. and Endou, H. 2001. Expression cloning of a Na^+-independent aromatic amino acid transporter with structural similarity to H+/monocarboxylate transporters. *J Biol Chem.* **276**: 17221-17228.

Kim, H. C. and Huibregtse, J. M. 2009. Polyubiquitination by HECT E3s and the determinants of chain type specificity. *Mol Cell Biol.* **29**: 3307-3318.

Kim, H. T., Kim, K. P., Lledias, F., Kisselev, A. F., Scaglione, K. M., Skowyra, D., Gygi, S. P. and Goldberg, A. L. 2007. Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. *J Biol Chem.* **282**: 17375-17386.

Kim, K. S., Rosenkrantz, M. S. and Guarente, L. 1986. *Saccharomyces cerevisiae* contains two functional citrate synthase genes. *Mol Cell Biol.* **6**: 1936-1942.

Kirk, P. M., P. F. Cannon, J. C. David, and J. A. Staplers (ed.) 2001. Ainsworth & Bisby's dictionary of the fungi, 9th ed. CABI Publishing.

Kloetzel, P. M. 2001. Antigen processing by the proteasome. *Nat Rev Mol Cell Biol.* **2**: 179-187.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U. and Jentsch, S. 1999. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell.* **96**: 635-644.

Kolling, R. and Hollenberg, C. P. 1994. The ABC-transporter Ste6 accumulates in the plasma membrane in a ubiquitinated form in endocytosis mutants. *EMBO J.* **13**: 3261-3271.

Koszul, R., Caburet, S., Dujon, B. and Fischer, G. 2004. Eucaryotic genome evolution through the spontaneous duplication of large chromosomal segments. *EMBO J.* **23**: 234-243.

Kreger-vav Rij, N. J. W. 1984. (ed.) The yeast—a taxonomic study, 3rd ed. Elsevier, Amsterdam.

Kresze, G. B. and Ronft, H. 1981. Pyruvate dehydrogenase complex from baker's yeast. 2. Molecular structure, dissociation, and implications for the origin of mitochondria. *Eur J Biochem.* **119**: 581-587.

Kuczynski, J. T. and Radler, F. 1982. The anaerobic metabolism of malate of *Saccharomyces bailii* and the partial purification and characterization of malic enzyme. *Arch Microbiol.* **131**: 266-270.

Kunze, M., Kragler, F., Binder, M., Hartig, A. and Gurvitz, A. 2002. Targeting of malate synthase 1 to the peroxisomes of *Saccharomyces cerevisiae* cells depends on growth on oleic acid medium. *Eur J Biochem.* **269**: 915-922.

Kurtzman, C. P., and Fell JW, eds. 1998. The yeasts, a taxonomic study, 4th ed. Amsterdam: Elsevier. 1055 p.

Labeyrie, F. and Slonimski, P. P. 1964. Mode of Action of Lactate Dehydrogenases Linked to Flavin and Cytochrome Systems. *Bull Soc Chim Biol (Paris).* **46**: 1793-1828.

Lachance, M.-A., and Starmer, W.T. 1998. Ecology and yeasts. In:Kurtzman CP, Fell JW, eds. The yeasts, a taxonomic study. 4th ed. Amsterdam: Elsevier. p 23–30.

Lam, M. H., Urban-Grimal, D., Bugnicourt, A., Greenblatt, J. F., Haguenauer-Tsapis, R. and Emili, A. 2009. Interaction of the deubiquitinating enzyme Ubp2 and the e3 ligase Rsp5 is required for transporter/receptor sorting in the multivesicular body pathway. *PLoS One.* **4**: e4259.

Lan, C. Y., Newport, G., Murillo, L. A., Jones, T., Scherer, S., Davis, R. W. and Agabian, N. 2002. Metabolic specialization associated with phenotypic switching in *Candida albicans*. *Proc Natl Acad Sci U S A.* **99**: 14907-14912.

Lan, C. Y., Rodarte, G., Murillo, L. A., Jones, T., Davis, R. W., Dungan, J., Newport, G. and Agabian, N. 2004. Regulatory networks affected by iron availability in *Candida albicans*. *Mol Microbiol.* **53**: 1451-1469.

Lawrence, C. L., Botting, C. H., Antrobus, R. and Coote, P. J. 2004. Evidence of a new role for the high-osmolarity glycerol mitogen-activated protein kinase pathway in yeast: regulating adaptation to citric acid stress. *Mol Cell Biol.* **24**: 3307-3323.

Lawson, J. E., Behal, R. H. and Reed, L. J. 1991. Disruption and mutagenesis of the *Saccharomyces cerevisiae PDX1* gene encoding the protein X component of the pyruvate dehydrogenase complex. *Biochemistry.* **30**: 2834-2839.

Lemire, B. D. and Oyedotun, K. S. 2002. The *Saccharomyces cerevisiae* mitochondrial succinate:ubiquinone oxidoreductase. *Biochim Biophys Acta.* **1553**: 102-116.

Leon, S. and Haguenauer-Tsapis, R. 2009. Ubiquitin ligase adaptors: regulators of ubiquitylation and endocytosis of plasma membrane proteins. *Exp Cell Res.* **315**: 1574-1583.

Leuker, C. E., Sonneborn, A., Delbruck, S. and Ernst, J. F. 1997. Sequence and promoter regulation of the *PCK1* gene encoding phosphoenolpyruvate carboxykinase of the fungal pathogen *Candida albicans*. *Gene.* **192**: 235-240.

Levy, F., Muehlethaler, K., Salvi, S., Peitrequin, A. L., Lindholm, C. K., Cerottini, J. C. and Rimoldi, D. 2005. Ubiquitylation of a melanosomal protein by HECT-E3 ligases serves as sorting signal for lysosomal degradation. *Mol Biol Cell.* **16**: 1777-1787.

Lin, R. Y., Vera, J. C., Chaganti, R. S. and Golde, D. W. 1998. Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter. *J Biol Chem.* **273**: 28959-28965.

Llorente, B., Durrens, P., Malpertuy, A., Aigle, M., Artiguenave, F., Blandin, G., Bolotin-Fukuhara, M., Bon, E., Brottier, P., Casaregola, *et al.* 2000a. Genomic exploration of the hemiascomycetous yeasts: 20. Evolution of gene redundancy compared to *Saccharomyces cerevisiae*. *FEBS Lett.* **487**: 122-133.

Llorente, B., Malpertuy, A., Neuveglise, C., de Montigny, J., Aigle, M., Artiguenave, F., Blandin, G., Bolotin-Fukuhara, M., Bon, E., Brottier, P., *et al.* 2000b. Genomic exploration of the hemiascomycetous yeasts: 18. Comparative analysis of chromosome maps and synteny with *Saccharomyces cerevisiae*. *FEBS Lett.* **487**: 101-112.

Lodi, T., Diffels, J., Goffeau, A. and Baret, P. V. 2007. Evolution of the carboxylate Jen transporters in fungi. *FEMS Yeast Res.* **7**: 646-656.

Lodi, T. and Ferrero, I. 1993. Isolation of the *DLD* gene of *Saccharomyces cerevisiae* encoding the mitochondrial enzyme D-lactate ferricytochrome c oxidoreductase. *Mol Gen Genet.* **238**: 315-324.

Lodi, T., Fontanesi, F., Ferrero, I. and Donnini, C. 2004. Carboxylic acids permeases in yeast: two genes in *Kluyveromyces lactis*. *Gene.* **339**: 111-119.

Lodi, T., Fontanesi, F. and Guiard, B. 2002. Co-ordinate regulation of lactate metabolism genes in yeast: the role of the lactate permease gene *JEN1*. *Mol Genet Genomics.* **266**: 838-847.

Lombardo, A., Carine, K. and Scheffler, I. E. 1990. Cloning and characterization of the iron-sulfur subunit gene of succinate dehydrogenase from *Saccharomyces cerevisiae*. *J Biol Chem.* **265**: 10419-10423.

Lorenz, M. C., Bender, J. A. and Fink, G. R. 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell.* **3**: 1076-1087.

Lorenz, M. C. and Fink, G. R. 2001. The glyoxylate cycle is required for fungal virulence. *Nature.* **412**: 83-86.

Lorenz, M. C. and Fink, G. R. 2002. Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryot Cell.* **1**: 657-662.

Luttik, M. A., Kotter, P., Salomons, F. A., van der Klei, I. J., van Dijken, J. P. and Pronk, J. T. 2000. The *Saccharomyces cerevisiae ICL2* gene encodes a mitochondrial 2-methylisocitrate lyase involved in propionyl-coenzyme A metabolism. *J Bacteriol.* **182**: 7007-7013.

Lutzoni, F., Kauff, F., Cox, C.J., McLaughlin, D., Celio, G., Dentinger, B.,, Padamsee, M., Hibbett, D.S., James, T.Y., Baloch, *et al.* and 2004. Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *American Journal of Botany*. **91**: 1446–1480.

Lynch, M. and Force, A. 2000. The probability of duplicate gene preservation by subfunctionalization. *Genetics.* **154**: 459-473.

Ma, Q. 2007. Aryl hydrocarbon receptor degradation-promoting factor (*ADPF*) and the control of the xenobiotic response. *Mol Interv.* **7**: 133-137.

Maeta, K., Izawa, S. and Inoue, Y. 2005. Methylglyoxal, a metabolite derived from glycolysis, functions as a signal initiator of the high osmolarity glycerol-mitogen-activated protein kinase cascade and calcineurin/Crz1-mediated pathway in *Saccharomyces cerevisiae*. *J Biol Chem.* **280**: 253-260.

Maeting, I., Schmidt, G., Sahm, H., Revuelta, J. L., Stierhof, Y. D. and Stahmann, K. P. 1999. Isocitrate lyase of Ashbya gossypii--transcriptional regulation and peroxisomal localization. *FEBS Lett.* **444**: 15-21.

Maglott, D., Ostell, J., Pruitt, K. D. and Tatusova, T. 2007. Entrez Gene: gene-centered information at NCBI. *Nucleic Acids Res.* **35**: D26-31.

Makuc, J., Paiva, S., Schauen, M., Kramer, R., Andre, B., Casal, M., Leao, C. and Boles, E. 2001. The putative monocarboxylate permeases of the yeast *Saccharomyces cerevisiae* do not transport monocarboxylic acids across the plasma membrane. *Yeast.* **18**: 1131-1143.

Marchal, C., Haguenauer-Tsapis, R. and Urban-Grimal, D. 1998. A PEST-like sequence mediates phosphorylation and efficient ubiquitination of yeast uracil permease. *Mol Cell Biol.* **18**: 314-321.

Marchal, C., Haguenauer-Tsapis, R. and Urban-Grimal, D. 2000. Casein kinase I-dependent phosphorylation within a PEST sequence and ubiquitination at nearby lysines signal endocytosis of yeast uracil permease. *J Biol Chem.* **275**: 23608-23614.

Marger, M. D. and Saier, M. H., Jr. 1993. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends Biochem Sci.* **18**: 13-20.

McAlister-Henn, L. and Thompson, L. M. 1987. Isolation and expression of the gene encoding yeast mitochondrial malate dehydrogenase. *J Bacteriol.* **169**: 5157-5166.

McCammon, M. T., Veenhuis, M., Trapp, S. B. and Goodman, J. M. 1990. Association of glyoxylate and beta-oxidation enzymes with peroxisomes of *Saccharomyces cerevisiae*. *J Bacteriol.* **172**: 5816-5827.

McKinney, J. D., Honer zu Bentrup, K., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr. and Russell, D. G. 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature.* **406**: 735-738.

Mewes, H. W., Albermann, K., Bahr, M., Frishman, D., Gleissner, A., Hani, J., Heumann, K., Kleine, K., Maierl, A., Oliver, S. G., Pfeiffer, F. and Zollner, A. 1997. Overview of the yeast genome. *Nature.* **387**: 7-65.

Meyer, W., Latouche, G. N., Daniel, H. M., Thanos, M., Mitchell, T. G., Yarrow, D., Schonian, G. and Sorrell, T. C. 1997. Identification of pathogenic yeasts of the imperfect genus *Candida* by polymerase chain reaction fingerprinting. *Electrophoresis.* **18**: 1548-1559.

Millard, S. M. and Wood, S. A. 2006. Riding the DUBway: regulation of protein trafficking by deubiquitylating enzymes. *J Cell Biol.* **173**: 463-468.

Minard, K. I. and McAlister-Henn, L. 1991. Isolation, nucleotide sequence analysis, and disruption of the *MDH2* gene from *Saccharomyces cerevisiae*: evidence for three isozymes of yeast malate dehydrogenase. *Mol Cell Biol.* **11**: 370-380.

Miran, S. G., Lawson, J. E. and Reed, L. J. 1993. Characterization of *PDH* beta 1, the structural gene for the pyruvate dehydrogenase beta subunit from *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A.* **90**: 1252-1256.

Miranda, M. and Sorkin, A. 2007. Regulation of receptors and transporters by ubiquitination: new insights into surprisingly similar mechanisms. *Mol Interv.* **7**: 157-167.

Mollapour, M. and Piper, P. W. 2006. Hog1p mitogen-activated protein kinase determines acetic acid resistance in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **6**: 1274-1280.

Mollapour, M. and Piper, P. W. 2007. Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Mol Cell Biol.* **27**: 6446-6456.

Morvan, J., Froissard, M., Haguenauer-Tsapis, R. and Urban-Grimal, D. 2004. The ubiquitin ligase Rsp5p is required for modification and sorting of membrane proteins into multivesicular bodies. *Traffic.* **5**: 383-392.

Mosesson, Y., Shtiegman, K., Katz, M., Zwang, Y., Vereb, G., Szollosi, J. and Yarden, Y. 2003. Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. *J Biol Chem.* **278**: 21323-21326.

Murad, A. M., d'Enfert, C., Gaillardin, C., Tournu, H., Tekaia, F., Talibi, D., Marechal, D., Marchais, V., Cottin, J. and Brown, A. J. 2001. Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors CaTup1, CaMig1 and CaNrg1. *Mol Microbiol.* **42**: 981-993.

Muratsubaki, H. and Enomoto, K. 1998. One of the fumarate reductase isoenzymes from *Saccharomyces cerevisiae* is encoded by the *OSM1* gene. *Arch Biochem Biophys.* **352**: 175-181.

Nagai, A., Takebe, K., Nio-Kobayashi, J., Takahashi-Iwanaga, H. and Iwanaga, T. 2009. Cellular Expression of the Monocarboxylate Transporter (MCT) Family in the Placenta of Mice. *Placenta.*

Nelissen, B., De Wachter, R. and Goffeau, A. 1997. Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. *FEMS Microbiol Rev.* **21**: 113-134.

Nikko, E., Sullivan, J. A. and Pelham, H. R. 2008. Arrestin-like proteins mediate ubiquitination and endocytosis of the yeast metal transporter Smf1. *EMBO Rep.* **9**: 1216-1221.

Nikolaou, E., Agrafioti, I., Stumpf, M., Quinn, J., Stansfield, I. and Brown, A. J. 2009. Phylogenetic diversity of stress signalling pathways in fungi. *BMC Evol Biol.* **9**: 44.

Nishida, H., and J. Sugiyama. 1994. Archiascomycetes: detection of a major new lineage within the ascomycota. *Mycoscience* **35**:361–366.

Niu, X. D., Browning, K. S., Behal, R. H. and Reed, L. J. 1988. Cloning and nucleotide sequence of the gene for dihydrolipoamide acetyltransferase from *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A.* **85**: 7546-7550.

Nygaard, A. P. 1961. Induction of D(-)- and L-lactic cytochrome c reductase in yeast. *J Biol Chem.* **236**: 1585-1588.

Obita, T., Saksena, S., Ghazi-Tabatabai, S., Gill, D. J., Perisic, O., Emr, S. D. and Williams, R. L. 2007. Structural basis for selective recognition of ESCRT-III by the AAA ATPase Vps4. *Nature.* **449**: 735-739.

Ochman, H., Daubin, V. and Lerat, E. 2005. A bunch of fun-guys: the whole-genome view of yeast evolution. *Trends Genet.* **21**: 1-3.

Odds, F. C., Rinaldi, M. G., Cooper, C. R., Jr., Fothergill, A., Pasarell, L. and McGinnis, M. R. 1997. *Candida* and *Torulopsis*: a blinded evaluation of use of pseudohypha formation as basis for identification of medically important yeasts. *J Clin Microbiol.* **35**: 313-316.

Ohno, S. 1970. Evolution by Gene Duplication. Allen & Unwin, London/Springer-Verlag, New York.

Ohno, S. 1998. The notion of the *Cambrian pananimalia* genome and a genomic difference that separated vertebrates from invertebrates. *Prog Mol Subcell Biol.* **21**: 97-117.

Osothsilp, C. and Subden, R. E. 1986. Malate transport in *Schizosaccharomyces pombe*. *J Bacteriol.* **168**: 1439-1443.

Oyedotun, K. S. and Lemire, B. D. 1997. The carboxyl terminus of the *Saccharomyces cerevisiae* succinate dehydrogenase membrane subunit, Sdh4p, is necessary for ubiquinone reduction and enzyme stability. *J Biol Chem.* **272**: 31382-31388.

Oyedotun, K. S. and Lemire, B. D. 1999. The *Saccharomyces cerevisiae* succinate-ubiquinone oxidoreductase. Identification of Sdh3p amino acid residues involved in ubiquinone binding. *J Biol Chem.* **274**: 23956-23962.

Paiva, S., Devaux, F., Barbosa, S., Jacq, C. and Casal, M. 2004. Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. *Yeast.* **21**: 201-210.

Paiva, S., Kruckeberg, A. L. and Casal, M. 2002. Utilization of green fluorescent protein as a marker for studying the expression and turnover of the monocarboxylate permease Jen1p of *Saccharomyces cerevisiae*. *Biochem J.* **363**: 737-744.

Paiva, S., Vieira, N., Nondier, I., Haguenauer-Tsapis, R., Casal, M. and Urban-Grimal, D. 2009. Glucose-induced ubiquitylation and endocytosis of the yeast Jen1 transporter: role of lysine 63-linked ubiquitin chains. *J Biol Chem.* **284**: 19228-19236.

Palkova, Z., Devaux, F., Icicova, M., Minarikova, L., Le Crom, S. and Jacq, C. 2002. Ammonia pulses and metabolic oscillations guide yeast colony development. *Mol Biol Cell.* **13**: 3901-3914.

Peleg, Y., Rokem, J. S. and Goldberg, I. 1990. A simple plate-assay for the screening of L-malic acid producing microorganisms. *FEMS Microbiol Lett.* **55**: 233-236.

Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D. and Gygi, S. P. 2003. A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol.* **21**: 921-926.

Pickart, C. M. and Fushman, D. 2004. Polyubiquitin chains: polymeric protein signals. *Curr Opin Chem Biol.* **8**: 610-616.

Piekarska, K., Hardy, G., Mol, E., van den Burg, J., Strijbis, K., van Roermund, C., van den Berg, M. and Distel, B. 2008. The activity of the glyoxylate cycle in peroxisomes of *Candida albicans* depends on a functional beta-oxidation pathway: evidence for reduced metabolite transport across the peroxisomal membrane. *Microbiology.* **154**: 3061-3072.

Piekarska, K., Mol, E., van den Berg, M., Hardy, G., van den Burg, J., van Roermund, C., MacCallum, D., Odds, F. and Distel, B. 2006. Peroxisomal fatty acid beta-oxidation is not essential for virulence of *Candida albicans*. *Eukaryot Cell.* **5**: 1847-1856.

Pines, O., Even-Ram, S., Elnathan, N., Battat, E., Aharonov, O., Gibson, D. and Goldberg, I. 1996. The cytosolic pathway of L-malic acid synthesis in *Saccharomyces cerevisiae*: the role of fumarase. *Appl Microbiol Biotechnol.* **46**: 393-399.

Pines, O., Shemesh, S., Battat, E. and Goldberg, I. 1997. Overexpression of cytosolic malate dehydrogenase (*MDH2*) causes overproduction of specific organic acids in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol.* **48**: 248-255.

Pitarch, A., Diez-Orejas, R., Molero, G., Pardo, M., Sanchez, M., Gil, C. and Nombela, C. 2001. Analysis of the serologic response to systemic *Candida albicans* infection in a murine model. *Proteomics.* **1**: 550-559.

Plant, P. J., Lafont, F., Lecat, S., Verkade, P., Simons, K. and Rotin, D. 2000. Apical membrane targeting of Nedd4 is mediated by an association of its C2 domain with annexin XIIIb. *J Cell Biol.* **149**: 1473-1484.

Poole, R. C. and Halestrap, A. P. 1992. Identification and partial purification of the erythrocyte L-lactate transporter. *Biochem J.* **283**: 855-862.

Poole, R. C. and Halestrap, A. P. 1993. Transport of lactate and other monocarboxylates across mammalian plasma membranes. *Am J Physiol*. **264**:761-782.

Poole, R. C., Sansom, C.E., and Halestrap, A.P 1996. Studies of the membrane topology of the rat erythrocyte H+/lactate cotransporter (MCT1). *Biochem J.* **320:**817-824.

Price, N. T., Jackson, V. N. and Halestrap, A. P. 1998. Cloning and sequencing of four new mammalian monocarboxylate transporter (MCT) homologues confirms the existence of a transporter family with an ancient past. *Biochem J.* **329 :**321-328.

Prigneau, O., Porta, A., Poudrier, J. A., Colonna-Romano, S., Noel, T. and Maresca, B. 2003. Genes involved in beta-oxidation, energy metabolism and glyoxylate cycle are induced by *Candida albicans* during macrophage infection. *Yeast.* **20**: 723-730.

Przybyla-Zawislak, B., Gadde, D. M., Ducharme, K. and McCammon, M. T. 1999. Genetic and biochemical interactions involving tricarboxylic acid cycle (TCA) function using a collection of mutants defective in all TCA cycle genes. *Genetics.* **152**: 153-166.

Queiros, O., Casal, M., Althoff, S., Moradas-Ferreira, P. and Leao, C. 1998. Isolation and characterization of *Kluyveromyces marxianus* mutants deficient in malate transport. *Yeast.* **14**: 401-407.

Queiros, O., Pereira, L., Paiva, S., Moradas-Ferreira, P. and Casal, M. 2007. Functional analysis of *Kluyveromyces lactis* carboxylic acids permeases: heterologous expression of *KlJEN1* and *KlJEN2* genes. *Curr Genet.* **51**: 161-169.

Rabitsch, K. P., Toth, A., Galova, M., Schleiffer, A., Schaffner, G., Aigner, E., Rupp, C., Penkner, A. M., Moreno-Borchart, A. C., Primig, M., Esposito, R. E., Klein, F., Knop, M. and Nasmyth, K. 2001. A screen for genes required for meiosis and spore formation based on whole-genome expression. *Curr Biol.* **11**: 1001-1009.

Radler, F., (1993) Yeasts—metabolism of organic acids. In: Fleet GH (ed) Wine microbiology and biotechnology. H. A. Publishers (ed). Switerzland, pp. 165–182.

Ramirez, M. A. and Lorenz, M. C. 2007. Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. *Eukaryot Cell.* **6**: 280-290.

Ramirez, M. A. and Lorenz, M. C. 2009. The transcription factor homolog *CTF1* regulates {beta}-oxidation in *Candida albicans*. *Eukaryot Cell.* **8**: 1604-1614.

Redzepovic, S., Orlic, S., Majdak, A., Kozina, B., Volschenk, H. and Viljoen-Bloom, M. 2003. Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation. *Int J Food Microbiol.* **83**: 49-61.

Reed, L., and Yeaman, S. J. 1987. Pyruvate dehydrogenase. *The Enzymes.* **18**: 77-95.

Reihl, P. and Stolz, J. 2005. The monocarboxylate transporter homolog Mch5p catalyzes riboflavin (vitamin B2) uptake in *Saccharomyces cerevisiae*. *J Biol Chem.* **280**: 39809-39817.

Robellet, X., Flipphi, M., Pegot, S., Maccabe, A. P. and Velot, C. 2008. AcpA, a member of the *GPR1/FUN34/YaaH* membrane protein family, is essential for acetate permease activity in the hyphal fungus *Aspergillus nidulans*. *Biochem J.* **412**: 485-493.

Robinson, K. M. and Lemire, B. D. 1992. Isolation and nucleotide sequence of the *Saccharomyces cerevisiae* gene for the succinate dehydrogenase flavoprotein subunit. *J Biol Chem.* **267**: 10101-10107.

Rodriguez, S. B. and Thornton, R. J. 1990. Factors influencing the utilisation of L-malate by yeasts. *FEMS Microbiol Lett.* **60**: 17-22.

Rohde, M., Lim, F. and Wallace, J. C. 1991. Electron microscopic localization of pyruvate carboxylase in rat liver and *Saccharomyces cerevisiae* by immunogold procedures. *Arch Biochem Biophys.* **290**: 197-201.

Roman, E., Nombela, C. and Pla, J. 2005. The Sho1 adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen *Candida albicans*. *Mol Cell Biol.* **25**: 10611-10627.

Ross, J., Reid, G. A. and Dawes, I. W. 1988. The nucleotide sequence of the *LPD1* gene encoding lipoamide dehydrogenase in *Saccharomyces cerevisiae*: comparison between eukaryotic and prokaryotic sequences for related enzymes and identification of potential upstream control sites. *J Gen Microbiol.* **134**: 1131-1139.

Rotin, D. and Kumar, S. 2009. Physiological functions of the HECT family of ubiquitin ligases. *Nat Rev Mol Cell Biol.* **10**: 398-409.

Saier, M. H., Jr. 2000. A functional-phylogenetic classification system for transmembrane solute transporters. *Microbiol Mol Biol Rev.* **64**: 354-411.

Saier, M. H., Jr. and Ren, Q. 2006. The bioinformatic study of transmembrane molecular transport. *J Mol Microbiol Biotechnol.* **11**: 289-290.

Saliola, M., Bartoccioni, P. C., De Maria, I., Lodi, T. and Falcone, C. 2004. The deletion of the succinate dehydrogenase gene *KlSDH1* in *Kluyveromyces lactis* does not lead to respiratory deficiency. *Eukaryot Cell.* **3**: 589-597.

Salmon, J. M. 1987. L-malic-acid permeation in resting cells of anaerobically grown *Saccharomyces cerevisiae*. *Biochim Biophys Acta.* **901**: 30-34.

Satyanarayana, T., Mandel, A. D. and Klein, H. P. 1974. Evidence for two immunologically distinct acetyl-co-enzyme A synthetase in yeast. *Biochim Biophys Acta.* **341**: 396-401.

Schuller, H. J. 2003. Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. *Curr Genet.* **43**: 139-160.

Schwartz, H., and Radler, F. 1988. Formation of L-malate by *Saccharomyces cerevisiae* during fermentation. *Appl Microbiol Biotechnol.* **27**: 553-560.

Semighini, C. P., Goldman, M. H. and Goldman, G. H. 2004. Multi-copy suppression of an *Aspergillus nidulans* mutant sensitive to camptothecin by a putative monocarboxylate transporter. *Curr Microbiol.* **49**: 229-233.

Sexton, J. A., Brown, V. and Johnston, M. 2007. Regulation of sugar transport and metabolism by the *Candida albicans* Rgt1 transcriptional repressor. *Yeast.* **24**: 847-860.

Shearwin-Whyatt, L., Dalton, H. E., Foot, N. and Kumar, S. 2006. Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. *Bioessays.* **28**: 617-628.

Shen, H. D., Choo, K. B., Lee, H. H., Hsieh, J. C., Lin, W. L., Lee, W. R. and Han, S. H. 1991. The 40-kilodalton allergen of *Candida albicans* is an alcohol dehydrogenase: molecular cloning and immunological analysis using monoclonal antibodies. *Clin Exp Allergy.* **21**: 675-681.

Shih, S. C., Katzmann, D. J., Schnell, J. D., Sutanto, M., Emr, S. D. and Hicke, L. 2002. Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. *Nat Cell Biol.* **4**: 389-393.

Singh, V., Sinha, I. and Sadhale, P. P. 2005. Global analysis of altered gene expression during morphogenesis of *Candida albicans* in vitro. *Biochem Biophys Res Commun.* **334**: 1149-1158.

Smith, D. A., Nicholls, S., Morgan, B. A., Brown, A. J. and Quinn, J. 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. *Mol Biol Cell.* **15**: 4179-4190.

Soares-Silva, I., Paiva, S., Kotter, P., Entian, K. D. and Casal, M. 2004. The disruption of *JEN1* from *Candida albicans* impairs the transport of lactate. *Mol Membr Biol.* **21**: 403-411.

Soetens, O., De Craene, J. O. and Andre, B. 2001. Ubiquitin is required for sorting to the vacuole of the yeast general amino acid permease, Gap1. *J Biol Chem.* **276**: 43949-43957.

Solomon, P. S., Lee, R. C., Wilson, T. J. and Oliver, R. P. 2004. Pathogenicity of *Stagonospora nodorum* requires malate synthase. *Mol Microbiol.* **53**: 1065-1073.

Sousa, M. J., Mota, M. and Leao, C. 1992. Transport of malic acid in the yeast *Schizosaccharomyces pombe*: evidence for a proton-dicarboxylate symport. *Yeast.* **8**: 1025-1031.

Staub, O. and Rotin, D. 2006. Role of ubiquitylation in cellular membrane transport. *Physiol Rev.* **86**: 669-707.

Steensma, H. Y., Holterman, L., Dekker, I., van Sluis, C. A. and Wenzel, T. J. 1990. Molecular cloning of the gene for the E1 alpha subunit of the pyruvate dehydrogenase complex from *Saccharomyces cerevisiae*. *Eur J Biochem.* **191**: 769-774.

Steffan, J. S. and McAlister-Henn, L. 1992. Isolation and characterization of the yeast gene encoding the *MDH3* isozyme of malate dehydrogenase. *J Biol Chem.* **267**: 24708-24715.

Stimpson, H. E., Lewis, M. J. and Pelham, H. R. 2006. Transferrin receptor-like proteins control the degradation of a yeast metal transporter. *EMBO J.* **25**: 662-672.

Stuchell-Brereton, M. D., Skalicky, J. J., Kieffer, C., Karren, M. A., Ghaffarian, S. and Sundquist, W. I. 2007. ESCRT-III recognition by VPS4 ATPases. *Nature.* **449**: 740-744.

Suh, S. O., Blackwell, M., Kurtzman, C. P. and Lachance, M. A. 2006. Phylogenetics of Saccharomycetales, the ascomycete yeasts. *Mycologia.* **98**: 1006-1017.

Tachibana, C., Yoo, J. Y., Tagne, J. B., Kacherovsky, N., Lee, T. I. and Young, E. T. 2005. Combined global localization analysis and transcriptome data identify genes that are directly coregulated by Adr1 and Cat8. *Mol Cell Biol.* **25**: 2138-2146.

Tamas, M. J., Luyten, K., Sutherland, F. C., Hernandez, A., Albertyn, J., Valadi, H., Li, H., Prior, B. A., Kilian, S. G., Ramos, J., Gustafsson, L., Thevelein, J. M. and Hohmann, S. 1999. Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol Microbiol.* **31**: 1087-1104.

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

Tanaka, A., and Ueda, M. 1993. Assimilation of alkanes by yeasts – function and biogenesis of peroxisomes. *Mycol Res.* **97**: 1025–1044.

Tanaka, N., Kyuuma, M. and Sugamura, K. 2008. Endosomal sorting complex required for transport proteins in cancer pathogenesis, vesicular transport, and non-endosomal functions. *Cancer Sci.* **99**: 1293-1303.

Tanner, W. and Caspari, T. 1996. Membrane Transport Carriers. *Annu Rev Plant Physiol Plant Mol Biol.* **47**: 595-626.

Taylor, J. W. and Berbee, M. L. 2006. Dating divergences in the Fungal Tree of Life: review and new analyses. *Mycologia.* **98**: 838-849.

Taylor, K. M., Kaplan, C. P., Gao, X. and Baker, A. 1996. Localization and targeting of isocitrate lyases in *Saccharomyces cerevisiae*. *Biochem J.* **319**: 255-262.

Tehler, A. 1988. A cladistic outline of the Eumycota. *Cladistics* **4**: 227–277.

Teo, H., Perisic, O., Gonzalez, B. and Williams, R. L. 2004. ESCRT-II, an endosome-associated complex required for protein sorting: crystal structure and interactions with ESCRT-III and membranes. *Dev Cell.* **7**: 559-569.

Titorenko, V. I., Smith, J. J., Szilard, R. K. and Rachubinski, R. A. 1998. Pex20p of the yeast *Yarrowia lipolytica* is required for the oligomerization of thiolase in the cytosol and for its targeting to the peroxisome. *J Cell Biol.* **142**: 403-420.

Todisco, S., Agrimi, G., Castegna, A. and Palmieri, F. 2006. Identification of the mitochondrial NAD+ transporter in *Saccharomyces cerevisiae*. *J Biol Chem.* **281**: 1524-1531.

Tournu, H., Tripathi, G., Bertram, G., Macaskill, S., Mavor, A., Walker, L., Odds, F. C., Gow, N. A. and Brown, A. J. 2005. Global role of the protein kinase Gcn2 in the human pathogen *Candida albicans*. *Eukaryot Cell.* **4**: 1687-1696.

Tsong, A. E., Miller, M. G., Raisner, R. M. and Johnson, A. D. 2003. Evolution of a combinatorial transcriptional circuit: a case study in yeasts. *Cell.* **115**: 389-399.

Tzschoppe, K., Augstein, A., Bauer, R., Kohlwein, S. D. and Barth, G. 1999. Trans-dominant mutations in the *GPR1* gene cause high sensitivity to acetic acid and ethanol in the yeast *Yarrowia lipolytica*. *Yeast.* **15**: 1645-1656.

Umebayashi, K., Stenmark, H. and Yoshimori, T. 2008. Ubc4/5 and c-Cbl continue to ubiquitinate EGF receptor after internalization to facilitate polyubiquitination and degradation. *Mol Biol Cell.* **19**: 3454-3462.

Urban, C., Sohn, K., Lottspeich, F., Brunner, H. and Rupp, S. 2003. Identification of cell surface determinants in *Candida albicans* reveals Tsa1p, a protein differentially localized in the cell. *FEBS Lett.* **544**: 228-235.

Urbanowski, J. L. and Piper, R. C. 2001. Ubiquitin sorts proteins into the intralumenal degradative compartment of the late-endosome/vacuole. *Traffic.* **2**: 622-630.

Usseglio-Tomasset, T. 1989. In: Chimie Oenologique. Technique et Documentation. Lavoisier (Eds.) pp122-123.

Vachova, L., Devaux, F., Kucerova, H., Ricicova, M., Jacq, C. and Palkova, Z. 2004. Sok2p transcription factor is involved in adaptive program relevant for long term survival of *Saccharomyces cerevisiae* colonies. *J Biol Chem.* **279**: 37973-37981.

Valdes-Hevia, M. D., de la Guerra, R. and Gancedo, C. 1989. Isolation and characterization of the gene encoding phosphoenolpyruvate carboxykinase from *Saccharomyces cerevisiae*. *FEBS Lett.* **258**: 313-316.

Valenciano, S., Lucas, J. R., Pedregosa, A., Monistrol, I. F. and Laborda, F. 1996. Induction of beta-oxidation enzymes and microbody proliferation in *Aspergillus nidulans*. *Arch Microbiol.* **166**: 336-341.

van den Berg, M. A., de Jong-Gubbels, P. and Steensma, H. Y. 1998. Transient mRNA responses in chemostat cultures as a method of defining putative regulatory elements: application to genes involved in *Saccharomyces cerevisiae* acetyl-coenzyme A metabolism. *Yeast.* **14**: 1089-1104.

Van den Berg, M. A. and Steensma, H. Y. 1995. ACS2, a *Saccharomyces cerevisiae* gene encoding acetyl-coenzyme A synthetase, essential for growth on glucose. *Eur J Biochem.* **231**: 704-713.

Van Der Walt, J. P. and Hopsu-Havu, V. K. 1976. A colour reaction for the differentiation of ascomycetous and hemibasidiomycetous yeasts. *Antonie Van Leeuwenhoek.* **42**: 157-163.

van Urk, H., Schipper, D., Breedveld, G. J., Mak, P. R., Scheffers, W. A. and van Dijken, J. P. 1989. Localization and kinetics of pyruvate-metabolizing enzymes in relation to aerobic alcoholic fermentation in *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621. *Biochim Biophys Acta.* **992**: 78-86.

Vellucci, V. F., Gygax, S. E. and Hostetter, M. K. 2007. Involvement of *Candida albicans* pyruvate dehydrogenase complex protein X (Pdx1) in filamentation. *Fungal Genet Biol.* **44**: 979-990.

Vereecke, D., Cornelis, K., Temmerman, W., Jaziri, M., Van Montagu, M., Holsters, M. and Goethals, K. 2002. Chromosomal locus that affects pathogenicity of *Rhodococcus fascians*. *J Bacteriol.* **184**: 1112-1120.

Vieira, N., Casal, M., Johansson, B., Maccallum, D. M., Brown, A. J. and Paiva, S. 2009. Functional specialization and differential regulation of short-chain carboxylic acid transporters in the pathogen *Candida albicans*. *Mol Microbiol.* **76:**1337-54.

Viljoen, M., Subden, R. E., Krizus, A. and Van Vuuren, H. J. 1994. Molecular analysis of the malic enzyme gene (*MAE2*) of *Schizosaccharomyces pombe*. *Yeast.* **10**: 613-624.

Voet, D., and Voet, J. D. 1990. Biochemistry. New York, John wiley and Sons.

Voet, D., and Voet, J. G. 2004. Biochemistry (3rd Edition). Wiley Intenationl Edition. United States of America.

Walker, M. E., Val, D. L., Rohde, M., Devenish, R. J. and Wallace, J. C. 1991. Yeast pyruvate carboxylase: identification of two genes encoding isoenzymes. *Biochem Biophys Res Commun.* **176**: 1210-1217.

Wang, Z. Y., Thornton, C. R., Kershaw, M. J., Debao, L. and Talbot, N. J. 2003. The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*. *Mol Microbiol.* **47**: 1601-1612.

Ward, C. L., Omura, S. and Kopito, R. R. 1995. Degradation of *CFTR* by the ubiquitin-proteasome pathway. *Cell.* **83**: 121-127.

Weissman, A. M. 2001. Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol.* **2**: 169-178.

White, T. J., Bruns, T.D., Lee, S., and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky J, White TJ (eds), PCR Protocols: a Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322

Wilkinson, K. D. 2000. Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. *Semin Cell Dev Biol.* **11**: 141-148.

Wills, C. 1990. Regulation of sugar and ethanol metabolism in *Saccharomyces cerevisiae*. *Crit Rev Biochem Mol Biol.* **25**: 245-280.

Wilson, M. C., Jackson, V. N., Heddle, C., Price, N. T., Pilegaard, H., Juel, C., Bonen, A., Montgomery, I., Hutter, O. F. and Halestrap, A. P. 1998. Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3. *J Biol Chem.* **273**: 15920-15926.

Winkler, A., Arkind, C., Mattison, C. P., Burkholder, A., Knoche, K. and Ota, I. 2002. Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. *Eukaryot Cell.* **1**: 163-173.

Wysocki, R., Chery, C. C., Wawrzycka, D., Van Hulle, M., Cornelis, R., Thevelein, J. M. and Tamas, M. J. 2001. The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. *Mol Microbiol.* **40**: 1391-1401.

Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D. and Peng, J. 2009. Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell.* **137**: 133-145.

Yin, Z., Stead, D., Selway, L., Walker, J., Riba-Garcia, I., McLnerney, T., Gaskell, S., Oliver, S. G., Cash, P. and Brown, A. J. 2004. Proteomic response to amino acid starvation in *Candida albicans* and *Saccharomyces cerevisiae*. *Proteomics.* **4**: 2425-2436.

Yoon, H. Y., Fanelli, A., Grollman, E. F., and Philp., N. J. 1997. Identification of a unique monocarboxylate transporter (MCT3) in retinal pigment epithelium. *Biochemical and Biophysical Research Communications.* **234**: 90-94.

Yorikawa, C., Shibata, H., Waguri, S., Hatta, K., Horii, M., Katoh, K., Kobayashi, T., Uchiyama, Y. and Maki, M. 2005. Human CHMP6, a myristoylated ESCRT-III protein, interacts directly with an ESCRT-II component EAP20 and regulates endosomal cargo sorting. *Biochem J.* **387**: 17-26.

Young, E. T., Dombek, K. M., Tachibana, C. and Ideker, T. 2003. Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. *J Biol Chem.* **278**: 26146-26158.

Zhou, H. and Lorenz, M. C. 2008. Carnitine acetyltransferases are required for growth on non-fermentable carbon sources but not for pathogenesis in *Candida albicans*. *Microbiology.* **154**: 500-509.

Zmijewski, M. J., Jr. and MacQuillan, A. M. 1975. Dual effects of glucose on dicarboxylic acid transport in *Kluyveromyces lactis*. *Can J Microbiol.* **21**: 473-480.

Section I

Carboxylate transporters homologs in different microorganisms

CHAPTER II – Functional specialization and differential regulation of short-chain carboxylic acids transporters in the pathogen *Candida albicans*

The work presented in this chapter has been published:
Neide Vieira, Margarida Casal, Björn Johansson, Donna M. MacCallum, Alistair JP Brown and Sandra Paiva, (2009) *Functional specialization and differential regulation of short-chain carboxylic acids transporters in the pathogen* Candida albicans.
Mol Microbiol 75(6):1337-54

Molecular Microbiology (2009) ■ doi:10.1111/j.1365-2958.2009.07003.x

Functional specialization and differential regulation of short-chain carboxylic acid transporters in the pathogen *Candida albicans*

Neide Vieira,[1] Margarida Casal,[1] Björn Johansson,[1] Donna M. MacCallum,[2] Alistair J.P. Brown[2] and Sandra Paiva[1]*

[1]*Department of Biology, Molecular and Environmental Biology Centre (CBMA), University of Minho, 4710-057 Braga, Portugal.*

[2]*Aberdeen Fungal Group, School of Medical Sciences, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK.*

Summary

The major fungal pathogen *Candida albicans* has the metabolic flexibility to assimilate a wide range of nutrients in its human host. Previous studies have suggested that *C. albicans* can encounter glucose-poor microenvironments during infection and that the ability to use alternative non-fermentable carbon sources contributes to its virulence. *JEN1* encodes a monocarboxylate transporter in *C. albicans* and we show that its paralogue, *JEN2*, encodes a novel dicarboxylate plasma membrane transporter, subjected to glucose repression. A strain deleted in both genes lost the ability to transport lactic, malic and succinic acids by a mediated mechanism and it displayed a growth defect on these substrates. Although no significant morphogenetic or virulence defects were found in the double mutant strain, both *JEN1* and *JEN2* were strongly induced during infection. Jen1-GFP (green fluorescent protein) and Jen2-GFP were upregulated following the phagocytosis of *C. albicans* cells by neutrophils and macrophages, displaying similar behaviour to an Icl1-GFP fusion. In the murine model of systemic candidiasis approximately 20–25% of *C. albicans* cells infecting the kidney expressed Jen1-GFP and Jen2-GFP. Our data suggest that Jen1 and Jen2 are expressed in glucose-poor niches within the host, and that these short-chain carboxylic acid transporters may be important in the early stages of infection.

Accepted 28 November, 2009. *For correspondence. E-mail spaiva@bio.uminho.pt; Tel. (+351) 25360 4310/1514; Fax (+351) 25367 8980.

Introduction

The opportunistic commensal organism, *Candida albicans,* is part of the normal gastrointestinal microflora in healthy individuals. *C. albicans* is a common cause of mucosal infections in immunodefective individuals, can be responsible for life-threatening systemic infections, in severely immunocompromised patients, and remains a common cause of nosocomial bloodstream infections in humans (Odds, 1988; Calderone, 2002; Perlroth *et al.*, 2007). As well as defective immune defences, a number of additional risk factors have been associated with invasive *C. albicans* infections. These include the use of broad spectrum antibiotics, the application of catheters or prosthetic devices, and the disruption of normal skin barriers (for a review see Perlroth *et al.*, 2007).

The pathogenicity of *C. albicans* is promoted by virulence factors such as adhesins, secretion of hydrolytic enzymes, morphogenesis and phenotypic switching (Odds, 1988; Brown and Gow, 1999; Calderone and Fonzi, 2001; Calderone, 2002). In addition, fitness attributes, such as its robust stress responses and metabolic flexibility, promote the pathogenicity of *C. albicans* (Wysong *et al.*, 1998; Alonso-Monge *et al.*, 1999; Hwang *et al.*, 2002). This fungus exhibits a notable capacity to sense and adjust to environmental changes, such as alterations in nutrient availability and extracellular pH, allowing it to survive within diverse niches such as the skin, mucous membranes, blood and internal organs of its human host (Odds, 1988; Calderone, 2002). Analyses of genome-wide expression profiles of *C. albicans* cells undergoing phagocytosis by mammalian macrophages showed a reprogramming of basal metabolism, which occurs in two successive steps (Prigneau *et al.*, 2003; Lorenz *et al.*, 2004). The first step involved the upregulation of genes encoding gluconeogenic, glyoxylate cycle and fatty acid β-oxidation enzymes, and the concomitant downregulation of glycolytic genes. The upregulation of genes encoding key glyoxylate cycle enzymes was also observed when *C. albicans* cells were exposed to human blood and neutrophils (Rubin-Bejerano *et al.*, 2003; Fradin *et al.*, 2005). The second step occurred as *C. albicans* cells escaped from macrophages and was

coincident with the morphogenetic switch from the budding yeast form to the hyphal form. This late response was characterized by a resumption of glycolysis and a derepression of the translation machinery. The early response appears to be a major feature of the adaptation to non-fermentable carbon sources and was presumed to reflect glucose deprivation. *C. albicans* mutants that lack isocitrate lyase (*icl1*) display attenuated virulence in a mouse model of systemic candidiasis (Lorenz and Fink, 2001; Barelle *et al.*, 2006). This suggests a requirement for the synthesis of C4 compounds from acetyl-CoA, at some stage in the infection process (Lorenz and Fink, 2001; Lorenz and Fink, 2002). Because the early response also included the upregulation of genes involved in lipid degradation, it was postulated that lipids could be the precursors of acetyl-CoA, and thus that peroxisomal fatty acid β-oxidation is important for fungal virulence (Lorenz and Fink, 2002).

The use of green fluorescent protein (GFP) fusions to monitor gene activity has reinforced these findings. Using this approach it was shown that glyoxylate cycle and gluconeogenic genes are induced in individual *C. albicans* cells following phagocytosis by macrophages and neutrophils, but not during cell-to-cell contact (Barelle *et al.*, 2006). However, it has now been shown that fatty acids are not the only sources of acetyl-CoA available to *C. albicans in vivo*. A *pex5* mutant, which is impaired in peroxisomal protein import and hence in fatty acid β-oxidation, is still virulent in the mouse model of systemic candidiasis (Piekarska *et al.*, 2006). Therefore it was postulated that this fungus uses non-fermentable carbon sources, such as acetate or lactate, to survive within the glucose-poor environment of the phagolysosome (Piekarska *et al.*, 2006). This idea was also supported by Ramirez and Lorenz (2007) who reported that the ability to assimilate alternative non-fermentable carbon sources contributes to the virulence of *C. albicans*. They showed that *C. albicans* requires carnitine acetyltransferases of the carnitine shuttle for growth on acetate as sole carbon source (Prigneau *et al.*, 2003; Strijbis *et al.*, 2008; Zhou and Lorenz, 2008).

The transport of carboxylic acids across the plasma membrane presumably is essential for the assimilation of these alternative carbon sources. These substrates are weak organic acids that partially dissociate in aqueous solution, according to their pKa(s) and to the pH value of the medium. The uptake of the undissociated form of these compounds can occur through the plasma membrane by a simple diffusion. However at pH of above 5, the carboxylic acids are predominantly present in their anionic form and their assimilation depends on transporter-mediated uptake (for a review see Casal *et al.*, 2008). In *Saccharomyces cerevisiae* it has been reported that the Fps1 channel promotes the facilitated diffusion of the undissociated form of acetic acid at low pH (4.5) (Mollapour and Piper, 2007), and the ScJen1 transporter is responsible for the active transport of lactate and pyruvate (Casal *et al.*, 1999; Akita *et al.*, 2000). *C. albicans* Jen1 was identified by sequence homology with ScJen1 (Casal *et al.*, 1999) and it was subsequently characterized as a lactate, pyruvate, propionate/proton symporter in this pathogen (Soares-Silva *et al.*, 2004). *JEN1* is upregulated about fivefold when *C. albicans* cells are phagocytosed by macrophages (Piekarska *et al.*, 2006). Similarly, *CYB2,* which encodes an L-lactate dehydrogenase, is upregulated following macrophage attack (Piekarska *et al.*, 2006), reinforcing the idea that carboxylate assimilation is an integral part of the *C. albicans* response to phagocytosis.

A recent study based on synteny analysis, sequence similarity and motif analysis revealed the existence of at least 35 fungal homologues of the *S. cerevisiae JEN1* gene in 13 different *Hemiascomycetes* and four *Euascomycetes* (Lodi *et al.*, 2007). A phylogenetic tree of ScJen1p homologues (Lodi *et al.*, 2007) showed the existence of two main clusters. The first cluster represents a Jen1 group of proteins that have been functionally characterized as monocarboxylate transporters. The second cluster comprises Jen2-like proteins. This cluster includes a new Jen1-like protein from *C. albicans,* Jen2, which has not been functionally characterized. In addition, the cluster contains KlJen2, which is a succinate and D,L-malate plasma membrane transporter in *Kluyveromyces lactis* (Lodi *et al.*, 2004; Queiros *et al.*, 2007). *Schizosaccharomyces pombe* also expresses a dicarboxylate transporter, but the Mae1 protein has no significant homology to the Jen protein family. Mae1 transporter is constitutively expressed and not subjected to glucose repression (Grobler *et al.*, 1995).

Candida albicans JEN2 is closely related to *JEN1* (Casal *et al.*, 2008). Furthermore, *JEN2* is strongly upregulated during phagocytosis by macrophages (160-fold, compared with fivefold for *JEN1*) (Piekarska *et al.*, 2006), and is strongly repressed by glucose (18-fold). Therefore, we reasoned that *JEN2* might play a significant role in the assimilation of short-chain carboxylic acids by *C. albicans.* We have confirmed this, showing that Jen2 executes a distinct function from Jen1 in *C. albicans*. Jen2 is a plasma membrane transporter, rapidly degraded following exposure to glucose, responsible for the saturable kinetics observed for malate and succinate uptake. Given the importance of non-fermentable carbon sources to the growth of *C. albicans* in some host niches (Prigneau *et al.*, 2003; Strijbis *et al.*, 2008; Zhou and Lorenz, 2008) and the potential importance of short-chain carboxylates as a carbon source *in vivo,* we have also compared the expression of Jen1 and Jen2 in disease models.

coincident with the morphogenetic switch from the budding yeast form to the hyphal form. This late response was characterized by a resumption of glycolysis and a derepression of the translation machinery. The early response appears to be a major feature of the adaptation to non-fermentable carbon sources and was presumed to reflect glucose deprivation. *C. albicans* mutants that lack isocitrate lyase (*icl1*) display attenuated virulence in a mouse model of systemic candidiasis (Lorenz and Fink, 2001; Barelle *et al.*, 2006). This suggests a requirement for the synthesis of C4 compounds from acetyl-CoA, at some stage in the infection process (Lorenz and Fink, 2001; Lorenz and Fink, 2002). Because the early response also included the upregulation of genes involved in lipid degradation, it was postulated that lipids could be the precursors of acetyl-CoA, and thus that peroxisomal fatty acid β-oxidation is important for fungal virulence (Lorenz and Fink, 2002).

The use of green fluorescent protein (GFP) fusions to monitor gene activity has reinforced these findings. Using this approach it was shown that glyoxylate cycle and gluconeogenic genes are induced in individual *C. albicans* cells following phagocytosis by macrophages and neutrophils, but not during cell-to-cell contact (Barelle *et al.*, 2006). However, it has now been shown that fatty acids are not the only sources of acetyl-CoA available to *C. albicans in vivo*. A *pex5* mutant, which is impaired in peroxisomal protein import and hence in fatty acid β-oxidation, is still virulent in the mouse model of systemic candidiasis (Piekarska *et al.*, 2006). Therefore it was postulated that this fungus uses non-fermentable carbon sources, such as acetate or lactate, to survive within the glucose-poor environment of the phagolysosome (Piekarska *et al.*, 2006). This idea was also supported by Ramirez and Lorenz (2007) who reported that the ability to assimilate alternative non-fermentable carbon sources contributes to the virulence of *C. albicans*. They showed that *C. albicans* requires carnitine acetyltransferases of the carnitine shuttle for growth on acetate as sole carbon source (Prigneau *et al.*, 2003; Strijbis *et al.*, 2008; Zhou and Lorenz, 2008).

The transport of carboxylic acids across the plasma membrane presumably is essential for the assimilation of these alternative carbon sources. These substrates are weak organic acids that partially dissociate in aqueous solution, according to their pKa(s) and to the pH value of the medium. The uptake of the undissociated form of these compounds can occur through the plasma membrane by a simple diffusion. However at pH of above 5, the carboxylic acids are predominantly present in their anionic form and their assimilation depends on transporter-mediated uptake (for a review see Casal *et al.*, 2008). In *Saccharomyces cerevisiae* it has been reported that the Fps1 channel promotes the facilitated diffusion of the undissociated form of acetic acid at low pH (4.5) (Mollapour and Piper, 2007), and the ScJen1 transporter is responsible for the active transport of lactate and pyruvate (Casal *et al.*, 1999; Akita *et al.*, 2000). *C. albicans* Jen1 was identified by sequence homology with ScJen1 (Casal *et al.*, 1999) and it was subsequently characterized as a lactate, pyruvate, propionate/proton symporter in this pathogen (Soares-Silva *et al.*, 2004). *JEN1* is upregulated about fivefold when *C. albicans* cells are phagocytosed by macrophages (Piekarska *et al.*, 2006). Similarly, *CYB2,* which encodes an L-lactate dehydrogenase, is upregulated following macrophage attack (Piekarska *et al.*, 2006), reinforcing the idea that carboxylate assimilation is an integral part of the *C. albicans* response to phagocytosis.

A recent study based on synteny analysis, sequence similarity and motif analysis revealed the existence of at least 35 fungal homologues of the *S. cerevisiae JEN1* gene in 13 different *Hemiascomycetes* and four *Euascomycetes* (Lodi *et al.*, 2007). A phylogenetic tree of ScJen1p homologues (Lodi *et al.*, 2007) showed the existence of two main clusters. The first cluster represents a Jen1 group of proteins that have been functionally characterized as monocarboxylate transporters. The second cluster comprises Jen2-like proteins. This cluster includes a new Jen1-like protein from *C. albicans,* Jen2, which has not been functionally characterized. In addition, the cluster contains KlJen2, which is a succinate and D,L-malate plasma membrane transporter in *Kluyveromyces lactis* (Lodi *et al.*, 2004; Queiros *et al.*, 2007). *Schizosaccharomyces pombe* also expresses a dicarboxylate transporter, but the Mae1 protein has no significant homology to the Jen protein family. Mae1 transporter is constitutively expressed and not subjected to glucose repression (Grobler *et al.*, 1995).

Candida albicans JEN2 is closely related to *JEN1* (Casal *et al.*, 2008). Furthermore, *JEN2* is strongly upregulated during phagocytosis by macrophages (160-fold, compared with fivefold for *JEN1*) (Piekarska *et al.*, 2006), and is strongly repressed by glucose (18-fold). Therefore, we reasoned that *JEN2* might play a significant role in the assimilation of short-chain carboxylic acids by *C. albicans.* We have confirmed this, showing that Jen2 executes a distinct function from Jen1 in *C. albicans*. Jen2 is a plasma membrane transporter, rapidly degraded following exposure to glucose, responsible for the saturable kinetics observed for malate and succinate uptake. Given the importance of non-fermentable carbon sources to the growth of *C. albicans* in some host niches (Prigneau *et al.*, 2003; Strijbis *et al.*, 2008; Zhou and Lorenz, 2008) and the potential importance of short-chain carboxylates as a carbon source *in vivo,* we have also compared the expression of Jen1 and Jen2 in disease models.

Results

JEN2 encodes a dicarboxylate transporter in C. albicans

The *C. albicans JEN2* (*orf19.5307*) *locus* was annotated as a putative carboxylic acid plasma membrane transporter on the basis of its sequence similarity to *ScJEN1* (http://www.candidagenome.org/). However, Jen2 function has not been tested experimentally. There is only one *JEN locus* in *S. cerevisiae,* even though this species has undergone whole genome duplication (Scannell *et al.*, 2007). Yet there are two *JEN loci* in *C. albicans,* although this pathogen has not undergone whole genome duplication. We reasoned therefore, that the functions of *C. albicans JEN1* and *JEN2* might differ in *C. albicans.* Consequently, to determine the physiological role of *JEN2* in *C. albicans*, both chromosomal copies of this gene were disrupted in the RM1000 strain, resulting in strain CNV3 (*jen2*Δ/*jen2*Δ: Table 1). Additionally, a double *jen1jen2* mutant was constructed, resulting in *C. albicans* strain CNV4 (*jen1*Δ/*jen1*Δ, *jen2*Δ/*jen2*Δ: Table 1).

The ability of these new *jen2* and *jen1jen2* mutants to take up succinic acid and L-malic acid was compared with wild-type and *jen1* cells. We examined the uptake of succinic acid and malic acid because *JEN2* is found in the same phylogenetic cluster as *KlJEN2,* which is a succinate and malate transporter in *Kluyveromyces lactis* (Lodi *et al.*, 2004; Queiros *et al.*, 2007). Cells were grown in succinic acid and malic acid containing medium, at pH 5.0, under derepressing conditions (i.e. in the absence of glucose), and then the initial uptake rates of labelled succinic acid and L-malic acid were measured. Computer-assisted non-linear regression analyses to the experimental data suggested the presence of a mediated transport system for succinic acid in wild-type *C. albicans* cells (RM1000: Table 1) with the following kinetic parameters: K_m 0.49 ± 0.27 mM and V_{max} 0.25 ± 0.040 nmol s^{-1} mg dry $wt.^{-1}$ (for concentrations between 0.1 mM and 4 mM, pH 5.0) (Fig. 1A). Malic acid uptake by wild-type cells also displayed saturable kinetics with a K_m of 0.12 ± 0.019 mM, and a V_{max} of 0.18 ± 0.0070 nmol s^{-1} mg dry $wt.^{-1}$ (for concentrations between 0.04 mM and 2 mM, pH 5.0) (Fig. 1B).

Similar observations were made for *C. albicans jen1* mutant (CPK2: Table 1). For succinic acid transport, this mutant displayed a K_m of 0.71 ± 0.27 and a V_{max} of 0.29 ± 0.038 nmol s^{-1} mg dry $wt.^{-1}$ (Fig. 1), and these *jen1* cells transported malic acid with a K_m of 0.096 ± 0.033 mM and a V_{max} of 0.18 ± 0.015 nmol s^{-1} mg dry $wt.^{-1}$ (Fig. 1). In contrast, the uptake of succinic and malic acid by *jen2* and *jen1jen2* cells fitted to a first order kinetics (Fig. 1). These results show that *JEN2* codes for a saturable (second order kinetics) transport system of succinic and malic acid across the plasma membrane in *C. albicans*. In contrast, the kinetics of lactic acid transport was not affected by deletion of *JEN2*, but fitted to a first order kinetics in the strains lacking *JEN1* (not shown). Therefore, the Jen1 and Jen2 transporters have different specificities in *C. albicans*: Jen1 transports short-chain monocarboxylic acids such as lactate, whereas Jen1 transports short-chain dicarboxylic acids such as succinate and malate.

Table 1. *Saccharomyces cerevisiae* and *Candida albicans* strains used in this study.

Strain	Genotype	Reference
S. cerevisiae		
W303-1A	*MATa ade2 leu2 his3 trp1 ura3*	Thomas and Rothstein, 1989
BLC 491-U2	*MATa ura3-52 JEN1: :GFP Kanr*	Paiva *et al.*, 2002
*jen1*Δ	*W303-1A jen1*::KanMx4	Paiva *et al.*, 2004
*jen1*Δ – p*416GPD*	*jen1*Δ *transformed with* p*416GPD*	Soares-Silva *et al.*, 2004
*jen1*Δ – p*416GPDCaJEN2*	*jen1*Δ *transformed with* p*416GPDCaJEN2*	This work
C. albicans		
RM1000	*ura3::imm434/ura3::imm434, his1::hisG/his1::hisG*	Negredo *et al.*, 1997
CPK2	*ura3::imm434/ura3::imm434 his1::hisG/his1::G jen1::HIS1/jen1::URA3*	Soares-Silva *et al.*, 2004
CNV3	*ura3::imm434/ura3::imm434 his1::hisG/his1::G jen2::HIS1/jen2::URA3*	This work
CNV4	*ura3::imm434/ura3::imm434 his1::hisG/his1::G jen1::HIS1/jen1::ura3-, jen2::ura3-/jen2::URA3*	This work
CNV2-1	CPK2 with *RPS1*-Clp20	This work
CNV2-2	CPK2 with *RPS1*-Clp20-*JEN1*	This work
CNV3-1	CNV3 with *RPS1*-Clp20	This work
CNV4-1	CNV4 with *RPS1*-Clp20	This work
CNV4-1	CNV4 with *RPS1*-Clp20-*JEN1*	This work
CPK20-5	RM1000 with *JEN1/JEN1-GFP-URA3*	Soares-Silva *et al.*, 2004
CNV30-5	RM1000 with *JEN2/JEN2-GFP-URA3*	This work
CJB	*ura3::λimm434/ura3::λ imm434, pACT1-GFP*	Barelle *et al.*, 2004
CJB-1	*ura3::λimm434/ura3::λ imm434,* pGFP	Barelle *et al.*, 2004
CJB-3	*ura3::λimm434/ura3::λ imm434, pICL1GFP*	Barelle *et al.*, 2004

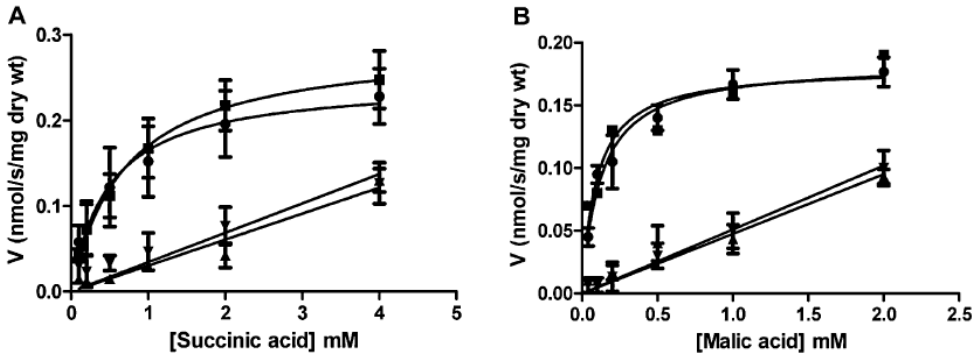

Fig. 1. Transport of carboxylic acids in *Candida albicans*.
A. Initial uptake rates of [2,3-^{14}C] succinic acid, at pH 5.0, as a function of succinic acid concentration, after growth in medium containing succinic acid: RM1000 (wild-type), ●; CPK2 (*jen1*), ■; CNV3 (*jen2*), ▲; CNV4 (*jen1jen2*), ▼.
B. Initial uptake rates of L-[1,4(2,3)-^{14}C] malic acid, at pH 5.0, as a function of malic acid concentration after growth in medium containing malic acid: symbols as for (A).

jen1jen2 *mutant is affected in the growth on mono- and dicarboxylic acids*

Given that *C. albicans jen* mutants have defects in carboxylic acid transport, it was conceivable that these mutants might also display growth defects on the corresponding carbon sources. Therefore, the growth of wild-type and *jen* mutants was evaluated on solid media containing different carbon and energy sources, such as glucose, lactic, pyruvic, malic and succinic acids. Growth was evaluated at pH 5.0 and pH 7.0, and the cells were incubated across a range of temperatures: 18°C, 30°C and 37°C. When incubated at 37°C, for 96 h, *jen1* and *jen1jen2* mutants displayed a growth defect on media containing lactic or pyruvic acids, as sole carbon source (Fig. 2). The *jen2* and *jen1jen2* mutants exhibited a growth defect on succinic and malic acids (Fig. 2). The residual growth observed for the mutant strains could be attributed to simple diffusion; however, because it also persisted in the media with pH 7.0, it is also likely due to the presence of other transport systems, not identified yet.

Heterologous expression of C. albicans JEN2 *in* S. cerevisiae

No transporters for dicarboxylic acids have been so far assigned in *S. cerevisiae,* being assumed that these substrates cross the plasma membrane by a simple diffusion mechanism of the undissociated molecules (Salmon, 1987; Queirós *et al.*, 2003). *S. cerevisiae jen1* cells that express *KlJEN2* in the plasmid p416-GPD (glyceraldehyde 3-phosphate dehydrogenase) acquire the ability to transport succinic and malic acids by a mediated mecha-

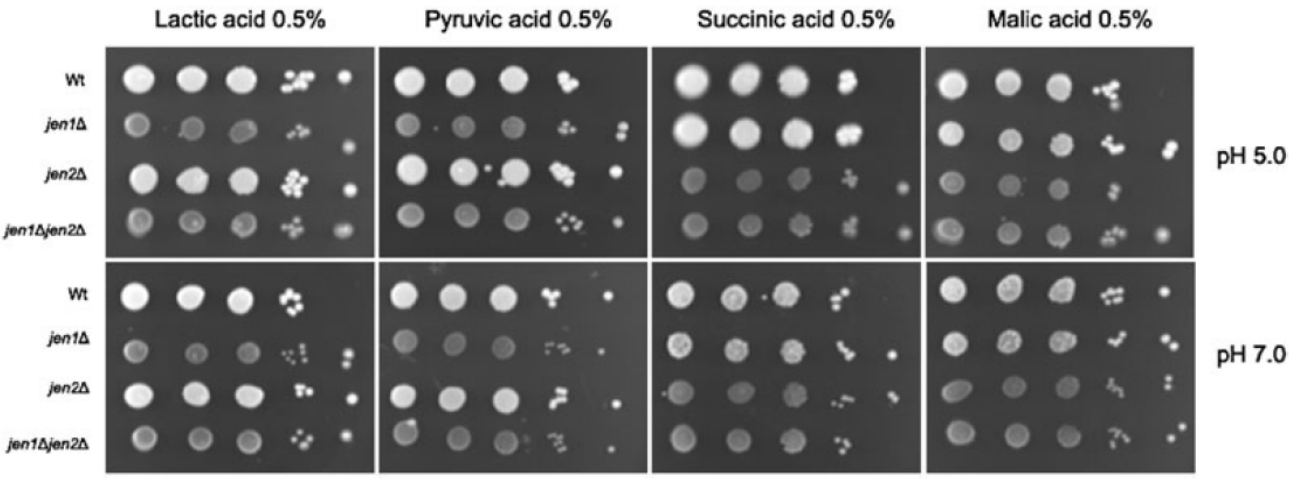

Fig. 2. Growth phenotypes, at 37°C, of *Candida albicans* RM1000 (wild-type), CPK2 (*jen1*), CNV3 (*jen2*), CNV4 (*jen1jen2*), incubated for 96 h, in the following solid media: SC-lactic acid (0.5%, w/v, pH 5.0 or 7.0); SC-pyruvic acid (0.5%, w/v, pH 5.0 or 7.0); SC-succinic acid (0.5%, w/v, pH 5.0 or 7.0); SC-malic acid (0.5%, w/v, pH 5.0 or 7.0). Cells were serially diluted; 3 µl of drops of each dilution were spotted onto the plates.

Table 2. Plasmids used in this study.

Plasmids	Source or reference
Clp20	Murad *et al.*, 2000
pNV1 (*CaJEN1* in Cip20)	This work
pGFP-URA3	Gerami-Nejad *et al.*, 2001
pLUL	Dennison *et al.*, 2005
pLHL	Dennison *et al.*, 2005
pDDB57	Wilson *et al.*, 2000
p416GPD	Mumberg *et al.*, 1995
pNV3 (*CaJEN2* in p416GPD)	This work

nism (Queiros *et al.*, 2007). Therefore *C. albicans JEN2* was cloned into the same *S. cerevisiae* expression vector, p416-GPD (Mumberg *et al.*, 1995), resulting in the plasmid pNV3 (Table 2). *S. cerevisiae jen1* cells were transformed with this plasmid (pNV3) and with the control empty vector (p416-GPD: Table 2). We then determined the kinetic parameters for succinate uptake at pH 5.0 by these strains. *S. cerevisiae jen1* cells expressing *C. albicans JEN2* acquired the ability to transport succinate by a mediated mechanism with a V_{max} of 0.17 ± 0.020 nmol s^{-1} mg dry $wt.^{-1}$ and a K_m of 0.50 ± 0.16 mM (Fig. 3). In contrast, control cells containing p416-GPD behaved as the wild-type. The kinetic parameters obtained were equivalent to the ones found in *C. albicans* wild-type cells. These data confirm the function of *C. albicans* Jen2 as a dicarboxylate transporter.

Expression of JEN1/JEN2 *and subcellular localization of Jen1-GPF and Jen2-GFP in living* C. albicans *cells in response to different carbon sources*

Candida albicans Jen2 was tagged with the reporter gene *GFP* at its carboxy-terminus (Table 1) to study the expression and sub-cellular localization of this transporter. The behaviour of this Jen2-GFP fusion in *C. albicans* was compared with a previously constructed Jen1-GFP fusion (Table 1). Cells expressing Jen1-GFP and Jen2-GFP were grown in minimal medium supplemented with 2% glucose to mid-exponential phase. Cells were then washed and transferred to fresh minimal media containing different carbon sources. After 4 h of incubation, at 30°C, samples were collected to examine cells by epifluorescence microscopy, to prepare extracts for further analyses by western immunoblotting with anti-GFP antibody and to prepare mRNA for quantitative real-time polymerase chain reaction (qRT-PCR) studies. The results show that the Jen2-GFP fusion was expressed and mainly localized to the plasma membrane, after derepression in lactic, succinic, pyruvic and malic acids (Fig. 4A), although some intracellular fluorescence was also observed. The levels of Jen2 protein expression were measured by Western blot analyses with an anti-GFP antibody, and compared with the internal control, Act1. The Jen2-GFP signal was detected in all of the conditions tested, except in glucose grown cells (Fig. 4B), which was consistent with the fluorescence data. We also quantified *JEN2* mRNA levels by qRT-PCR (Fig. 4C). The data show lower *JEN2* mRNA levels during growth on lactic or pyruvic acid compared with succinic or malic acid grown cells. This result correlates reasonably well with the levels of the corresponding Jen2-GFP fusions (Fig. 4B and C), although some post-translational regulation of Jen2-GFP levels cannot be excluded.

With respect to Jen1-GFP subcellular expression, the images show a faint plasma membrane localization in lactic and pyruvic acid derepressed cells, although some intracellular fluorescence was also detected (Fig. 4A). Very low Jen1-GFP fluorescence levels were observed in malic and succinic acid derepressed cells. This was consistent with the Jen1-GFP signals on Western blots, which were lower for malic and succinic acid-derepressed cells relative to the Act1 loading control (Fig. 4B). Furthermore *JEN1* transcript levels were lower in these cells compared with those in cells derepressed on lactic or pyruvic acids (Fig. 4C). However, in lactic and succinic acids derepressed cells there appeared to be a lack of correlation between the levels of *JEN1* mRNA and Jen1-GFP protein (Fig. 4B and C) in that the relatively high levels of *JEN1* mRNA in lactic acid were not reflected in equivalent Jen1-GFP levels; also the signal obtained for Jen1-GFP by Western blot in succinic acid was not consistent with the very low levels of *JEN1* mRNA and the weak fluorescence found (Fig. 4A–C). Similar observations were reproduced in three independent experiments. We have, previously, confirmed that Jen1 fused with GFP is a functional lactate transporter with identical K_m and V_{max} as the wild-type. Therefore, the GFP fusion does not seem to affect the

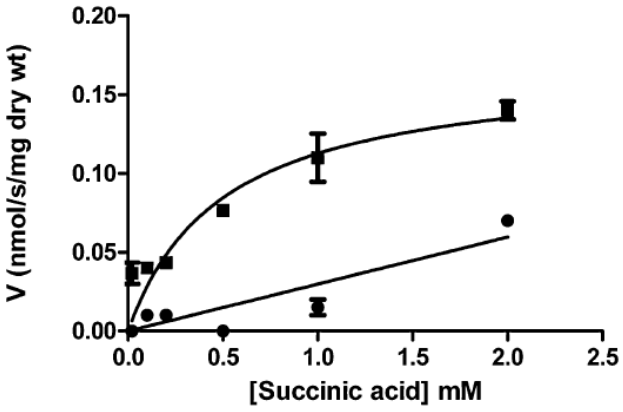

Fig. 3. Heterologous expression of *Candida albicans JEN2* in *Saccharomyces cerevisiae*. Initial uptake rates of [2,3-^{14}C] succinic acid at pH 5.0, as a function of succinic acid concentration, by YNB-glucose-grown cells of *S. cerevisiae* W303-1A *jen1Δ* strain transformed with p416GPD (●) or pNV3 (■).

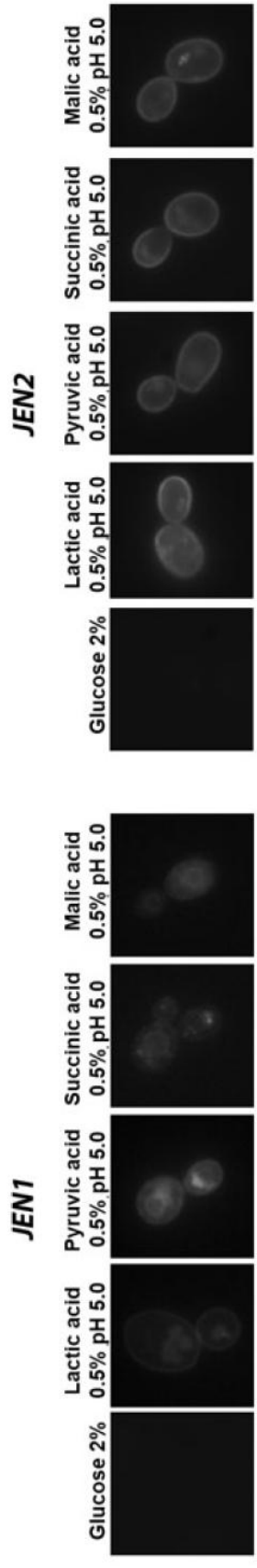

Fig. 4. Expression of *JEN1*/*JEN2* and localization of Jen1-GFP and Jen2-GFP in living *Candida albicans* cells grown on different carbon sources. Mid-exponential *C. albicans* cells grown in minimal medium containing 2% w/v glucose were washed twice with deionized water and then transferred, for 4 h at 30°C, to fresh minimal media containing different carbon sources: glucose 2%, w/v (glu); lactic acid 0.5%, v/v (lac); pyruvic acid 0.5%, v/v (pyr); succinic acid 0.5%, v/v (suc); malic acid 0.5%, v/v (mal). Samples were collected after induction, to examine cells by epifluorescence microscopy, to pepare mRNA and protein extracts.
A. Subcellular localization of Jen1-GFP and Jen2-GFP in living cells.
B. Protein extracts were separated by SDS-PAGE and analysed for Jen1-GFP and Jen2-GFP by western immunoblotting with an anti-GFP antibody. The blots were reprobed with an anti-ACT1 to provide loading controls.
C. Expression analysis of *JEN1* and *JEN2* was followed by qRT-PCR. *JEN1* and *JEN2* mRNA expression levels were normalized to *ACT1*. The results are presented as the mean ± SD of two independent experiments with duplicates for each experiment.

folding and/or localization of Jen1. This apparent inconsistency between *JEN1* mRNA and proteins levels in lactic and succinic acid-grown cells could be explained by post-translational control of Jen1 expression levels, but this remains to be tested. Nevertheless, we have already shown that post-translational control mechanisms exist at the level of Jen1/Jen2 protein turnover in response to glucose (Andrade and Casal, 2001; Paiva *et al.*, 2002; Queirós *et al.*, 2003).

Taken together these results indicate that monocarboxylic acids induce *JEN1* expression to a greater degree than *JEN2*, whereas *JEN2* is induced more strongly than *JEN1* during growth on dicarboxylic acids. These data, which suggest that the Jen transporters play distinct physiological roles in *C. albicans* cell, are consistent with the results obtained from the transport and growth assays. It still remains to be explained why *JEN* genes and proteins are also expressed under conditions where they do not appear to be functional: Jen1 is expressed in the presence of dicarboxylic acids and Jen2 in monocarboxylic acids.

Inactivation of Jen1-GFP and Jen2-GFP by glucose in C. albicans

Jen proteins are generally subjected to tight glucose repression (Paiva *et al.*, 2002; Lodi *et al.*, 2004; Queiros *et al.*, 2007; Soares-Silva *et al.*, 2007). In *S. cerevisiae* the addition of glucose to lactic acid-grown cells triggers the loss of ScJen1p activity and the repression of *ScJEN1* expression (Andrade and Casal, 2001; Paiva *et al.*, 2002). The loss of ScJen1-GFP is the result of its End3-dependent internalization and its subsequent targeting to the vacuole for degradation (Paiva *et al.*, 2002). This glucose-regulated endocytosis of ScJen1 in *S. cerevisiae* has been characterized in detail and it is one of the few examples for which ubiquitin-K63 linked chain(s) have been shown to be required for correct trafficking at two stages of endocytosis: endocytic internalization and sorting at Multi Vesicular Bodies (MVBs) (Paiva *et al.*, 2009). To compare the impact of glucose upon *C. albicans* Jen proteins with ScJen1, *C. albicans* cells carrying the *JEN1-GFP* or *JEN2-GFP* fusions were compared with *S. cerevisiae* cells expressing *ScJEN1-GFP.* These strains were grown in minimal medium containing 2% glucose and then derepressed for 4 h in minimal medium containing 0.5% lactic acid at pH 5.0, a condition where all transporters were shown to be expressed and localized to the plasma membrane (Fig. 4A). These cells were then treated with glucose, at final concentrations ranging from 0.01% to 1%. These conditions were selected to cover the range of glucose concentrations found in human blood, which generally varies between 3 and 5 mM (equivalent to about 0.06–0.1% glucose) (Barelle *et al.*, 2006). Control cells were treated with sorbitol (final concentration 110 mM) as a control for the osmotic changes imposed by the glucose addition. After these treatments the cells were examined by epifluorescence microscopy (Fig. 5).

The first observation of note in these experiments was that fluorescence levels were generally higher in *S. cerevisiae* than *C. albicans* (Fig. 5). This was consistent with published comparisons of GFP levels in these two yeasts (Cormack *et al.*, 1997). The next observation was that even 1 h after the addition of 0.01% glucose to the *C. albicans* and *S. cerevisiae* cells, Jen1-GFP, Jen2-GFP and ScJen1-GFP levels persisted in their plasma membranes (Fig. 5). However, within 20 min of incubation with 0.1 or 1% glucose, all the Jen-GFP fluorescence in *C. albicans* cells had disappeared from the plasma membrane (Fig. 5). In the *S. cerevisiae* cells incubated with 0.1% glucose, not all of the ScJen1-GFP signal was removed from the plasma membrane, but punctuate structures were observed in the cytoplasm (Fig. 5). When *S. cerevisiae* cells were treated with 1% glucose the ScJen1-GFP fluorescence signal finally disappeared from the plasma membrane after 60 min of incubation (Fig. 5). This indicates that the Jen1 and Jen2 transporters are internalized more quickly in response to glucose in *C. albicans* than ScJen1 in *S. cerevisiae.* We set to determine the threshold glucose concentration which triggers *C. albicans* Jen degradation. Because the results showed that Jen1 and Jen2 respond similarly to glucose in *C. albicans*, we carried out a Western blot analysis to follow Jen2-GFP expression, over time, after a pulse of 0.01% and 0.05% glucose, to lactic acid induced cells. In the presence of 0.05% (close to the minimal blood glucose concentration) the level of Jen2-GFP remained stable, even after 60 min of the pulse of glucose (data not shown). However, the addition of 0.1% glucose triggered Jen2-GFP degradation after 20 min of incubation. This indicates that Jen proteins may still be expressed in niches where only low amounts of glucose are present ($\leq$ 0.05%). This is consistent with the view that *C. albicans* cells may be able to utilize some alternative carbon sources in such microenvironments. This view is strengthened by the observation that gluconeogenic and glyoxylate cycle genes are expressed by some *C. albicans* cells infecting the kidney (Barelle *et al.*, 2006).

The inactivation of JEN1 *or* JEN2 *does not affect* C. albicans *morphogenesis*

The ability of *C. albicans* to switch between hypha and yeast growth forms is considered to be a virulence attribute of this fungus (Berman and Sudbery, 2002). The hyphal form facilitates the adhesion and invasion of human tissues as well as the evasion of phagocytic cells. In contrast, the yeast form is better adapted to dissemi-

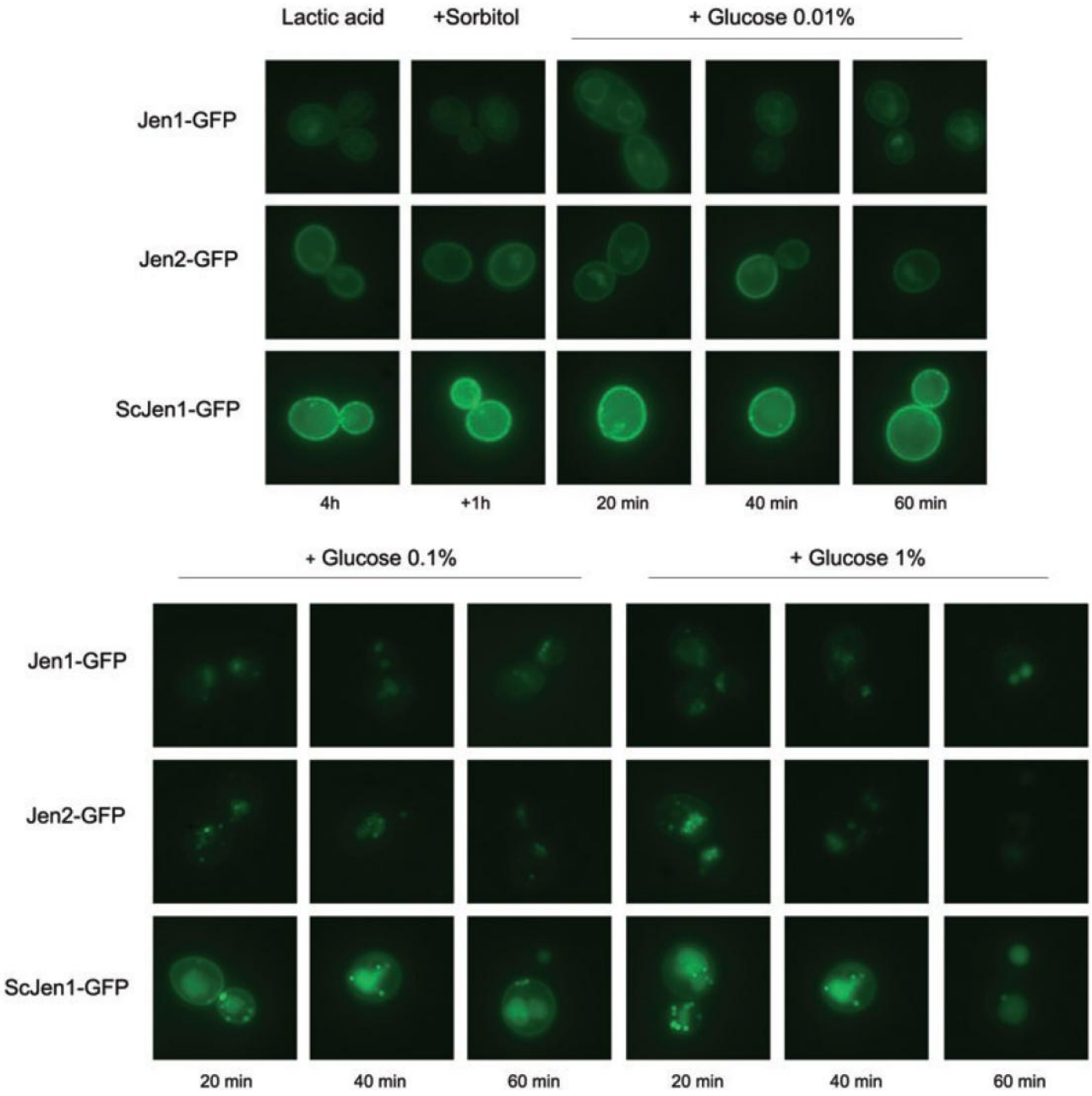

Fig. 5. Timecourse of Jen1-GFP, Jen2-GFP and ScJen1-GFP inactivation at different glucose concentrations. Induced cells were treated with 0.01% (0.55 mM), 0.1% (5.5 mM) and 1% (55 mM) glucose or with sorbitol at the same concentrations (as a control for osmotic shock) and examined by fluorescence microscopy, after continued incubation: CPK20-5 (*Candida albicans JEN1-GFP*); CNV30-5 (*C. albicans JEN2-GFP*); BLC 491-U2 (*Saccharomyces cerevisiae JEN1-GFP*).

nation (Gow *et al.*, 2002). Hence the ability to switch between morphological forms is important for *C. albicans* virulence (Berman and Sudbery, 2002; Gow *et al.*, 2002). Environmental pH is a trigger for *C. albicans* morphological differentiation (Buffo *et al.*, 1984) and influences nutrient uptake via the functionality of plasma membrane transporters. The responses of *C. albicans* to changes in extracellular pH have been analysed by global expression analysis, revealing links between extracellular pH and iron acquisition (Bensen *et al.*, 2004), another virulence determinant. Furthermore, the activities of plasma membrane proton transport systems are important in controlling internal pH and these are also associated with the regulation of dimorphism (Stewart *et al.*, 1988; 1989; Kaur and Mishra, 1994). The yeast form is favoured at low ambient pHs, for example around pH 4.0 when undisso-

ciated forms of carboxylic acids predominate and the simple diffusion of these acids into the cell is favoured. On the other hand, hyphal growth is stimulated at neutral ambient pH, when the mediated transport systems are more active (Buffo *et al.*, 1984; El Barkani *et al.*, 2000). Therefore, environmental pH, morphogenesis and carboxylic acid uptake are linked, a reason why we tested whether the inactivation of *JEN1* or *JEN2* affects morphogenesis in *C. albicans.*

Morphogenesis can be triggered by several different treatments such as exposure of *C. albicans* cells to temperatures higher than 37°C, ambient pH above 6.5, serum and low concentrations of dissolved O_2. Serum has been shown to be the strongest inducer of hyphal development, when combined with temperatures of 37°C. For that reason, *C. albicans* RM1000 (wild-type), CPK2 (*jen1*), CNV3 (*jen2*) and CNV4 (*jen1jen2*) cells were grown under conditions that normally induce *JEN1* and *JEN2* expression (minimal medium containing 0.5% lactic acid at pH 5.0). These cells were then transferred to hypha inducing conditions by supplementing the medium with 10% (v/v) fetal calf serum and incubating at 37°C. The morphology of cells in these cultures was then monitored after 1, 2 and 3 h. Filamentation was also assessed in solid media supplemented with 10% (v/v) fetal bovine serum (FBS). Both wild-type and mutant strains formed hyphae at similar rates (data not shown). To strengthen these findings *C. albicans* cells were also subjected to pH (Buffo *et al.*, 1984) and GlcNAc switch (Mattia *et al.*, 1982). Again, both at higher pHs and in the presence of poor carbon and nitrogen sources, at temperatures above 37°C, yeast to hypha transition was induced in a similar way in all the strains tested. Therefore, the Jen1 and Jen2 carboxylate transporters were not required for hyphal development under the conditions tested.

Regulation of JEN1 *and* JEN2 *following contact with the host*

Human blood is a complex and hostile environment for microorganisms. *C. albicans* cells adapt rapidly to this hostile environment, displaying dramatic changes in its transcript profile (Fradin *et al.*, 2003). The first line of defence against *C. albicans* is provided by phagocytic cells such as macrophages and neutrophils (Lorenz and Fink, 2002). Therefore, we examined the impact of phagocytosis upon the expression of the *C. albicans* carboxylate transporters.

First we examined the behaviour of control *C. albicans* strains carrying pGFP, *ACT1-GFP* or *ICL1-GFP* fusions. The strains were incubated with murine macrophages or human neutrophils for 2.5–3 h at 37°C, and GFP expression was detected by fluorescence microscopy of the phagocytosed and non-phagocytosed *C. albicans* cells. All of the control strains behaved as expected (Fig. 6). No GFP fluorescence was detected in phagocytosed or non-phagocytosed cells in the negative control (pGFP). In contrast, the positive control (*ACT1-GFP*) was expressed under both conditions. Furthermore, *ICL1-GFP* was induced following phagocytosis, as reported previously (Lorenz *et al.*, 2004; Fradin *et al.*, 2005).

The fluorescence of *C. albicans* CPK20-5 (Jen1-GFP) and CNV30-5 (Jen2-GFP) cells was examined under equivalent conditions. Both Jen1-GFP and Jen2-GFP were expressed, although weakly, following phagocytosis by macrophages or neutrophils. In contrast, the non-phagocytosed cells in human plasma (a glucose-containing medium) displayed no significant Jen1-GFP and Jen2-GFP expression. As a further control, CPK20-5 and CNV30-5 cells were examined after 3 h of incubation at 37°C, 5% CO_2, in glucose-containing cell culture medium [Dulbecco's modified Eagle's medium (DMEM)]. No fluorescence was observed (not shown). Therefore Jen1-GFP and Jen2-GFP expression mirrored that of the Icl1-GFP fusion. Expression of Jen1 and Jen2 carboxylate transporters was only observed following phagocytosis, presumably as a result of the transfer of *C. albicans* cells into the glucose poor environment of the phagolysosome (Fig. 6A and B).

If Jen1 and Jen2 are expressed following phagocytosis, do these carboxylate transporters promote the survival of phagocytosed *C. albicans* cells? We tested this by comparing the survival of *jen1* and *jen2* mutants with wild-type cells following exposure to human blood. RM1000 (wild-type), CPK2 (*jen1*), CNV3 (*jen2*) and CNV4 (*jen1jen2*) cells were grown to mid-exponential phase in minimal media at pH 5.0 containing either 0.5% lactic acid or 1% succinic acid. These mid-exponential cells were incubated with human blood for 30 min at 37°C, and then viable cell counts determined. As a negative control cells were plated after incubation in sterile water. In independent replicate experiments using blood from several different donors, we observed no significant difference in the blood killing between the wild-type cells and the *jen* mutant cells (60–65% survival). We conclude that the survival of phagocytosed *C. albicans* cells is not dependent upon Jen1 or Jen2.

Single cell profiling of C. albicans *carboxylate transporter genes during systemic infections and virulence of* C. albicans *null jen mutants*

After assessing the induction of the Jen1 and Jen2 carboxylate transporters in *ex vivo* infection models, we examined their expression *in vivo* in the mouse model of systemic candidiasis. Mice were infected with *C. albicans* strains containing *JEN1-GFP, JEN2-GFP, ACT1-GFP* or pGFP and humanely terminated after the animals lost

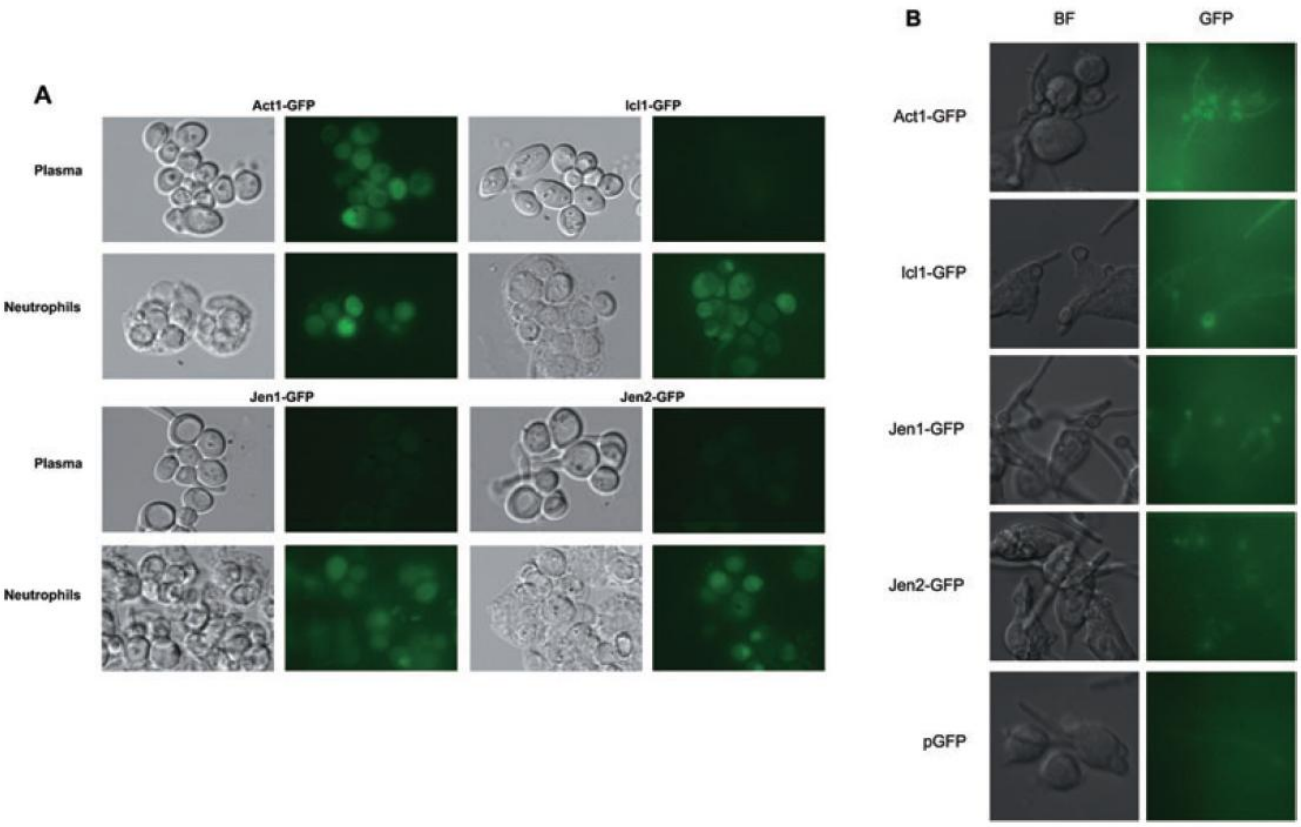

Fig. 6. Differential regulation of Jen1-GFP and Jen2-GFP in *Candida albicans* following phagocytosis by immune cells.
A. Interaction of *C. albicans* with neutrophils. *C. albicans* cells containing *ACT1-GFP, ICL1-GFP, JEN1-GFP, JEN2-GFP* or the control pGFP plasmid were mixed in a 1:1 ratio with human neutrophils, and examined microscopically after 2.5 h at 37°C. Corresponding light and fluorescence images are shown.
B. Interaction of *C. albicans* with macrophages. Cultured murine macrophages (RAW.264.7) were mixed in a 1:3 ratio with the *C. albicans* strains, incubated for 3 h and analysed by light and fluorescence microscopy.

approximately 20% of their body weight and/or showed signs of illness (3 or 4 days). The kidneys were removed aseptically and kidney sections prepared. Fungal cells in these sections were identified by Calcofluor white staining, and then GFP fluorescence in these cells detected by fluorescence microscopy (Gow and Gooday, 1982). As expected no GFP flucorescence was observed for the negative control (pGFP) (Fig. 7A). In contrast, over 80% of *ACT1-GFP* containing cells displayed fluorescence in the kidney sections. Jen1-GFP and Jen2-GFP were expressed in approximately 20–25% of the cells in infected kidneys (Fig. 7A and B). This is consistent with a previous report which suggested that about one-third of *C. albicans* cells infecting the mouse kidney assimilate carbon via gluconeogenesis (Barelle *et al.*, 2006). Interestingly, the intensity of Jen1-GFP and Jen2-GFP fluorescence was comparable *in vivo* and *in vitro* grown fungal cells, suggesting that in those *C. albicans* cells that activate *JEN1* and *JEN2* in the kidney, the level of *JEN* expression is similar to that observed in gluconeogenic cells *in vitro*.

Additionally, we determined the impact of *jen* mutations upon *C. albicans* virulence. Prototrophic wild-type cells and the *jen1jen2* double mutant were tested in a murine model of systemic candidiasis (MacCallum and Odds, 2005). No statistically significant differences in the virulence of the wild-type and *jen1jen2* cells were found. Furthermore similar fungal burdens were observed in the kidneys of infected animals. Therefore, the inactivation of Jen1 and Jen2 does not impair the virulence of *C. albicans*, at least in the mouse model of systemic candidiasis (Fig. 7C).

Discussion

Candida albicans has the ability to survive within different host niches in part due to its metabolic flexibility. This versatile microorganism is a Krebs-positive yeast (Rao *et al.*, 1962) that can use different nutrients, including short-chain carboxylic acids, as sole carbon and energy sources. In this study we have shown that Jen2 is a dicarboxylate transporter that mediates the uptake of

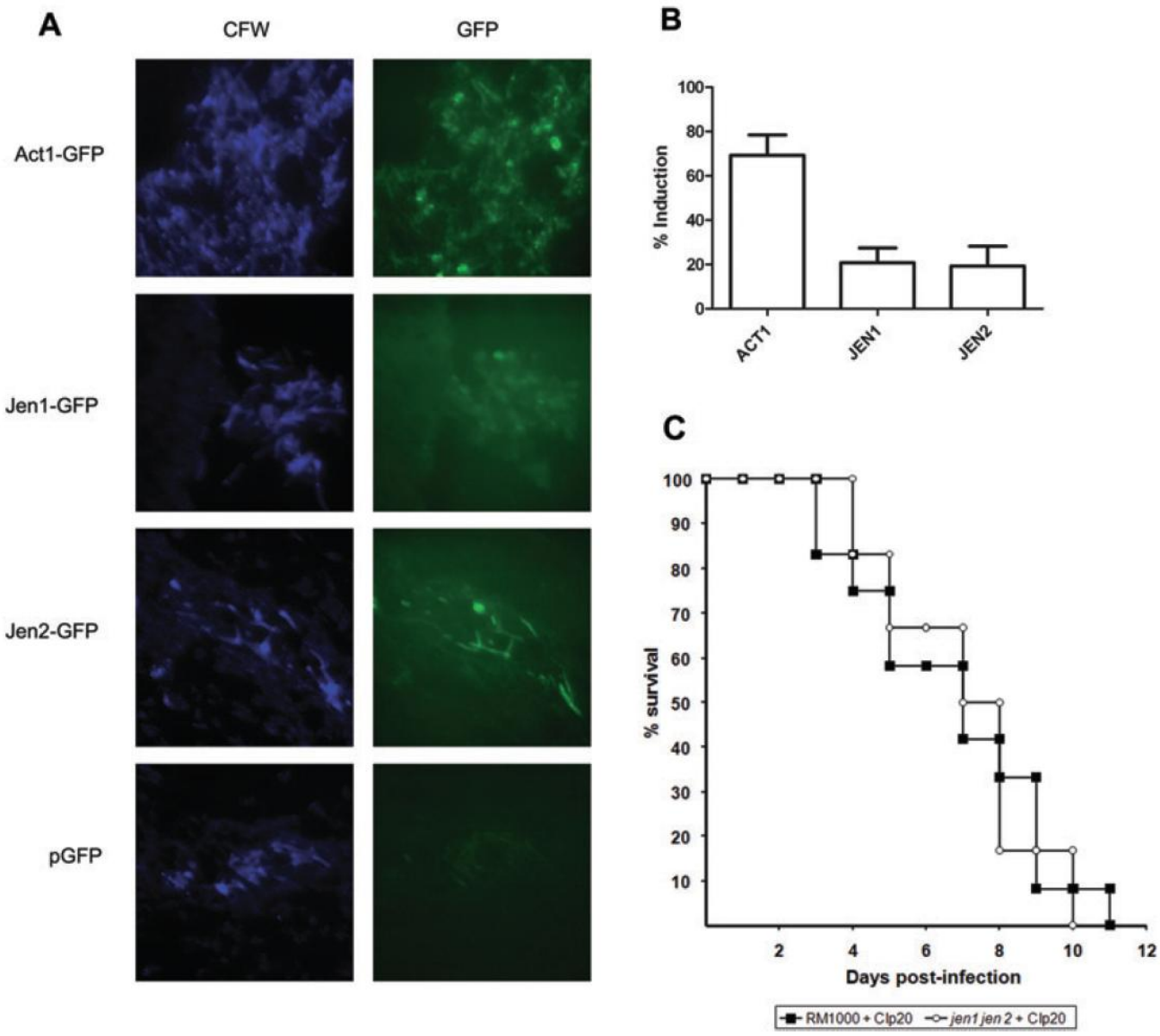

Fig. 7. Expression of *Jen1-GFP* and *Jen2-GFP* in *Candida albicans* cells infecting the kidney.
A. *C. albicans* cells infecting the kidneys of mice were visualized by staining with Calcofluor white (CFW), and then those that expressed their GFP fusions were imaged by fluorescence microscopy (GFP): Act1-GFP, Jen1-GFP and Jen2-GFP.
B. The proportion of *C. albicans* cells infecting the kidney that display GFP fluorescence above background levels.
C. Comparison of the virulence of wild-type and *jen1jen2* cells in the mouse model of systemic candidiasis.

malate and succinate through the plasma membrane. This is in contrast to its *C. albicans* paralogue, Jen1, which transports lactate, pyruvate and propionatec (Soares-Silva *et al.*, 2004). This conclusion was based on three complementary observations. First, Jen2 localized to the plasma membrane (Fig. 4A). Second, the inactivation of *JEN2* (but not *JEN1*) was shown to abolish the saturable uptake of malic acid and succinic acid by *C. albicans* cells (Fig. 1) and to affect the growth in media containing those substrates, as sole carbon and energy sources (Fig. 2). Third, the heterologous expression of *C. albicans JEN2* in *S. cerevisiae* cells conferred upon them the ability to take up succinic acid by a Michaelis-Menten kinetics (Fig. 3). These experiments confirmed that *JEN1* and *JEN2* execute distinct transport functions in *C. albicans*.

The Jen1 and Jen2 transporters displayed distinct regulatory profiles in response to carbon source, both at the protein and at the mRNA level. Both were expressed during growth on lactic, pyruvic acid, succinic and malic acids (Fig. 4B and C). However, the monocarboxylic acids tested induced the expression of *JEN1* stronger than of *JEN2* whereas *JEN2* is more induced than *JEN1* in the presence of dicarboxylic acids (Fig. 4C). This differential

regulation reinforces the notion that these transporters play distinct roles in *C. albicans*.

Both Jen1 and Jen2 are sensitive to glucose (Fig. 5). Following the addition of glucose at concentrations ≥ 0.1% to *C. albicans* cells growing on lactic acid, both Jen1-GFP and Jen2-GFP were rapidly internalized. Interestingly, the rates of Jen1-GFP and Jen2-GFP internalization in *C. albicans* were significantly faster than the rate of ScJen1-GFP internalization in *S. cerevisiae* (Fig. 5). Therefore, mechanistic differences might exist between *C. albicans* and *S. cerevisiae* with regard to the glucose mediated downregulation of Jen transporters in these yeasts. These results corroborate suggestions that *C. albicans* and *S. cerevisiae* may sense sugars differently (Brown *et al.*, 2006) despite both of these yeasts being sensitive to low concentrations of glucose (Rodaki *et al.*, 2009).

Considerable advances have been made in our understanding of how *C. albicans* responds to the metabolic stimuli encountered in its human host, but large gaps in our knowledge remain. Genome-wide expression profiling of *C. albicans* cells in *ex vivo* infection models have highlighted the significance of central metabolic pathways for the adaptation of this organism to its host (Lorenz and Fink, 2001; Lorenz and Fink, 2002; Fradin *et al.*, 2003), and it is now clear that these pathways are regulated in a niche-specific manner during infection (Barelle *et al.*, 2006). It has been postulated that glucose levels are low in the phagolysosome, but this microenvironment is rich in fatty acids and their products (Lorenz and Fink, 2001). These compounds may be sources of acetyl-CoA that feed the glyoxylate cycle (Lorenz and Fink, 2001; Lorenz *et al.*, 2004). However, acetate and lactate may be the main sources of acetyl-coA. This idea is consistent with microarray data, which reveal an upregulation of *JEN1* and *JEN2* in *C. albicans* cells phagocytosed by macrophages and neutrophils (Lorenz *et al.*, 2004; Fradin *et al.*, 2005; Piekarska *et al.*, 2006). Moreover, *S. cerevisiae* and *C. albicans* acetyl-CoA synthases, which are responsible for the conversion of acetate to acetyl-CoA, are upregulated (8.7- and 6.1-fold, respectively) after phagocytosis by macrophages and by neutrophils (Fradin *et al.*, 2005). It was suggested that acetate might be a product of lactate degradation that sustains *C. albicans* inside the phagolysosome (Piekarska *et al.*, 2006). Our results revealed that both Jen1-GFP and Jen2-GFP are expressed inside macrophages and neutrophils, but not in the bloodstream, a glucose-rich environment (Fig. 6). Moreover, in the murine model of systemic candidiasis about 20–25% of *C. albicans* cells infecting the kidney express Jen1 and Jen2 (Fig. 7), a similar value to Icl1 (Barelle *et al.*, 2006). These results support the idea that carboxylic acids such as lactate are present inside the phagolysosome, and suggest that both monocarboxylates and dicarboxylates, such as succinate and malate, may act as carbon sources that help to sustain *C. albicans* following phagocytosis. However, the inactivation of Jen1 and Jen2 does not attenuate the virulence of *C. albicans* (Fig. 7C). This result implies that maybe other, yet uncharacterized, carboxylate transporters play a role in the uptake of further carboxylic acids, such as acetic or citric acids.

The biochemical pathways that mediate monocarboxylate and dicarboxylate anabolism in *C. albicans* during the infection process remain to be confirmed. For example, it is not clear how malic acid is metabolized in this organism. According to the Candida Genome Database, *orf19.1867*, which displays homology to *S. pombe MAE1* (Grobler *et al.*, 1995), encodes a putative malate transporter that is induced during phagocytosis by macrophages (Prigneau *et al.*, 2003). Also, *orf19.3419* encodes a putative mitochondrial malic enzyme, with homology to *S. cerevisiae MAE1* (Boles *et al.*, 1998). This has resulted in a nomenclature conflict, because both *orf19.1867* and *orf19.3419* are referred to *MAE1* in *C. albicans*. In *S. pombe* the malic enzyme is encoded by *MAE2*, and it is a cytoplasmatic protein that, unlike ScMae1, has a high affinity for its substrate (Viljoen *et al.*, 1994). Our results suggest that those *C. albicans* genes currently presumed to be malate transporters are not involved in the uptake of dicarboxylic acids across the plasma membrane, because deletion of *JEN2* abolished the mediated uptake of succinate and malate under the conditions tested (Fig. 1). Nevertheless, it is conceivable that another malate transporter exists in *C. albicans*, with a different pattern of regulation. Alternatively, *C. albicans* might express other malic acid transporters at alternative cellular locations, for example in the peroxisome (Tournu *et al.*, 2005; Piekarska *et al.*, 2008).

The same intriguing questions arise for a malic acid enzyme, whose activity has been reported in *C. albicans in vitro* (Rao *et al.*, 1962). This enzyme converts malic acid into pyruvate. Depending upon yeast species and growth conditions, malic acid can be further converted into oxaloacetate and enter the Krebs cycle or can be metabolized to ethanol via acetaldehyde during maloalcoholic fermentation (Rodriguez and Thornton, 1990). *S. cerevisiae* can only use small amounts of malic acid under anaerobic conditions in the presence of glucose, by fermenting it to ethanol or to succinate via fumarate (Kuczynski and Radler, 1982). In *S. cereviase* it has been speculated that malic enzyme could be involved in the anaplerotic supply of pyruvate during growth on ethanol and acetate (Boles *et al.*, 1998). These and other questions have arisen, particularly in the light of recent work that suggests that significant differences exist between the regulatory networks governing carbon metabolism in *S. cerevisiae* and *C. albicans* (Ramirez and Lorenz, 2007; 2009; Carman *et al.*, 2008). A complete understanding of the precise function and regulation of *C. albicans* carboxylate transporters will require quantitative knowledge of how respon-

sive *C. albicans* is to changes in the activity of these transporters, how responsive these transporters are to changes in the environment, and how they interact with other proteins. These answers will be essential to have a detailed view of the metabolic flexibility of this important pathogen.

Experimental procedures

Yeast strains, plasmids and growth conditions

The yeast strains and the plasmids used in this work are listed in Tables 1 and 2 respectively. Cultures were maintained on YPD (Sherman, 1991). Yeast cells were grown at 30°C in minimal medium (yeast nitrogen base 0.67% w/v) supplemented with the appropriate requirements for prototrophic growth (Sherman, 1991) and containing carbon sources at the following concentrations: glucose (2% w/v: SD), lactic acid (0.5% v/v, pH 5.0), pyruvic acid (0.5% w/v, pH 5.0), succinic acid (1% v/v, pH 5.0) or malic acid (1% v/v, pH 5.0). Solid media was prepared adding agar (2% w/v) to the respective liquid media, and the pH for media containg carboxylic acids was always set either to 5.0 or 7.0. Cultures were harvested during the exponential phase (OD_{640nm} = 0.5). Yeast cells were grown under repression conditions in SD. For derepression conditions, glucose-grown cells were harvested, washed twice in ice-cold deionized water and inoculated into fresh minimal medium supplemented with the carbon source of choice.

C. albicans *mutant construction*

The *C. albicans JEN2* gene was identified through homology to *ScJEN1* using the BLAST program. The two *JEN2* alleles in *C. albicans* strains RM1000 (*JEN1/JEN1*: Table 1) were inactivated sequentially with the *loxP-URA3-loxP* (LUL) and *loxP-HIS1-loxP* (LHL) markers (Dennison *et al.*, 2005). *jen2::LUL* and *jen2::LHL* disruption cassettes designed to delete the complete *JEN2* open reading frame were generated by PCR amplification with the primers CaJEN2-S1Fwd and CaJEN2-S2Rev (Table 3). The *jen2::LUL* cassette was transformed into *C. albicans* (Gietz and Woods, 1998), transformants selected on the basis of their uridine protrophy, and correct integration confirmed by diagnostic PCR with primers DCaURA3-1Rev and DCaJEN2IcFwd (Table 3) (Wilson *et al.*, 1999). The resultant heterozygote (*jen2::loxP-URA3-loxP/JEN2*) was then transformed with the *jen2::LHL* cassette, and accurate disruption of the remaining *JEN2* allele confirmed by PCR using primers DCaHIS1-1Rev and DCaJEN2IcFwd, and CaJEN2A1 and CaJEN2A2 (Table 3). This yielded the homozygous *null* mutant, CNV3 (*jen2::LUL/jen2::LHL*: Table 1). The genotypes of this mutant and all other mutants made in this study were confirmed by Southern blotting (not shown).

The *JEN2 locus* was also disrupted in *C. albicans* strain CPK2 (*jen1/jen1*) to create the double *jen1jen2* mutant, CNV4 (Table 1). First, *ura3*- segregants of CPK2 were selected using 5-fluoroorotic acid (Boeke *et al.*, 1984). The first *JEN2* allele was disrupted using a cassette created by PCR amplification of the mini-*URA3* blaster (Wilson *et al.*, 2000) with primers CaJEN2-DB1 Fwd and CaJEN2-DB2 Rev (Table 3). *Ura3*- segregants were selected 5-fluoroorotic acid to create a *jen1/jen1, jen2/JEN2* heterozygote. The remaining *JEN2* allele in this strain was then disrupted using a second cassette created by PCR amplification of the mini-*URA3* blaster (Wilson *et al.*, 2000) with primers CaJEN2-DB3 Fwd and CaJEN2-DB4 (Table 3). Once again, *ura3*- segregants were selected 5-fluoroorotic acid to create a *jen1/jen1, jen2/jen2* double mutant, CNV4 (Table 1). Correct integration of the cassettes and loss of the wild-type *JEN2* allele were confirmed by diagnostic PCR with primers DCaJEN2IcFwd and DCaURA3-1Rev, DCaJEN2IcFwd and DminiURA3Rev, URA3-dpl200fwd and URA3-dpl200rev, and CaJEN2A1 and CaJEN2A2 (Table 3).

To reintroduce a functional *JEN1* gene into strains CPK2 and CNV4, the *JEN1* gene, plus approximately 2000 bp upstream and 600 bp downstream of its coding sequence were PCR amplified using primers CaJEN1Fwd and CaJEN1Rev (Table 3). The resulting PCR fragment was digested with SalI and MluI and ligated into CIp20 (Dennison *et al.*, 2005) to create CIp20-JEN1. This plasmid was then digested with StuI and integrated at the *RPS1* locus in *C. albicans* CPK2 and CNV4 (Murad *et al.*, 2000), thereby generating CNV2-2 and CNV4-2 (Table 1). Correct integration at the *RPS1* locus was confirmed by diagnostic PCR, using primers RPS10 and InsCaJEN1 (Table 3), and by Southern blot analysis. As controls the *C. albicans* strains CPK2, CNV3 and CNV4 were also transformed with the empty CIp20 plasmid. *JEN1* reintegration suppressed all *jen1/jen1* phenotypes, as expected (not shown). Multiple attempts were made to reintegrate *JEN2* into *jen2* mutant strains without success. It was not possible to clone the *JEN2* locus into several different types of *Escherichia coli* or *C. albicans* vectors using distinct PCR strategies or by using the Clonetech Cloning System (In-Fusion™ 2.0 Dry-Down PCR Cloning Kit; http://www.clontech.com). Additionally several attempts to clone PCR-amplified *JEN2* directly in *S. cerevisiae* and into *C. albicans* genomic locus proved unsuccessful. We conclude that some feature of the *JEN2* locus precludes its cloning using a range of standard procedures. This meant that it was not possible to restoring *JEN2* in the *jen2* mutant. In the absence of a *JEN2* reintegrant we compared the phenotypes of five independent *jen2* mutants. These *jen2* mutants displayed identical phenotypes under all the conditions tested.

Construction of the JEN2-GFP *fusion in* C. albicans

To tag *C. albicans JEN2* at its 3-end, the GFP ORF was PCR amplified from pGFP-URA3 (Gerami-Nejad *et al.*, 2001) using primers CaJEN2GFPFwd and CaJEN2GFPRev (Table 3). The resultant PCR product was used to transform *C. albicans* RM1000, and transformants were screened for correct integration by diagnostic PCR using primers DJEN2GFPFwd and GFPRev (Table 3).

Heterologous expression of C. albicans JEN2 *in* S. cerevisiae

Candida albicans JEN2 was cloned into the plasmid p416GPD (Mumberg *et al.*, 1995) by gap repair (Orr-Weaver

Table 3. Oligonucleotides used in this study.

Name	Sequence
CaJEN1Fwd	GCATGCTTAGCACTGGCACTGACGCGTATTAGTAAATAGACTTTAATTTAG CTTTTACCC
CaJEN1Rev	ATCGAATGCTTAACTGATCACGTCGACTTTTTAGTATTTGATTGAATT GAATTGGTTATAAGA
CaJEN2Fwd	GCATGCTTAGCACTGGCACTGACGCGTGAGCACTAACAATTAGTTGTACAGTTCAAAACTCCG
CaJEN2Rev	ATCGAATGCTTAACTGATCACGTCGACCCGTCTCATATTTCTAACCGATTGTGCCAGTGGCTC
DRPS10	GTGGTTGGAGCTTTGATG
DCaJEN2Rev	AGCCATGAGAGCCATCTC
CaJEN2A1	GGTGATACATATGGTAGA
CaJEN2A2	GTGATCCACATTGGATGG
CaJEN2-S1Fwd	GGATGAATTGAAACAATATTCCTGGCACGAAGTTCTTAATCCGTTTGAACCATTAGTGGA **CCTCTTCGCTATTACGCCAG**
CaJEN2-S2Rev	CGGCACCTCTGTTTTCAGGACCAATAAAAACAACAAACATCAAGTAAGCCAAAACAGCAC **GCAGATTACCCTGTTATCCCTA**
DCaURA3-1Rev	CTGCTCTCTCACTATAGGTC
DCaHIS1-1Rev	CGGTCTGGTAAATGATTGAC
DCaJEN2lcFwd	CCCAATACATCACATTAC
CaJEN2-DB1Fwd	CTAACCATAGAAATATTATGACTGCTGCTGATACTCATTCTATCACTAGTGCTGATG TTC **TTTTCCCAGTCACGACGTT**
CaJEN2-DB2Rev	CCACCAACACTTACTCTTTATGTTCAACTTCTGGTTTTTCCAAAGTCTTTTGAG TAATATTAC **TGTGGAATTGTGAGCGGATA**
CaJEN2-DB3Fwd	CATAATAGACACATTATTCGTCCACCAAAATTCACTTGGCCCGCTATTCGAAAATATGCC **TTTTCCCAGTCACGACGTT**
CaJEN2-DB4Rev	CTTCAAGATCAGAATCACCTCTGTCTTCCCTATCACTATCGTACGCACTGTATTCGTCATC **TGTGGAATTGTGAGCGGATA**
DminiURA3Rev	TAGAAGGACCACCTTTGATTGT
CaJEN2GFPFwd	GAAGAAGGTAATATTACTCAAAAGACTTTGGAAAAACCAGAA GTTGAACATAAAGAG **GGTGGTGGTTCTAAAGGTGAAGAATTATTC**
CaJEN2GFPRev	CACACACATACTATTTTAACAAATCATAAACCCATTTATTATCAAAATAAACTATACTTG **TCTAGAAGGACCACCTTTGATTG**
DJEN2GFP_Fwd	CTTGGTCAGTGGTGCCAA
GFP Rev	AACATCACCATCTAATTCAAC
JEN2_416For	GGGATCCAATATTATGACTGCTGCTGATACTCATTCTATC
JEN2_416Rev	GAAGCTTTTAGTGATGGTGATGGTGATGCTCTTTATGTTCAACTTCTGGTTTTTC
JEN2416ForREQ	AAAACACCAAGAACTTAGTTTCGACGGATTCTAGAACTAGTGGATCCAATATTATGACTGCTGCTGATACTCATTCTATC
JEN2416RevREQ	CATGACTCGAGGTCGACGGTATCGATAAGCTTTTAGTGATGGTGATGGTGATGCTCTTTATGTTCAACTTCTGGTTTTTC
URA3-dpl200fwd	TAAAACGACGGCCAGTGAAT
URA3-dpl200rev	ACCATGATTACGCCAAGCTC
RPS10	ACTAATTCTTCTCTTCAG
Ins CaJEN1	AAGTCTATTTACTAATACG
TDH promoter	ACAAGGCAATTGACCCACGCATGTATCTA
CYC Terminator	GAATGTAAGCGTGACATAACTAATTACATG

DNA sequences complementary to the sequences of pLUL and pLHL (S1/S2), pDDB57 (DB1/DB2/DB3/DB4) and pGFP-URA3 (F1/R1) are shown in bold.

and Szostak, 1983). To achieve this, the *JEN2* ORF was PCR amplified with primers JEN2416ForREQ and JEN2416RevREQ (Table 3). Both the plasmid and the PCR product were digested with BamHI and HindIII, purified from an agarose gel, and co-transformed into the *S. cerevisiae jen1* mutant (Table 1). Correct clones were identified by colony PCR using primers TDH promoter and CYC terminator (Table 3).

DNA manipulations

Cloning, PCR amplification and Southern analysis were performed as described previously (Sambrook *et al*., 1989; Dennison *et al*., 2005).

Transport assays

Cells incubated under derepressing conditions were harvested by centrifugation, washed twice in ice-cold deionized water and resuspended in ice-cold deionized water to a final concentration of about 25–40 mg dry wt. ml^{-1}. 10 µl of yeast cell suspension were mixed in 10 ml of conical tubes with 30 µl of 0.1 M potassium phosphate, pH 5.0. After 2 min of incubation at 30°C in a water bath, the reaction was started by the addition of 10 µl of an aqueous solution of labelled carboxylic acid at the desired concentration and pH value, and stopped by dilution with 5 ml of ice-cold water. The reaction mixtures were filtered immediately through Whatman GF/C membranes, the filters washed with 10 ml of ice-cold water and transferred to scintillation fluid (Opti-phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter. The following radiolabelled substrates were utilized: D,L-[U-^{14}C] lactic acid, sodium salt (CFB97–Amersham Biosciences); [2,3-^{14}C] succinic acid (NEN Life Science); and L-[1,4(2,3)-^{14}C] malic acid (CFB42-Amersham). Nonspecific ^{14}C adsorption to the filters and to the cells was determined by adding labelled acid after ice-cold water. Background values represented less than 5% of

the total incorporated radioactivity. The transport kinetics best fitting the experimental initial uptake rates and the kinetic parameters were determined by a computer-assisted nonlinear regression analysis (GraphPAD Software, San Diego, CA, USA).

Microscopy

Candida albicans and *S. cerevisiae* cells were examined with a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

RNA isolation and qRT-PCR analysis

Candida albicans wild-type and mutant cells were grown in YNB media supplemented with 2% glucose, till an OD_{640nm} of approximately 0.5, and derepressed for 4 h in media containing different carbon sources, prepared as previously described. Total RNA was then isolated using the standard hot acidic phenol protocol. qRT-PCR was carried out to analyse the expression of *JEN1/JEN2* and *ACT1* in the conditions tested. qRT-PCR was performed in an LC480 equipment using the standard real-time PCR conditions. Ct values were transformed in expression values using standard curves made on a pool of samples. Both cDNA samples and RT-minus reactions (RNA samples treated in the same way but without the addition of the RT enzyme in the reverse transcription reaction) were analysed, and the level of cDNA was considered as the expression signal of each sample substracting the RT-minus signal. Finally, relative expression was calculated [(Gene of interest cDNA conc)/(calibrator cDNA conc)].

Cell extracts and immunoblotting

Cells were grown in glucose 2% and derepressed for 4 h in media containing different carbon sources. Lactic acid derepressed cells were also subjected to pulses of 0.01, 0.05 and 0.1% glucose. Preparation of total protein extracts followed the NaOHTCA lysis technique (Volland *et al.*, 1994). Sample buffer was added to extracted proteins, heated at 37°C and resolved by SDS polyacrylamide gel electrophoresis in 10% acrylamide gels. The gels were run using a tricine buffer and transferred to PVDF membranes that were probed with monoclonal anti-GFP antiserum (Roche diagnostics) and anti-ACT1 (abcam, Cambrige, UK). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G was used as the secondary antibody (Sigma, St Louis, MO USA) and was detected by enhanced chemiluminescence (ECL).

C. albicans *morphogenesis*

Different environmental signals were used to induce hyphal development in *C. albicans.* To trigger hyphal development by serum yeast cells were grown in minimal medium containing 0.5% lactic acid, pH 5.0, and then they were incubated with 0, 10 or 20% FBS at 37°C, with agitation, for 3 h (Swoboda *et al.*, 1994). Cells were also platted in YPD agar supplemented with 10% FBS. To monitor the effect of pH, growth was also carried out in minimal medium, but at pH 6.5. The starter culture was divided into two flasks with lactic acid 0.5%, at pH 4.5 and pH 6.5. The culture at pH 6.5 was incubated at 37°C, whereas the one at pH 4.5 at 25°C, for 5 h. Finally, a nutrient limitation stress was imposed by incubating the cells with media containing N-acetylglucosamine (Mattia *et al.*, 1982). The cells were grown in Lee's medium at pH 4.5, 37°C, for several days, and then starved for 24 h, at 37°C. Afterwards, they were ressuspended in BSM medium with or without the addition of 4 mM GlcNAc. All flasks were then incubated for 5 h, at 37°C.

Ex vivo *models of* C. albicans *phagocytosis*

Human Blood from several donors was collected by venepuncture using heparinized tubes. For blood killing assays, *C. albicans* cells were grown in the appropriate carbon source to an $OD_{640} = 0.5$, and then incubated with 100 µl of whole blood (100 cells: 100 µl of whole blood/neutrophils) at 37°C for 1 h. Cells were then plated on YPD and colony forming units (CFU) counted after 24 h, at 30°C. Neutrophils were isolated from human blood (Fradin *et al.*, 2005), *C. albicans* cells were washed with phosphate-buffered saline (PBS) and incubated with neutrophils in a 1:1 ratio. GFP fluorescence was measured after 2.5 h at 37°C (Barelle *et al.*, 2004). Control *C. albicans* cells were incubated with human plasma. Cultured murine RAW 264.7 macrophage (ECACC, Salisbury, UK), kindly provided by Leanne Clift (University of Aberdeen, UK), were diluted to 1×10^6 cells ml^{-1} in supplemented DMEM media, plated in six well plates, and grown overnight at 37°C, under 5% CO_2. *C. albicans* CPK20-5 and CNV30-5 (Table 1) were grown also overnight in minimal medium containing 0.5% lactic acid, pH 5.0 to an $OD_{640} = 0.5$, and then counted with a haemocytometer. Approximately 3×10^8 cells were then added to the wells containing macrophages to give a *C. albicans* macrophage ratio of 3:1. Samples were then incubated for 3 h, at 37°C, under 5% CO_2. Control cultures of *C. albicans* strains containing pICL1-GFP or the empty pGFP vector (Barelle *et al.*, 2004) were also incubated with macrophages (3:1). To provide a further control, *C. albicans* cells were incubated in macrophage growth medium (DMEM containing FBS and glutamine) with no macrophages. Cells were fixed, mounted and GFP quantified as described previously (Barelle *et al.*, 2004; 2006).

Murine model of systemic candidiasis

Female BALB/c mice (Harlan, UK) were handled and maintained according to the conditions specified by the Home Office (UK) regulations. As described previously (Barelle *et al.*, 2006), mice of approximately 6–8 weeks were infected with $2–6 \times 10^4$ *C. albicans* cells/g body weight by lateral tail vein injection. Actual levels of inoculation were assayed by viable plate counting. Fungal burdens and *in vivo* kidney sections were analysed after 3 or 4 days of infection (Fradin *et al.*, 2005). Kidneys were removed aseptically. Half of each

kidney was used for determination of fungal burdens, and the other half was fixed in 4% paraformaldehyde. Fixed kidneys were embedded in Cryo-M-Bed (Bright, Huntingdon, UK) and flash-frozen. Sections (5 μm) were cut and stained with Calcofluor white to identify fungal cells (Barelle *et al*., 2004). Images were generated at 461 nm (Calcofluor white staining), 516 nm (GFP) and 573 nm (Rhodamine as a control for GFP specificity). For the virulence assay strains were grown for 16 h in NGY medium (0.1% Neopeptone 0.4% glucose, 0.1% yeast extract), washed twice in sterile physiological saline and resuspended in saline to produce inocula of 5×10^4 CFU g^{-1} bodyweight per mouse. Actual inocula were determined from viable plating of the inocula. Fungal burdens were determined for the kidneys and spleen of all mice. Organs were homogenized in 0.5 ml of saline and dilutions of the resulting homogenate plated onto Saboraud agar. Plates were incubated overnight at 35°C, and then colonies counted. Survival of mice was analysed by log rank/Kaplan–Meier statistics. Organ burdens were compared by the Mann–Whitney *U*-test.

Acknowledgements

We thank Alexandra Rodaki, Brice Enjalbert and Susan Nicholls for their helpful advice, and all the members of the Aberdeen Fungal Group for their support. We also acknowledge Drs Bernard Hube and Mike Lorenz for fruitful discussions. This study was supported by the Portuguese grant POCI/BIA-BCM/57812/2004 (Eixo 2, Medida 2.3, QCAIII – FEDER). N. V. received FCT PhD fellowship (SFRH/BD/23503/2005). The work performed in Aberdeen was funded by the BBSRC (BB/C510391/1), and by the Wellcome Trust (080088).

References

Akita, O., Nishimori, C., Shimamoto, T., Fujii, T., and Iefuji, H. (2000) Transport of pyruvate in *Saccharomyces cerevisiae* and cloning of the gene encoded pyruvate permease. *Biosci Biotechnol Biochem* **64:** 980–984.

Alonso-Monge, R., Navarro-Garcia, F., Molero, G., Diez-Orejas, R., Gustin, M., Pla, J., *et al.* (1999) Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J Bacteriol* **181:** 3058–3068.

Andrade, R.P., and Casal, M. (2001) Expression of the lactate permease gene *JEN1* from the yeast *Saccharomyces cerevisiae*. *Fungal Genet Biol* **32:** 105–111.

Barelle, C.J., Manson, C.L., MacCallum, D.M., Odds, F.C., Gow, N.A., and Brown, A.J. (2004) GFP as a quantitative reporter of gene regulation in *Candida albicans*. *Yeast* **21:** 333–340.

Barelle, C.J., Priest, C.L., Maccallum, D.M., Gow, N.A., Odds, F.C., and Brown, A.J. (2006) Niche-specific regulation of central metabolic pathways in a fungal pathogen. *Cell Microbiol* **8:** 961–971.

Bensen, E.S., Martin, S.J., Li, M., Berman, J., and Davis, D.A. (2004) Transcriptional profiling in *Candida albicans* reveals new adaptive responses to extracellular pH and functions for Rim101p. *Mol Microbiol* **54:** 1335–1351.

Berman, J., and Sudbery, P.E. (2002) *Candida albicans*: a molecular revolution built on lessons from budding yeast. *Nat Rev Genet* **3:** 918–930.

Boeke, J.D., LaCroute, F., and Fink, G.R. (1984) A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet* **197:** 345–346.

Boles, E., de Jong-Gubbels, P., and Pronk, J.T. (1998) Identification and characterization of *MAE1*, the *Saccharomyces cerevisiae* structural gene encoding mitochondrial malic enzyme. *J Bacteriol* **180:** 2875–2882.

Brown, A.J., and Gow, N.A. (1999) Regulatory networks controlling *Candida albicans* morphogenesis. *Trends Microbiol* **7:** 333–338.

Brown, V., Sexton, J.A., and Johnston, M. (2006) A glucose sensor in *Candida albicans*. *Eukaryot Cell* **5:** 1726–1737.

Buffo, J., Herman, M.A., and Soll, D.R. (1984) A characterization of pH-regulated dimorphism in *Candida albicans*. *Mycopathologia* **85:** 21–30.

Calderone, R.A. (2002) *Candida and Candidiasis*. Washington, DC: American Society for Micobiology Press.

Calderone, R.A., and Fonzi, W.A. (2001) Virulence factors of *Candida albicans*. *Trends Microbiol* **9:** 327–335.

Carman, A.J., Vylkova, S., and Lorenz, M.C. (2008) Role of acetyl coenzyme A synthesis and breakdown in alternative carbon source utilization in *Candida albicans*. *Eukaryot Cell* **7:** 1733–1741.

Casal, M., Paiva, S., Andrade, R.P., Gancedo, C., and Leao, C. (1999) The lactate-proton symport of *Saccharomyces cerevisiae* is encoded by *JEN1*. *J Bacteriol* **181:** 2620–2623.

Casal, M., Paiva, S., Queiros, O., and Soares-Silva, I. (2008) Transport of carboxylic acids in yeasts. *FEMS Microbiol Rev* **32:** 974–994.

Cormack, B.P., Bertram, G., Egerton, M., Gow, N.A., Falkow, S., and Brown, A.J. (1997) Yeast-enhanced green fluorescent protein (yEGFP) a reporter of gene expression in *Candida albicans*. *Microbiology* **143:** 303–311.

Dennison, P.M., Ramsdale, M., Manson, C.L., and Brown, A.J. (2005) Gene disruption in *Candida albicans* using a synthetic, codon-optimised Cre-*loxP* system. *Fungal Genet Biol* **42:** 737–748.

El Barkani, A., Kurzai, O., Fonzi, W.A., Ramon, A., Porta, A., Frosch, M., and Muhlschlegel, F.A. (2000) Dominant active alleles of *RIM101* (*PRR2*) bypass the pH restriction on filamentation of *Candida albicans*. *Mol Cell Biol* **20:** 4635–4647.

Fradin, C., Kretschmar, M., Nichterlein, T., Gaillardin, C., d'Enfert, C., and Hube, B. (2003) Stage-specific gene expression of *Candida albicans* in human blood. *Mol Microbiol* **47:** 1523–1543.

Fradin, C., De Groot, P., MacCallum, D., Schaller, M., Klis, F., Odds, F.C., and Hube, B. (2005) Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol Microbiol* **56:** 397–415.

Gerami-Nejad, M., Berman, J., and Gale, C.A. (2001) Cassettes for PCR-mediated construction of green, yellow, and cyan fluorescent protein fusions in *Candida albicans*. *Yeast* **18:** 859–864.

Gietz, R.D., and Woods, R.A. (1998) Transformation of yeast by the lithium acetate/single-stranded carrier DNA/PEG

method. In *Methods in Microbiology: Yeast Gene Analysis*. Brown, A.J.P., and Tuite, M.F. (eds). London: Academic Stress, 53–66.
Gow, N.A., and Gooday, G.W. (1982) Growth kinetics and morphology of colonies of the filamentous form of *Candida albicans*. *J Gen Microbiol* **128:** 2187–2194.
Gow, N.A., Brown, A.J., and Odds, F.C. (2002) Fungal morphogenesis and host invasion. *Curr Opin Microbiol* **5:** 366–371.
Grobler, J., Bauer, F., Subden, R.E., and Van Vuuren, H.J. (1995) The *mae1* gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C4 dicarboxylic acids. *Yeast* **11:** 1485–1491.
Hwang, C.S., Rhie, G.E., Oh, J.H., Huh, W.K., Yim, H.S., and Kang, S.O. (2002) Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. *Microbiology* **148:** 3705–3713.
Kaur, S., and Mishra, P. (1994) Differential increase in cytoplasmic pH at bud and germ tube formation in *Candida albicans*: studies of a nongerminative variant. *Can J Microbiol* **40:** 720–723.
Kuczynski, J.T., and Radler, F. (1982) The anaerobic metabolism of malate of *Saccharomyces bailii* and the partial purification and characterization of malic enzyme. *Arch Microbiol* **131:** 266–270.
Lodi, T., Fontanesi, F., Ferrero, I., and Donnini, C. (2004) Carboxylic acids permeases in yeast: two genes in *Kluyveromyces lactis*. *Gene* **339:** 111–119.
Lodi, T., Diffels, J., Goffeau, A., and Baret, P.V. (2007) Evolution of the carboxylate Jen transporters in fungi. *FEMS Yeast Res* **7:** 646–656.
Lorenz, M.C., and Fink, G.R. (2001) The glyoxylate cycle is required for fungal virulence. *Nature* **412:** 83–86.
Lorenz, M.C., and Fink, G.R. (2002) Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryot Cell* **1:** 657–662.
Lorenz, M.C., Bender, J.A., and Fink, G.R. (2004) Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **3:** 1076–1087.
MacCallum, D.M., and Odds, F.C. (2005) Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice. *Mycoses* **48:** 151–161.
Mattia, E., Carruba, G., Angiolella, L., and Cassone, A. (1982) Induction of germ tube formation by N-acetyl-D-glucosamine in *Candida albicans*: uptake of inducer and germinative response. *J Bacteriol* **152:** 555–562.
Mollapour, M., and Piper, P.W. (2007) Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Mol Cell Biol* **27:** 6446–6456.
Mumberg, D., Muller, R., and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156:** 119–122.
Murad, A.M., Lee, P.R., Broadbent, I.D., Barelle, C.J., and Brown, A.J. (2000) CIp10, an efficient and convenient integrating vector for *Candida albicans*. *Yeast* **16:** 325–327.
Negredo, A., Monteoliva, L., Gil, C., Pla, J., and Nombela, C. (1997) Cloning, analysis and one-step disruption of the *ARG5,6* gene of *Candida albicans*. *Microbiology* **143:** 297–302.
Odds, F.C. (1988) *Candida and Candidosis*, 2nd edn. London: Bailliere Tindall.
Orr-Weaver, T.L., and Szostak, J.W. (1983) Multiple, tandem plasmid integration in *Saccharomyces cerevisiae*. *Mol Cell Biol* **3:** 747–749.
Paiva, S., Kruckeberg, A.L., and Casal, M. (2002) Utilization of green fluorescent protein as a marker for studying the expression and turnover of the monocarboxylate permease Jen1p of *Saccharomyces cerevisiae*. *Biochem J* **363:** 737–744.
Paiva, S., Devaux, F., Barbosa, S., Jacq, C., and Casal, M. (2004) Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. *Yeast* **21:** 201–210.
Paiva, S., Vieira, N., Nondier, I., Haguenauer-Tsapis, R., Casal, M., and Urban-Grimal, D. (2009) Glucose-induced ubiquitylation and endocytosis of the yeast Jen1 transporter: role of lysine 63-linked ubiquitin chains. *J Biol Chem* **284:** 19228–19236.
Perlroth, J., Choi, B., and Spellberg, B. (2007) Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med Mycol* **45:** 321–346.
Piekarska, K., Mol, E., van den Berg, M., Hardy, G., van den Burg, J., van Roermund, C., *et al.* (2006) Peroxisomal fatty acid beta-oxidation is not essential for virulence of *Candida albicans*. *Eukaryot Cell* **5:** 1847–1856.
Piekarska, K., Hardy, G., Mol, E., van den Burg, J., Strijbis, K., van Roermund, C., *et al.* (2008) The activity of the glyoxylate cycle in peroxisomes of *Candida albicans* depends on a functional beta-oxidation pathway: evidence for reduced metabolite transport across the peroxisomal membrane. *Microbiology* **154:** 3061–3072.
Prigneau, O., Porta, A., Poudrier, J.A., Colonna-Romano, S., Noel, T., and Maresca, B. (2003) Genes involved in beta-oxidation, energy metabolism and glyoxylate cycle are induced by *Candida albicans* during macrophage infection. *Yeast* **20:** 723–730.
Queirós, O., Paiva, S., Moradas-Ferreira, P., and Casal, M. (2003) Molecular and physiological characterization of monocarboxylic acids permeases in the yeast *Kluyveromyces lactis*. *Yeast* **20:** S237.
Queiros, O., Pereira, L., Paiva, S., Moradas-Ferreira, P., and Casal, M. (2007) Functional analysis of *Kluyveromyces lactis* carboxylic acids permeases: heterologous expression of *KlJEN1* and *KlJEN2* genes. *Curr Genet* **51:** 161–169.
Ramirez, M.A., and Lorenz, M.C. (2007) Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. *Eukaryot Cell* **6:** 280–290.
Ramirez, M.A., and Lorenz, M.C. (2009) The transcription factor homolog *CTF1* regulates β-oxidation in *Candida albicans*. *Eukaryot Cell* **8:** 1604–1614.
Rao, G.R., Sirsi, M., and Ramakrishnan, T. (1962) Enzymes in *Candida albicans*. II. Tricarboxylic acid cycle and related enzymes. *J Bacteriol* **84:** 778–783.
Rodaki, A., Enjalbert, B., Young, T., Odds, F.C., Gow, N.A.R., and Brown, A.J.P. (2009) Glucose promotes stress resistance in the fungal pathogen, *Candida albicans*. *Mol Biol Cell* **20:** 4845–4855.

Rodriguez, S.B., and Thornton, R.J. (1990) Factors influencing the utilisation of L-malate by yeasts. *FEMS Microbiol Lett* **60:** 17–22.

Rubin-Bejerano, I., Fraser, I., Grisafi, P., and Fink, G.R. (2003) Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. *Proc Natl Acad Sci USA* **100:** 11007–11012.

Salmon, J.M. (1987) L-malic-acid permeation in resting cells of anaerobically grown *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **901:** 30–34.

Sambrook, J., Fritsch, E.F., and Maniatis, T., (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Scannell, D.R., Butler, G., and Wolfe, K.H. (2007) Yeast genome evolution – the origin of the species. *Yeast* **24:** 929–942.

Sherman, F. (1991) Getting started with yeast. *Methods Enzymol* **194:** 3–21.

Soares-Silva, I., Paiva, S., Kotter, P., Entian, K.D., and Casal, M. (2004) The disruption of *JEN1* from *Candida albicans* impairs the transport of lactate. *Mol Membr Biol* **21:** 403–411.

Soares-Silva, I., Paiva, S., Diallinas, G., and Casal, M. (2007) The conserved sequence NXX[S/T]HX[S/T]QDXXXT of the lactate/pyruvate:H(+) symporter subfamily defines the function of the substrate translocation pathway. *Mol Membr Biol* **24:** 464–474.

Stewart, E., Gow, N.A., and Bowen, D.V. (1988) Cytoplasmic alkalinization during germ tube formation in *Candida albicans*. *J Gen Microbiol* **134:** 1079–1087.

Stewart, E., Hawser, S., and Gow, N.A. (1989) Changes in internal and external pH accompanying growth of *Candida albicans*: studies of non-dimorphic variants. *Arch Microbiol* **151:** 149–153.

Strijbis, K., van Roermund, C.W., Visser, W.F., Mol, E.C., van den Burg, J., MacCallum, D.M., *et al.* (2008) Carnitine-dependent transport of acetyl coenzyme A in *Candida albicans* is essential for growth on nonfermentable carbon sources and contributes to biofilm formation. *Eukaryot Cell* **7:** 610–618.

Swoboda, R.K., Bertram, G., Delbruck, S., Ernst, J.F., Gow, N.A., Gooday, G.W., and Brown, A.J. (1994) Fluctuations in glycolytic mRNA levels during morphogenesis in *Candida albicans* reflect underlying changes in growth and are not a response to cellular dimorphism. *Mol Microbiol* **13:** 663–672.

Thomas, B.J., and Rothstein, R. (1989) Elevated recombination rates in transcriptionally active DNA. *Cell* **56:** 619–630.

Tournu, H., Serneels, J., and Van Dijck, P. (2005) Fungal pathogens research: novel and improved molecular approaches for the discovery of antifungal drug targets. *Curr Drug Targets* **6:** 909–922.

Viljoen, M., Subden, R.E., Krizus, A., and Van Vuuren, H.J. (1994) Molecular analysis of the malic enzyme gene (*mae2*) of *Schizosaccharomyces pombe*. *Yeast* **10:** 613–624.

Volland, C., Urban-Grimal, D., Geraud, G., and Haguenauer-Tsapis, R. (1994) Endocytosis and degradation of the yeast uracil permease under adverse conditions. *J Biol Chem* **269:** 9833–9841.

Wilson, R.B., Davis, D., and Mitchell, A.P. (1999) Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J Bacteriol* **181:** 1868–1874.

Wilson, R.B., Davis, D., Enloe, B.M., and Mitchell, A.P. (2000) A recyclable *Candida albicans URA3* cassette for PCR product-directed gene disruptions. *Yeast* **16:** 65–70.

Wysong, D.R., Christin, L., Sugar, A.M., Robbins, P.W., and Diamond, R.D. (1998) Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. *Infect Immun* **66:** 1953–1961.

Zhou, H., and Lorenz, M.C. (2008) Carnitine acetyltransferases are required for growth on non-fermentable carbon sources but not for pathogenesis in *Candida albicans*. *Microbiology* **154:** 500–509.

CHAPTER III – Plasmids for *in vivo* construction of integrative *Candida albicans* vectors in *Saccharomyces cerevisiae*

The work presented in this chapter has been accepted for publication:
Neide Vieira, Filipa Pereira, Margarida Casal, Alistair JP Brown, Sandra Paiva and Björn Johansson (2010). *A set of plasmids for* in vivo *construction of integrative* Candida albicans *vectors in* Saccharomyces cerevisiae. Yeast.

Abstract

A general system has been devised for the *in vivo* construction of *Candida albicans* integrative vectors in *Saccharomyces cerevisiae*. The system is especially useful for the integration of genes in *C. albicans* that cannot be propagated in *Escherichia coli* possibly because of their toxic effects. The ligation of *S. cerevisiae* 2µ sequences to a *C. albicans* integrative vector permits *in vivo* maintenance and gap repair cloning within *S. cerevisiae.* After the vector assembly, it can be purified from *S. cerevisiae* or amplified by PCR and then used for transformation of *C. albicans.* The *S. cerevisiae* 2µ sequence is completely removed by linearization prior to *C. albicans* transformation, such that no unwanted DNA is transferred in the final construct. The system was successfully used to clone and reintegrate the *C. albicans JEN2* gene, which encodes a membrane protein that is apparently toxic to *E. coli*. Three popular *C. albicans* integrative vectors CIp10, CIp20 and CIp30 are now available in versions that permit gap repair in *S. cerevisiae.*

GenBank Accession Nos: CIp10-2µ (GU550119), CIp20-2µ (GU550120) and CIp30-2µ (GU550121).

Introduction

Candida albicans is a major fungal pathogen especially for patients with compromised immune systems (Odds, 1988). Many of the molecular tools that have been developed for this organism are essentially based on those for the distantly related *Saccharomyces cerevisiae.* However, since *C. albicans* is an obligate diploid, gene disruptions must be carried out twice to inactivate both alleles for each gene (Berman and Sudbery, 2002; Noble and Johnson, 2007). Autonomously replicating plasmids exist for *C. albicans* (Cannon *et al.*, 1992), but their use is limited since they are less stable than their *S. cerevisiae* counterparts. The construction of expression plasmids for *C. albicans* is most often achieved by cloning the gene of interest into these plasmids using *Escherichia coli,* before integrating them into the *C. albicans* genome by homologous recombination (Sonneborn *et al.*, 2000; Tripathi *et al.*, 2002).

In *C. albicans,* the position of marker genes (such as *URA3*) may substantially affect the expression level of the marker gene (Lay *et al.*, 1998) and more importantly, virulence in animal models of *candidiasis* (Sundström *et al.*, 2002). Further research has

shown that integration of the *URA3* in the *RPS1* locus leads to expression levels that can restore virulence levels comparable to that of *URA3* at its wild type locus (Brand *et al.*, 2004). A series of vectors have been developed for integrating sequences at the *RPS1* locus (Dennison *et al.*, 2005; Murad *et al.*, 2000), to prevent *URA3* expression levels from influencing the virulence of these transformants. Since the effect of marker expression level on virulence is likely to be complex, the same vector integrated at the same genetic locus should be used for comparisons across genetic modifications if virulence is to be measured.

The use of this system to overcome these positional effects of marker genes creates an implicit need to assemble genetic constructions in *E. coli.* However, this creates a problem if the *C. albicans* genetic modification cannot be propagated in this organism. Genes encoding eukaryotic membrane proteins are sometimes toxic to the extent that cloning is not possible in *E. coli*. Examples of these include *S. cerevisiae* hexose transporters *HXT15* and *HXT16* (Wieczorke *et al.*, 1999) and the *C. albicans JEN2* (Vieira *et al.*, 2009).

In *S. cerevisiae*, this can be overcome by direct cloning in yeast by *in vivo* gap repair. Orr-Weaver and Szostak were the first to report *in vivo* gap repair of two linear fragments into an episomal plasmid (Orr-Weaver and Szostak, 1983). This method has been widely used for direct high throughput cloning of genes and libraries in yeast, where omitting *E. coli* in the cloning step simplifies the procedure and may increase the coverage of libraries. Therefore, we have created a set of three integrative *C. albicans* vectors, CIp10-2µ, CIp20-2µ and CIp30-2µ which permit *in vivo* assembly in *S. cerevisiae.* These vectors are based on the popular *C. albicans* integrative vectors CIp10 (Murad *et al.*, 2000), CIp20 and CIp30 (Dennison *et al.*, 2005). These vectors have one, two or three nutritional markers, respectively, for complementation of auxotrophic mutations (*URA3, HIS1, ARG4*), and they contain the *C. albicans RPS1* gene which is used to direct integration of the plasmid to this genomic locus.

The CIp20-2µ vector was used to clone the *C. albicans JEN2* gene in *S. cerevisiae* using gap repair, resulting in CIp20-2µ-*JEN2*. Despite many attempts and the use of a range of alternative cloning strategies, we were unable to clone the *JEN2* in *E. coli*. We tried unsuccessfully an *E. coli* strain that maintain a low copy number of the vector (CopyCutter™ EPI400™, EPICENTRE Biotechnologies, Madison, WI, USA), as well as constructing the vector by fusion PCR to avoid the *E. coli* cloning step.

However, we were able to make CIp20-2µ-*JEN2* in *S. cerevisiae* and the *JEN2* gene did not show any adverse effects in the yeast transformants. The cloned *JEN2* was then reintegrated in the *RPS1* genomic locus of a *C. albicans jen2* knockout mutant (Vieira *et al.*, 2009). The *JEN2* reintegration, which was confirmed by diagnostic PCR, suppressed the phenotypes of the *C. albicans jen2* knockout mutant. Growth on media containing succinic acid as sole carbon and energy source, as well as the ability to transport radiolabelled succinic acid by a mediated mechanism was restored.

Materials and methods

Yeast strains, plasmids and growth conditions

Yeast strains and the plasmids used in this work are listed in tables 1 and 2, respectively. Strains were maintained on solid YPD medium (Sherman, 1991). Growth of *C. albicans* strains was performed at 30ºC in SC (Synthetic Complete) medium without uracil or histidine (2% w/v glucose, 0.67% w/v Yeast Nitrogen Base w/o aminoacids and 2 g/l complete amino acid mixture w/o histidine or uracil (Kaiser, 1994)). The same medium was also prepared with succinic acid (1% v/v, pH 5.0) as carbon source. Growth of *S. cerevisiae* was performed in SD (Synthetic Defined) medium with the same composition, but leaving out the amino acid mixture. Solid media was prepared by adding agar (2% w/v).

Table 1 – *Saccharomyces cerevisiae, Escherichia coli* and *Candida albicans* strains used in this study.

Strains	Genotype	Reference
S. cerevisiae		
CEN.PK 113-5D	*MAT***a** ura3	(van Dijken *et al.*, 2000)
E. coli		
XL1Blue	*endA1 gyrA96(nal^R) thi-1 recA1relA1 lac glnV44 F'[:: Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17 (r_K^- m_K^+)*	(Bullock, 1987)
C. albicans		
RM1000	*ura3::imm434/ura3::imm434, his1::hisG/his1::hisG*	(Negredo *et al.*, 1997)
CNV3	*ura3::imm434/ura3::imm434,his1::hisG/his1::G jen2::HIS1/jen2::URA3*	(Vieira *et al.*, 2009)
CNV3-1	CNV3 with *RPS1*-CIp20	(Vieira *et al.*, 2009)
CNF5	CNV3 with *RPS1*-CIp20-*JEN2*	This study

Table 2 – Plasmids used in this study.

Plasmids	Source or references
CIp20	(Dennison *et al.*, 2005)
CIp20-2µ-*JEN2*	This work
YEplac112	(Gietz and Sugino, 1988)

Transport assays

Yeast cells were grown in SC medium to obtain glucose repressed cells. For conditions of glucose derepression, cells grown in SC medium were washed twice in ice-cold deionized water and inoculated into fresh SC medium with succinic acid instead of glucose. Cells were harvested during the exponential phase (OD_{640nm} = 0.5) by centrifugation, washed twice and resuspended in ice-cold deionized water to a final concentration of 25-35 mg dry weight/ml. Uptake rates of labelled succinic acid, [(2,3-^{14}C) succinic acid (NEN Life Science); 5000 dpm/nmol, pH 5.0] were estimated as described by Vieira and co-workers (Vieira *et al.*, 2009). A computer assisted non-linear regression analysis program (GraphPad software, San Diego, CA, USA) was used in order to determine the best-fitting transport kinetics to the experimental data and to estimate the kinetic parameters. All the experiments were performed in biological triplicates, and the data presented represent average values with error bars corresponding to the standard deviation.

Construction of the CIpX0-2µ vectors

CIp10, CIp20 and CIp30 were digested with *Stu* I (AGG^CCT), a restriction enzyme producing blunt ends that is normally used to linearize the vector before integration. The linearized vectors were ligated *in vitro* to a blunt PCR product of the *S. cerevisiae* 2µ replication origin, containing one half *Stu* I site at each end. The PCR product was amplified from the vector YEplac112 (Gietz and Sugino, 1988) with the primers 2u-CENARS_AarI_f and 2u-CENARS_AarI_r (Table 3). Although many vectors carry the 2µ sequence, the one in YEplac112 lacks *Xba* I site due to directed mutagenesis, making this site available for manipulation elsewhere in the resulting vector. The ligation mixture was transformed directly to *S. cerevisiae* CEN.PK113-5D (ura3-52), using the high efficiency LiAc/ssDNA protocol (Gietz and Schiestl, 2007). Transformants were selected on solid SD medium on the rationale that the *C. albicans URA3* gene is able to

complement the *S. cerevisiae ura3* mutation. Transformants were found to be almost exclusively the correct plasmids, which is expected since none of the DNA fragments alone should be able to both propagate in *S. cerevisiae* and complement the *ura3* mutation. The vectors were rescued from yeast by a combination of glass beads and *E. coli* plasmid mini preparation columns and transformed to *E. coli*. The vectors were purified from *E. coli* and analyzed by digestion with *Stu* I to confirm the release of the 2µ sequence. The location and orientation of the 2µ sequence was confirmed by PCR using the primers pairs cip20_3551_fwd/2my_4507_rev and cip20_4936_fwd/2my_5676_rev producing PCR products of 764 bp and 525 bp across the vector insert junctions, respectively. The vectors were given the names CIp10-2µ, CIp20-2µ and CIp30-2µ (Fig. 1). The sequences are available from GenBank with the Accession numbers: GU550119, GU550120 and GU550121, respectively.

Table 3 – Oligonucleotides used in this study.

Name	Sequence
2u-CENARS_AarI_f	CCT TTG CAG GTG GTA TTT CAC ACC GCA TAT ATC G
2u-CENARS_AarI_r	CCT TTG CAG GTG TTT TGA AAA GCA AGC ATA AAA GAT C
cip20_3551_fwd	TTT TCA ATT TCA CGG CCA AT
2my_4936_fwd	GCA CAG AGA TAT ATA GCA AAG AGA TAC
cip20_5676 _rev	CAA CAG ATC TAC CGG TTT AAA GAA
2my_4057_rev	GAA CCG GGG ATG CGA CGT
CaJEN2_rv_lo	AAA GGG AAC AAA AGC TGG GTA CCG GGC CCC CCC TCG AGG TCG ACC CGT CTC ATA TTT CTA ACC GAT TGT GCC AGT GGC TC
CaJEN2_fw_sh	CTT GTT TTT ACC GAC AGC CAT GTT GTA CTT GAG TTG GAT CTA CGC GTG AGC ACT AAC AAT TAG TTG TAC AGT TCA AA ACT
CaJEN2A1fwd	GGT GAT ACA TAT GGT AGA
CaJEN2A2rev	GTG ATC CAC ATT GGA TGG
DRPS10fwd	GTG GTT GGA GCT TTG ATG
DCaJEN2Rev	AGC CAT GAG AGC CAT CTC
HIS1_fw	TCA TCC TCC AGG TAC CGG ATC
RPS10_5ORF fwd	ACT TTG ATC AAC AGA TCT AC

Construction of the Cip20-2µ-CaJEN2 vector

The CIp20-2µ vector was digested with *Mlu* I and *Sal* I in the presence of shrimp alkaline phosphatase to prevent religation of the vector. The enzymes were heat inactivated according to the manufacturer's instructions. A 4295 bp fragment containing the *C. albicans JEN2* locus was PCR amplified from *C. albicans* genomic DNA using the primers CaJEN2_rv_lo and CaJEN2_fw_sh (Table 3). The primers add 41 and 45 bp

of homology to the CIp20-2µ vector, up and downstream of the *Mlu* I and *Sal* I sites. The linear vector and the PCR product were co-transformed into *S. cerevisiae* CEN.PK113-5D *(ura3-52)* and transformants were selected for uracil prototrophy on SD medium. The presence of the *C. albicans JEN2* gene sequence in the transformants was confirmed by colony PCR using the primers CaJEN2A1fwd and CaJEN2A2rev (Table 3) which amplify a 764 bp PCR product internally in the *JEN2* locus. The correct location of the *JEN2* relative to the CIp20-2µ vector was confirmed by PCR with the DRPS10fwd and DCaJEN2Rev primers (Table 3) resulting in a 387 bp PCR product across the junction between the *RPS1* gene of the vector and the *JEN2* fragment.

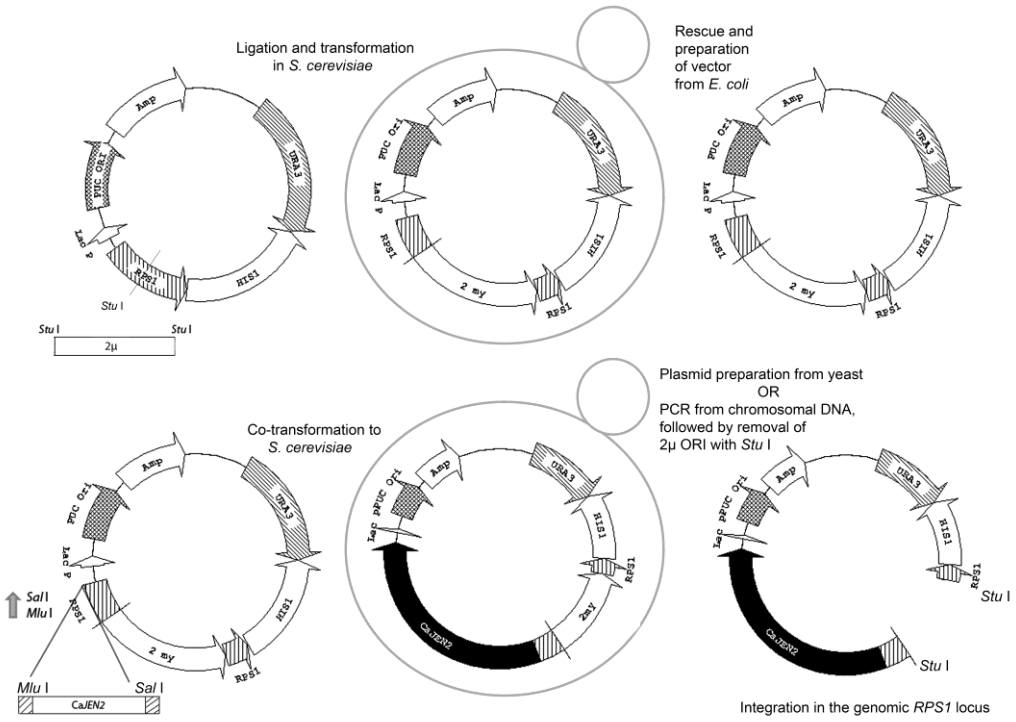

Figure 1 – Schematic representation of the *in vivo* construction of CIp20-2µ in *S. cerevisiae.* Only CIp20-2µ is shown in this schematic overview but the methodology is similar for the CIp10-2µ and CIp30-2µ construction. CIp20 was initially digested with *Stu* I and ligated to the 2µ sequence from YEplac112 *in vitro*. The CIp20-2µ vector was isolated from yeast and amplified in *E. coli*. CIp20-2µ was then digested with *Mlu* I and *Sal* I and co-transformed with a PCR amplified *JEN2* fragment into *S. cerevisiae*. Transformants were selected for uracil prototrophy. The circular vector was purified and used as template for PCR amplification. Finally, *Stu* I digestion enabled the integration of the CIp20-2µ plasmids at the *RPS1* genomic locus. The image was made with the vector drawing software "Simvector" from Premier Biosoft International (http://www.premierbiosoft.com/plasmid_maps/index.html).

*Isolation of the CIp20-2*µ*-CaJEN2 vector from S. cerevisiae*

Several protocols have been described for isolation of plasmids from *S. cerevisiae*. Most protocols rely on transformation of *E. coli* of a crude yeast DNA preparation. Since it

was not possible to propagate the *JEN2* gene, a method was needed for direct isolation of plasmid DNA from yeast. *S. cerevisiae* has a strong cell wall and expresses more nuclease activities than the normally used laboratory strains of *E. coli*, so direct plasmid purification pose a technical problem. Direct isolation of plasmid DNA and separation of circular and linear DNA has been described using CsCl density gradient centrifugation, alkaline lysis of spheroplasts and partitioning of linear DNA using acid phenol. In our hands it was difficult to prepare DNA of sufficient quantity and quality using these methods. We therefore chose to use a combination of plasmid DNA preparation from *S. cerevisiae* and amplification of the vector using long PCR. We prepared a small quantity of plasmid DNA from the *S. cerevisiae* strain using the same protocol combining glass beads and plasmid mini preparation columns described earlier. We used 4% (v/v) of the isolated DNA as template for PCR using the Long PCR Enzyme Mix (FERMENTAS) according to the manufacturer's recommendations and the primers 2my_4936_fwd and 2my_4057_rev. Four 50 µl PCR reaction were pooled, purified using the SureClean DNA cleanup kit (BIOLINE) and resuspended in 50 µl TE buffer. The PCR product was then digested with *Stu* I by adding 7.4 µl buffer 2.5 µl *Stu* I to 17.1 µl of the concentrated PCR product and used to transform *C. albicans* (Walther and Wendland, 2003). Three independent transformants were obtained, the correct localization of the CIp20-*JEN2* vector was confirmed by PCR using primers HIS1_fw and RPS10_5ORF fwd (Table 3). The first primer is plasmid specific whereas the second is located in the genomic *RPS1* locus producing a PCR product of 672 bp from the correct integrated plasmid (Fig. 2).

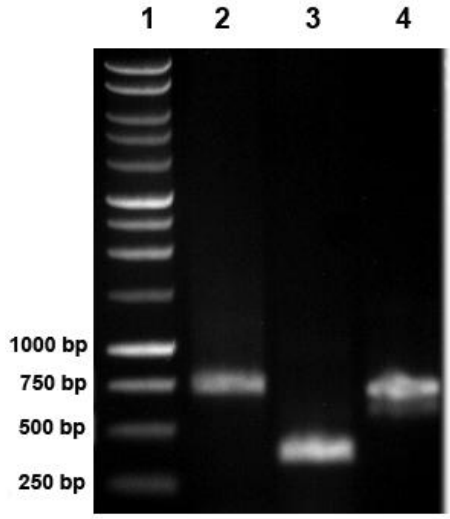

Figure 2 – Verification of *JEN2* ligation to CIp20-2µ and of plasmid integration in the *RPS1* genomic locus by analytical PCR. Correct recombination of *JEN2* in CIp20-2µ was confirmed

by diagnostic PCR with primers CaJEN2A1fwd/CaJEN2A2rev (764 bp) and DRPS10fwd/DCaJEN2Rev (387 bp) as represented in lanes 2 and 3, respectively. Additionally, correct integration of the CIp20-JEN2 in the *RPS1* genomic locus was confirmed with primers HIS1_fw/RPS10_5ORF (672 bp) fwd and is represented in lane 4.

Results and Discussion

We have constructed a series of *C. albicans* integrative vectors (CIp10-2µ, CIp20-2µ and CIp30-2µ) that permit plasmid construction *in vivo* by gap repair in *S. cerevisiae*. The vectors were constructed by combining a series of widely used *C. albicans* vectors (CIp10, 20 and 30) and a PCR product of the yeast 2µ sequence. This sequence was inserted within the part of the vector that normally directs the chromosomal integration to a specific locus by ends-in homologous recombination. The enzyme used to linearize the vector before transformation in *C. albicans* was retained on each side of the 2µ sequence, so that it is lost by linearization of the vector. This means that no *S. cerevisiae* sequences are transferred to *C. albicans* and the resulting integrated construct is not affected by the strategy. These vectors facilitate the cloning and integration of genes that are toxic in *E. coli* in *C. albicans*. A schematic view of the strategy is showed in figure 1.

The gene *JEN2* encodes a *C. albicans* dicarboxylate transporter, the deletion of which from *C. albicans* impairs the mediated transport of succinic and malic acid and leads to a growth defect on these substrates (Vieira *et al.*, 2009). We have previously tried unsuccessfully to clone this gene in *E. coli* by ligating a *JEN2* PCR product into a linearized CIp20 vector (Vieira *et al.*, 2009). Exhaustive attempts to clone *JEN2* in *E. coli* using alternative strategies were also unsuccessful in our hands (unpublished). In the current study we amplified the same *JEN2* fragment with primers adding a short stretch of homology to each side of the cloning site in CIp20-2µ. The CIp20-2µ and the PCR product was assembled *in vivo* and given the name CIp20-2µ-*JEN2*. CIp20-2µ-*JEN2* was amplified by long PCR from *S. cerevisiae*, digested and integrated in a *C. albicans jen2/jen2* double mutant, as described in materials and methods. The correct integration was confirmed by colony PCR (Fig. 2).

The growth phenotypes of *C. albicans* RM1000 (wild-type), CNV3 (*jen2/jen2*) and the reintegrant CNF5 (*jen2/jen2*, *RPS1*-CIp20-*JEN2*) were evaluated both on SC glucose 2% and SC succinic acid 1% solid media. No growth defect was found in SC glucose medium (Fig. 3A left panel), as expected. A growth defect was observed on succinic acid for *jen2/jen2* cells, and the insertion of CIp20-2µ-*JEN2* fully restored the

ability to grow on this carbon source (Fig. 3A right panel). In addition, we measured initial uptake rates of labelled succinic acid, pH 5.0 in cells grown on succinic acid. Like wild type *JEN2/JEN2* cells, the *jen2/jen2/JEN2* strain displayed the ability to transport succinate by a mediated mechanism, indicating that the reintegration of *JEN2* had suppressed this phenotype of the *jen2/jen2* mutant.

This *in vivo* gap repair strategy is not limited to the three vectors described here, but can in principle be applied to any integrative *C. albicans* vectors, provided that the enzyme used to linearize the vector is added to each side of the 2µ sequence before ligation to the vector. The plasmid isolation of the assembled vectors by PCR from *S. cerevisiae* could be done more efficiently using primers annealing immediately up and downstream of the 2µ sequences, so that the PCR product is devoid of *S. cerevisiae* sequences and no subsequent digestion is necessary. We chose to use the 2my_4936_fwd and 2my_4057_rev primers, which meant that the extraneous *S. cerevisiae* sequences had to be removed by *Stu* I digestion, since this avoided the synthesis of new oligonucleotides. This novel strategy, which has proved to be a very useful tool for the construction of an integrative vector of a toxic gene, can be used for the reintegration of any gene in *C. albicans*.

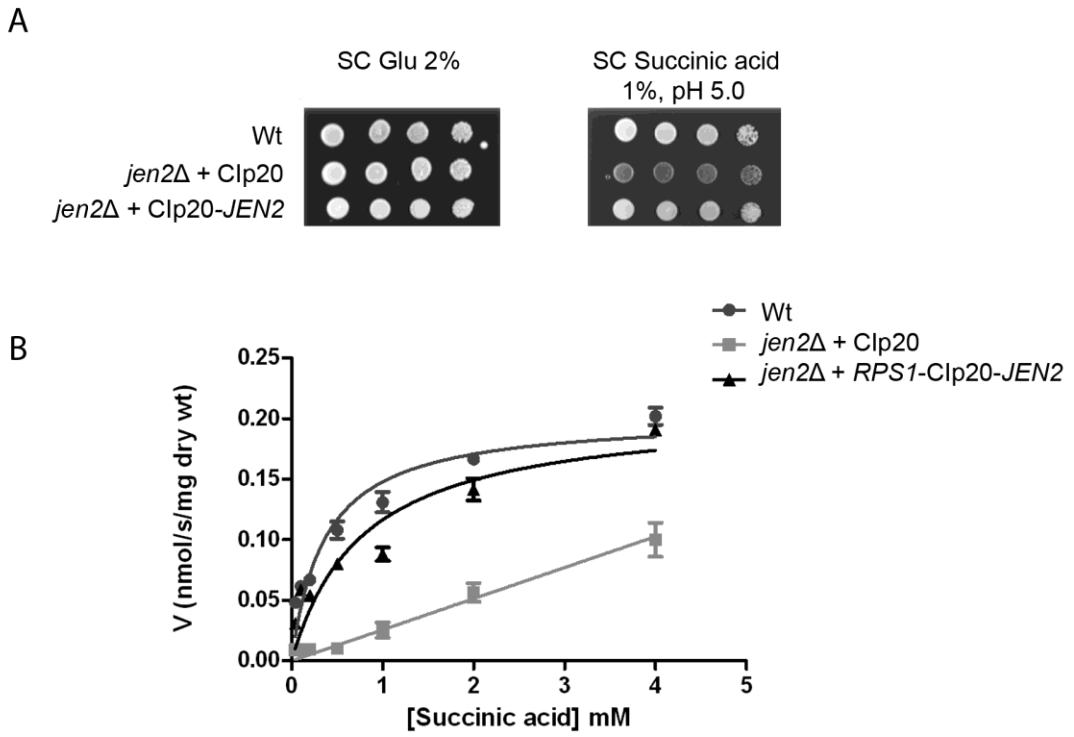

Figure 3 – Growth phenotypes and transport of succinic acid in *C. albicans* jen2 mutants. RM1000 (wild-type), CNV3 (*jen2*) and the reintegrant CNF5 (*jen2*, *RPS1*-CIp20-*JEN2*). A – *C.*

albicans strains were incubated for 48 h, at 37°C, in the following solid media: SC Glu (2 %, w/v) and SC succinic acid (1%, w/v, pH 5.0). Cells were serially diluted and 5 µl drops of each dilution were spotted onto the plates: Wt, *C. albicans* RM1000 (*JEN2/JEN2*); *jen2*Δ + CIp20, CNV3 (*jen2/jen2*, *RPS1*-CIp20); *jen2*Δ + CIp20-*JEN2*, CNF5 (*jen2/jen2*, *RPS1*-CIp20-*JEN2*). B –. Initial uptake rates of [2,3-^{14}C] succinic acid, at pH 5.0, as a function of succinic acid concentration, after growth in medium containing succinic acid: *C. albicans* RM1000 (*JEN2/JEN2*), ●; CNV3 (*jen2/jen2*), ■; CNF5 (*jen2/jen2*, *RPS1*-CIp20-*JEN2*), ▲. Error bars represent standard deviation of experiments carried out in biological triplicates.

References

Berman, J., Sudbery, P. 2002. *Candida albicans*: a molecular revolution built on lessons from budding yeast. *Nat Rev Genet.* **3:** 918-30.

Brand, A., MacCallum, D., Brown, A., *et al.* 2004. Ectopic expression of *URA3* can influence the virulence phenotypes and proteome of *Candida albicans* but can be overcome by targeted reintegration of *URA3* at the *RPS10* locus. *Eukaryot Cell* **3:** 900-9.

Bullock, O., Fernandez, J. and Short, J. 1987. XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with b-galactosidase selection *BioTechniques.* **5:** 376-378.

Cannon, R., Jenkinson, H., Shepherd, M. 1992. Cloning and expression of *Candida albicans ADE2* and proteinase genes on a replicative plasmid in *C. albicans* and in *Saccharomyces cerevisiae. Mol Gen Genet* **235:** 453-7.

Dennison, P., Ramsdale, M., Manson, C., *et al.* 2005. Gene disruption in *Candida albicans* using a synthetic, codon-optimised Cre-*loxP* system. *Fungal Genet Biol* **42:** 737-48.

Gietz, R., Schiestl, R. 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* **2:** 31-4.

Gietz, R., Sugino, A. 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74:** 527-34.

Kaiser, C., Michaelis, S. and Mitchell, A. (1994). *Methods in Yeast Genetics*. Cold Spring Harbor. Laboratory Press.

Lay, J., Henry, L., Clifford, J., *et al.* 1998. Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. *Infect Immun* **66:** 5301-6.

Murad, A., Lee, P., Broadbent, I., *et al.* 2000. CIp10, an efficient and convenient integrating vector for *Candida albicans*. *Yeast* **16:** 325-7.

Negredo, A., Monteoliva, L., Gil, C., *et al.* 1997. Cloning, analysis and one-step disruption of the *ARG5,6* gene of *Candida albicans*. *Microbiology* **143:** 297-302.

Noble, S., Johnson, A. 2007. Genetics of *Candida albicans*, a diploid human fungal pathogen. *Annu Rev Genet* **41:** 193-211.

Odds, F. 1988. *Candida and Candidosis*, 2nd edn. London: Bailliere Tindall.

Orr-Weaver, T., Szostak, J. 1983. Yeast recombination: the association between double-strand gap repair and crossing-over. *Proc Natl Acad Sci U S A* **80:** 4417-21.

Sherman, F. 1991. Getting started with yeast. *Methods Enzymol* **194:** 3-21.

Sonneborn, A., Bockmuhl, D., Gerads, M., *et al.* 2000. Protein kinase A encoded by *TPK2* regulates dimorphism of *Candida albicans*. *Mol Microbiol* **35:** 386-96.

Sundström, P., Cutler, J., Staab, J. 2002. Reevaluation of the role of *HWP1* in systemic candidiasis by use of *Candida albicans* strains with selectable marker *URA3* targeted to the *ENO1* locus. *Infect Immun* **70:** 3281-3.

Tripathi, G., Wiltshire, C., Macaskill, S., *et al.* 2002. Gcn4 co-ordinates morphogenetic and metabolic responses to amino acid starvation in *Candida albicans*. *EMBO J* **21:** 5448-56.

van Dijken, J., Bauer, J., Brambilla, L., *et al.* 2000. An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb Technol* **26:** 706-714.

Vieira, N., Casal, M., Johansson, B., *et al.* 2009. Functional specialization and differential regulation of short-chain carboxylic acid transporters in the pathogen *Candida albicans*. *Mol Microbiol* (ahead of print).

Walther, A., Wendland, J. 2003. An improved transformation protocol for the human fungal pathogen *Candida albicans*. *Curr Genet* **42:** 339-43.

Wieczorke, R., Krampe, S., Weierstall, T., *et al.* 1999. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Lett* **464:** 123-8.

Acknowledgments

This study was supported by the Portuguese grant POCI/BIA-BCM/57812/2004 (Eixo 2, Medida 45 2.3, QCAIII – FEDER). N. V. received FCT PhD fellowship (SFRH/BD/23503/2005). F. P. and B. J. were supported by EU project NILE (FP6-019882). AJPB is funded by the BBSRC (BB/F000111/1; BB/D009308/1; BB/F010826/1; BB/FO0513X/1) and the European Commission (PITN-GA-2008-214004; ERC-2009-AdG- 249793).

CHAPTER IV – *Candida glabrata ADY2* homologs, a step away from *Saccharomyces cerevisiae* monocarboxylate transporters

Abstract

Candida glabrata is the second most common cause of Candida infections, especially in immunocompromised individuals. This yeast can thrive both in glucose rich niches, such as rotting fruits, and in glucose poor niches, like the gastrointestinal (GI) tract, in its human host. This flexibility, certainly, relies in the ability to efficiently utilize different carbon sources, within the different niches it occupies. In *Candida albicans*, this ability has been proven to be determinant for its full virulence. Transcriptional studies on *C. glabrata* cells, phagocytosed by murine macrophages, identified a response that mirrors *C. albicans* behaviour, upon nutrient deprivation. Although *C. glabrata* preferentially assimilates glucose, it is also able to assimilate carboxylic acids, namely mono- di- and tricarboxylic acids, although to a lower extent. This pathogen encodes in its genome two homologs of the *Saccharomyces cerevisiae* acetate transporter, Ady2, which were shown to be induced in an *ex vivo* model of infection. Disruption of the phylogenetically closest homolog influenced the ability of *C. glabrata* to transport acetate, through the plasma membrane, by a saturable transport mechanism, but had no effect on the growth rate of the mutant strain, in distinct media, when compared to the wild-type cells.

Introduction

In the past years, *Candida* fungal infections have increased significantly, all over the world. The emergence of other *Candida* species, such as *C. glabrata*, has contributed markedly to that augment (Banerjee *et al.*, 1991; Nguyen *et al.*, 1996; Kao *et al.*, 1999). The haploid, asexual, ascomycetous yeast, *C. glabrata* is currently recognized as the second most common cause of *Candida* infections, after *C. albicans*, accounting for 15% of all symptomatic mucosal and bloodstream *Candida* infections worldwide (Pfaller *et al.*, 1998; Pfaller and Diekema, 2004; Richter *et al.*, 2005; Messer *et al.*, 2006). This opportunistic yeast can proliferate in the environment, for instance in rotten fruits, but most commonly in the gastrointestinal and genitourinary tracts of healthy human beings. Immune susceptibility of patients together with the broad use of spectrum antibiotics and of plastic catheters, which enable biofilm formation (Iraqui *et*

al., 2005) are predisposing risk factors that contribute to the invasiveness of *C. glabrata*, in the human host (Perlroth *et al.*, 2007). Additionally, *C. glabrata* exhibits an extreme natural resistance to azole antifungals (Richter *et al.*, 2005), therefore, the infections caused by this microorganism are hard to eradicate.

The ability to efficiently utilize non-fermentable carbon sources was reported as an important virulence attribute in *C. albicans* (Ramirez and Lorenz, 2007). The glyoxylate cycle enzymes seem to play a crucial role in the virulence of several pathogens and the disruption of this cycle led to an attenuated virulence of several pathogens, namely, in the human pathogen *C. albicans*, in bacterial pathogens of plants and mammals (McKinney *et al.*, 2000; Vereecke *et al.*, 2002) and in plant-pathogenic fungi (Idnurm and Howlett, 2002; Wang *et al.*, 2003; Solomon *et al.*, 2004). In this manner, the assimilation of acetyl-CoA, derived from lipid catabolism and from other simple carbon sources, such as acetate, must assume an important role in the survival of these pathogens in the different hosts (Lorenz and Fink, 2002; Boshoff and Barry, 2005; Piekarska *et al.*, 2006; Vieira *et al.*, 2009).

A transcriptional study was performed in wild-type *C. glabrata* cells, infecting a murine macrophage cell line (Kaur *et al.*, 2007). The results pointed to a very similar pattern of gene repression/induction between *C. glabrata* and *C. albicans* phagocytosed cells. Overall, in phagocytosed *C. glabrata* cells it was shown that genes involved in the glycolytic pathway were repressed and genes involved in gluconeogenesis, β-oxidation of fatty acids and in the glyoxylate cycle were induced. Moreover, the putative acetate transporter encoding genes were reported to be induced, indicating that acetate can be an available carbon source within the phagolysosome (Kaur *et al.*, 2007).

C. glabrata experienced a reductive evolution after the WGD, with a severe loss of duplicated genes (Dujon *et al.*, 2004), which is believed to reflect its pathogenicity. Several families of membrane transporters are absent in this pathogen, including the Sialate:H+ Symporter (SHS) family (2.A.1.12), where the lactate/pyruvate:H^+ symporter, Jen1 is included (De Hertogh *et al.*, 2006). Nevertheless, this species encodes in its genome two homologs of the Sc*ADY2* gene, an acetate transporter in *S. cerevisiae*, and one homolog of Sc*ATO3*, of unknown function. Little is known regarding carbon assimilation and metabolism in *C. glabrata.* Therefore, in this work we aim at physiologically characterizing *C. glabrata* ATCC2001 strain in respect to its growth behaviour, on non-fermentable carbon sources, and transport capacity of

labelled monocarboxylic acids. Ultimately, the functional role of ScAdy2 homologs in this species will be explored.

Materials and methods

Yeast strains construction

All *C. glabrata* strains, used in this study were obtained from Professor Karl Kuchler, Medical University of Vienna, Austria (Table 1). Gene deletion was carried out in the ATCC2001 background, 500 bp long homology flanking regions were amplified from genomic DNA, adding *Apa*I/*Xho*I RE sites, for the 5' fragment, and *Sac*I/*Sac*II RE sites, for the 3' fragment, and ligated into plasmid pSFS2 (Reuss *et al.*, 2004). The strain HTL was constructed from the ATCC2001 background, through the deletion of *HIS3*, *LEU2*, *TRP1*, using the SAT1 flipper method (Shen *et al.*, 2005), and transformed by electroporation into *C. glabrata* ATCC2001 strain, as described elsewhere (Reuss *et al.*, 2004). For construction of Cg*FPS1* (*CAGL0C03267g*) and Cg*ADY2a* (*CAGLOMO3465g*) genomic deletion cassettes, the nourseothricin marker gene, *NAT1* was amplified from plasmid pJK863 (Shen *et al.*, 2005), using primers fp_NAT1_U2 and rp_NAT1_D2. Barcodes and overlap sequences were added to the marker fragment and used to construct the disruption cassettes, by fusion PCR, as described previously (Noble and Johnson, 2005). Several attempts were performed to disrupt the other Sc*ADY2* homolog, *CAGL0L07766g* and the Sc*ATO3* homolog, however all the consecutive transformations were unsuccessful.

Table 1 – *C. glabrata* strains used in this study.

Strains	Genotype	Reference
ATCC2001 (CBS138)	Wild-type	ATCC collection (available at www.atcc.org)
HTL	Derived from ATCC2001, *his3*:: FRT, *leu2*::FRT and *trp1*::FRT	Jungwirth, H. and Lechner, S. 2009
FI1	Derived from HTL, *ady2*a::*NAT1*	Provided by Karl Kuchler
TS453	Derived from HTL, *fps1*::*NAT1*	Provided by Karl Kuchler

Growth conditions

Yeast cells were grown in a 0.7% (w/v) Difco yeast nitrogen base mineral medium (SC medium), supplemented with the adequate requirements for prototrophic growth. For growth under repression conditions, the yeast cells were cultivated in SC medium with 2% glucose (Sherman, 1991). For derepression conditions, glucose grown cells were

collected, washed twice with ice-cold deionized water and inoculated into SC medium with either: lactic acid (0.5% v/v; pH 5.0), acetic acid (0.5% v/v; pH 6.0), malic acid (1% w/v; pH 5.0), succinic acid (1% w/v; pH 5.0), citric acid (1%, w/v; pH 5.0) or ethanol (1% v/v), as sole carbon and energy sources. Solid media were prepared adding agar (2%, w/v) to the respective liquid media, and the pH set to either 5.0 or 6.0, as indicated in the results. Growth was carried out with shaking (200 rpm), at 30°C, in liquid media, and at 30ºC, 37ºC and 42ºC in solid media.

RT-PCR analysis

C. glabrata wild-type ATCC2001 cells were grown in SC media supplemented with 2% glucose, till an OD_{640nm} of approximately 0.5. Cells were then derepressed for 4h, in different carbon sources containing media, as described above. Total RNA was isolated using the standard hot acidic phenol protocol. The RNA obtained was quantified using nanodrop and analysed on an agarose 0.5% gel electrophoresis, to infer its quality. Total RNA was then subjected to a DNAse I digestion (SIGMA, Deoxyribonuclease I, AMP-D1), for 3h, at 37ºC, to eliminate any genomic DNA contamination. Real time PCR was performed using Invitrogen Superscript III, One-Step RT-PCR System with Platinum Taq DNA Polymerase (Cat. No 12574-026), which allowed both cDNA synthesis and PCR amplification, in a single tube, using gene-specific primers (Table 2) and total RNA. A primer BLAST was performed to confirm the absence of hybridizations with non specific RNAs. Additionally, primers were drawn to yield different PCR products, to allow the individual detection of the distinct genes. Control reactions were performed without SS III RT/Platinum Taq mix, adding only Taq polymerase, to verify the absence of genomic DNA, and without mRNA to confirm the purity of the PCR mixture used.

Table 2 – Oligonucleotides used in this study.

Name	Sequence
Fp_NAT1_U2	CGTACGCTGCAGGTCGACAGCTTGCCTCGTCCCCGCCG
Rp_NAT1_D2	CTACGAGACCGACACCGCTGGATGGCGGCGTTAGTATCG
CgACT1fwd	TCTACGTTTCCATTCAAGCTG
CgACT1rev	GGAGCTCTGAATCTTTCGTTAC
CgADY2a fwd	GTGGTACTTTGAACCCAGGTCTTG
CgADY2a rev	CCAACTTTGCCTGAGAATTC
CgADY2b fwd	GCACAGGACTGTAATTCTG
CgADY2b rev	CAGGAATGTTACCGCTAGCAG
CgATO3 fwd	GAGCAGATCACAACCTATTC
CgATO3 rev	CACATACACTTGCCAGAATAC

Transport assays

Cells incubated under derepression conditions were harvested by centrifugation, washed twice, in ice-cold deionized water, and resuspended in ice-cold deionized water to a final concentration of about 25–35 mg dry weight/ml. 10 µl of yeast cell suspension were mixed in 10 ml conical tubes with 30 µl of 0.1 M potassium phosphate, pH 5.0. After 2 minutes of incubation at 30ºC, in a water bath, the reaction was started by the addition of 10 µl of an aqueous solution of labelled carboxylic acid, at the desired concentration and pH value, and stopped by dilution with 5 ml of ice-cold water. Radiolabelled acetic acid, [^{14}C-acetic acid, sodium salt, Amersham; 3000 dpm/nmol, pH 5.0] or [^{14}C-lactic acid, sodium salt, Amersham; 4000 dpm/nmol, pH 5.0] were used, as substrates. The reaction mixtures were filtered immediately through Whatman GF/C membranes, the filters washed with 10 ml of ice-cold water and transferred to scintillation fluid (Opti-phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter. A computer assisted non-linear regression analysis program (GraphPAD software, San Diego, CA, USA) was used in order to determine the best-fitting transport kinetics to the experimental data and to estimate the kinetic parameters. All the experiments were performed in triplicate, and the data represent average values.

Results

Growth of ATCC2001 in different carbon sources

Given that little information is available on carbon assimilation in *C. glabrata*, we followed the growth phenotypes of the wild-type ATCC2001 strain, in different carbon sources. The same assays were performed for the mutant strains deleted in *CgADY2a* (*CAGLOMO3465g*) and in the *CgFPS1* (*CAGL0C03267g*). Consecutive attempts were performed to disrupt *CgADY2b* and *CgATO3*, however no clones were obtained, in all the performed transformations, indicating that these genes might be essential

Growth of the wild-type and mutant strains was then evaluated on solid media, as indicated in figure 1. Plates were incubated at 37ºC and 42ºC, for 24, 48 and 96h. Both wild-type strains (ATCC2001 and the derived HTL) had similar growth phenotypes, when compared to the mutant strains lacking *CgADY2a* (two individual clones) or *CgFPS1*, in all the tested media. The mutant strain *Cgfps1* was included in

the growth assays in order to assess its role in *C. glabrata*, since its *S. cerevisiae* homolog is known to be involved in the facilitated diffusion of undissociated acetic acid, in *S. cerevisiae* cells (Mollapour and Piper, 2007).

Regarding temperature dependent phenotypes, all strains had a similar behaviour, in all the tested conditions (Fig. 1). After 24h of incubation, growth was reduced in all the media supplemented with carboxylic acids, especially at 42ºC. At this high temperature there was an accentuated growth decrease in the presence of all the non-fermentable carbon sources, whereas in the presence of glucose the growth persisted. This is probably due to a significant decrease in the diffusion rate, of all the undissociated forms of these acids, across the plasma membrane, and of other putative transport mechanisms. Only after 48 h of incubation, at 42ºC, growth was evident in the presence of lactic, citric, succinic and malic acids, although still less pronounced than of the plates incubated at 37ºC (Fig. 1). For some reason, growth on acetic acid containing media was still very poor, especially when the pH was set to 5.0. After 96 h of incubation, growth on acetic acid at pH 5.0 remained extremely slow, whereas at pH 6.0 it was almost identical to the growth observed in the media containing the other carboxylic acids (Fig. 1). Most probably, besides the diffusion of acetic acid, a mediated transport mechanism involved in the uptake of acetate can be present, since growth on acetate was higher at pH 6.0, when more dissociated form of the acid is present in the media.

Growth was also assessed in liquid media during 4 days, at 30 ºC. Cells were grown in synthetic media supplemented either with glucose or acetic acid, pH 6.0. Growth curves of the wild-type strains and the mutant *Cgady2*a and *Cgfps1* strains are represented in figure 2, as well as the final OD_{640nm}, and the respective growth rates are described in table 3. Glucose-grown cells presented higher growth rates, in comparison to acetic acid-grown cells, indicating that *C. glabrata*, wild-type and mutant cells, grow better in the presence of a sugar (glucose), than in the presence of a monocarboxylic acid, as expected. Again, no significant differences were encountered between the growth rates in the wild-type and the mutant strains, in both tested conditions.

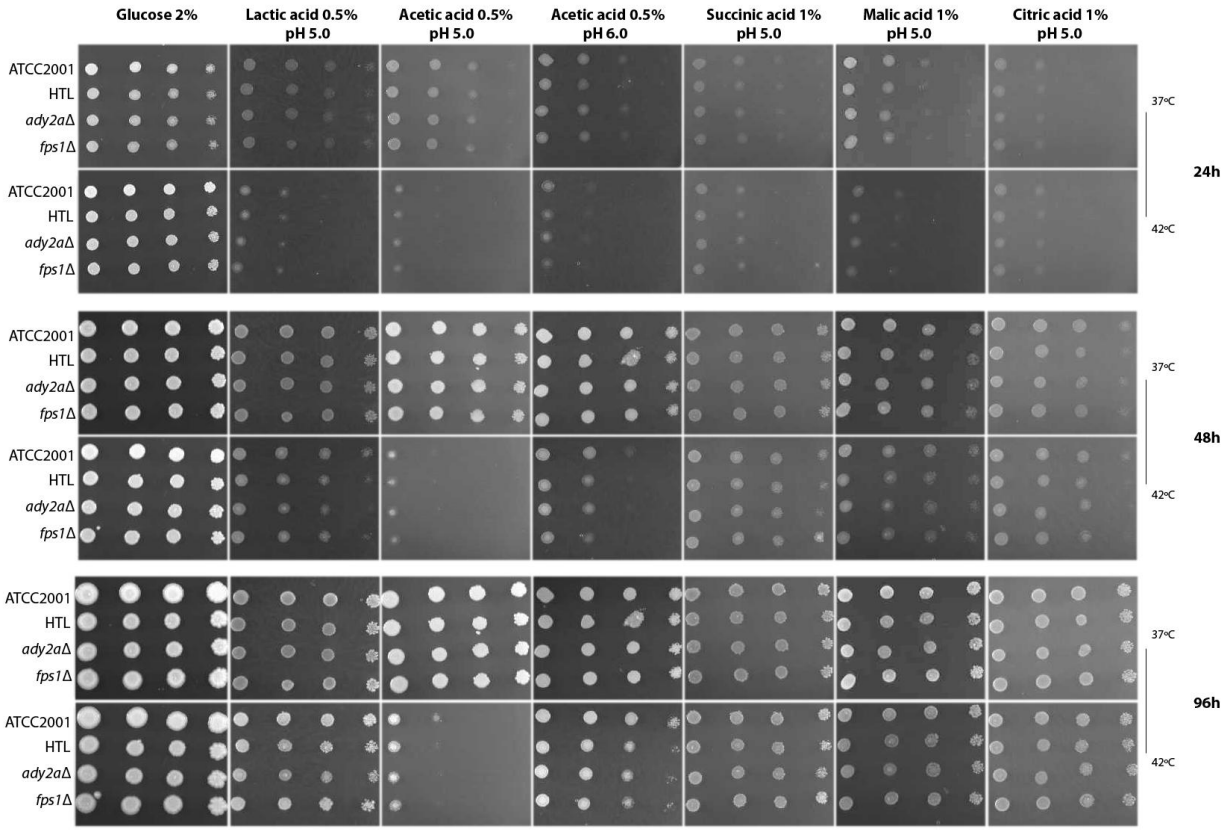

Figure 1 – Growth phenotypes, at 37ºC and 42ºC, of *C. glabrata* ATCC2001 and HTL (wild-types), FI1 (*ady2a*) and TS453 (*fps1*), incubated for 24, 48 and 96 h, in the following solid media: SC-glucose 2%; SC-lactic acid (0.5%, w/v, pH 5.0); SC-acetic acid (0.5%, w/v, pH 5.0 and 6.0); SC-succinic acid (1%, w/v, pH 5.0); SC-malic acid (1%, w/v, pH 5.0) and SC-citric acid (1%, w/v, pH 5.0). Cells were serially diluted; 3 µl drops of each dilution were spotted onto the plates.

Table 3 – Specific growth rates, µ (h^{-1}), and final OD_{640nm} (OD_{final}) for *C. glabrata* strains: ATCC2001, HTL, *fps1* and *ady2* obtained after growth in synthetic complete media supplemented either with glucose 2% (w/v) or acetic acid 0.5% (v/v). Growth was performed for four days at 30ºC.

Culture Media	**ATCC2001**		**HTL**		***fps1***		***ady2a***	
	µ	OD_{final}	µ	OD_{final}	µ	OD_{final}	µ	OD_{final}
Sc Glucose 2%	0,46	8,22	0,49	7,29	0,49	8,34	0,49	8,19
Sc Acetic acid 0.5% pH 6.0	0,30	1,13	0,26	1,03	0,26	0,96	0,29	0,85

*Transport of acetate and lactate in ATCC2001 and Cg*ady2 *mutant strain*

In the literature we have found no data regarding transport assays of radioactive labelled carboxylic acids in *C. glabrata*. The presence of two putative homologs of Sc*ADY2*, suggests that most probably this pathogenic yeast possesses a saturable transport system for the uptake of acetic acid. To determine the physiological role of these genes, we intended to disrupt them individually and in combination in the

ATCC2001 background. However, the disruption of Cg*ADY2b* proved to be difficult, and till date unsuccessful.

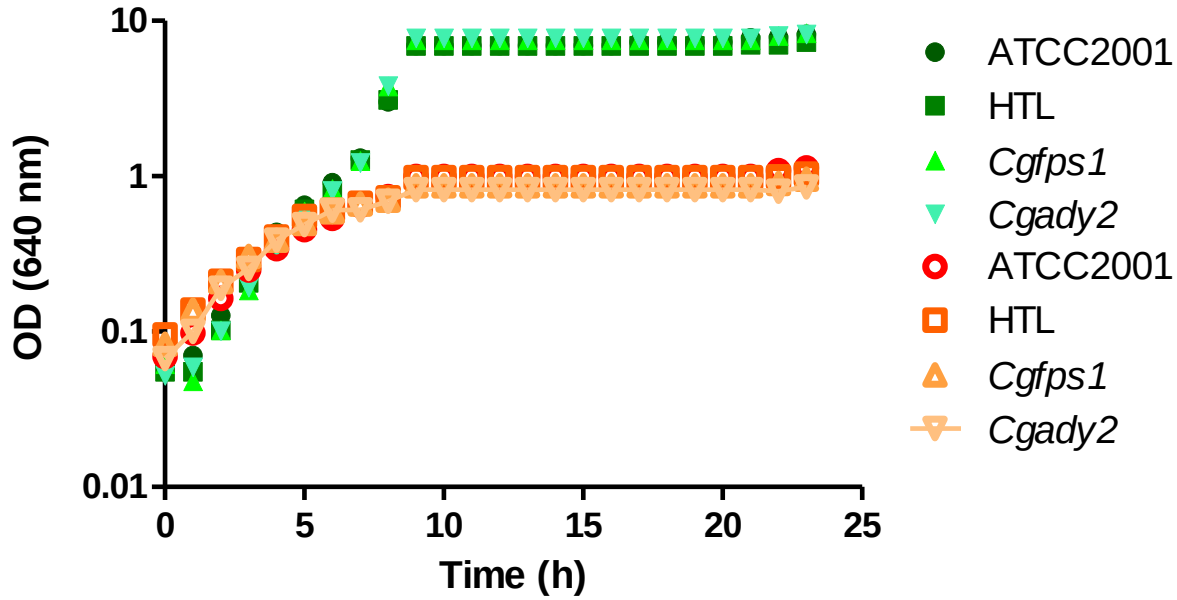

Figure 2 – Growth curves, at 30°C, of *C. glabrata* strains: ATCC2001(●); HTL (■); TS453 (*Cgfps1*) (▲), and FI1 (*Cgady2a*) (▼) in SC-glucose 2% (closed symbols) and SC-acetic acid media (open symbols).

A preliminary analysis of the capacity of *C. glabrata* cells to transport acetic and lactic acids was performed in wild-type cells grown in YP medium, supplemented either with acetic acid (pH 6.0), or lactic acid (pH 5.0). A computer-assisted non-linear regression analyses to the experimental data indicated the presence of a saturable transport mechanism involved in the uptake of labelled acetic acid, in YP acetic acid-grown cells (for concentrations between 0.1 mM and 5.54 mM) (Fig. 3). No measurable initial uptake rates were found, for labelled lactic acid, in YP lactic acid-grown cells, indicating that *C. glabrata* cells do not present a mediated transport system for lactic acid (Fig. 3). This observation explains the delayed growth on minimal liquid media, supplemented with lactic acid, as the only carbon and energy source, even after 24h of incubation (data not shown). However, in lactic acid derepressed cells, a saturable transport system for labelled acetic acid could be detected (data not shown).

The uptake of acetic acid was monitored in glucose or acetic acid (pH 6.0) grown-cells. A computer-assisted non-linear regression analyses applied to the experimental data indicated the presence of a first order kinetic, involved in the uptake of acetic acid, in glucose-grown cells (Fig. 4A) and of a second order kinetics in acetic acid-grown cells, with the following kinetic parameters: V_{max} of 8,08±1,21 nmol s-1 mg

dry wt.-1; and a K_m of 8,16±2,13 mM (for concentrations between 0.5 mM and 10 mM) (Fig. 4A). Moreover, initial uptake rates of labelled lactic acid were followed in the wild-type cells, grown in minimal media supplemented with acetic acid (Fig. 4B). Again, no significant values were found, indicating that *C. glabrata* cells do not display measurable transport for lactic acid after derepression in acetic acid (or after derepression in lactic acid, as described previously). Overall, these results suggest that *C. glabrata* presents a saturable transport system for the uptake of acetic acid, pH 5.0, albeit with an affinity lower than the one observed in *S. cerevisiae*.

We followed the uptake of radioactive labelled acetic acid, pH 5.0, in the *Cgady2a* mutant strain, in an effort to unravel the role of this protein in *C. glabrata*. A computer assisted analyses applied to the experimental data, from four independent experiments, pointed to the presence of a first order kinetics (for concentrations between 0.5 mM and 10 mM). In this manner, the disruption of C*gady2a* affected the mediated uptake of acetic acid, nevertheless, the standard deviation values are quite significant and imply an instable behaviour of this mutant (Fig. 4).

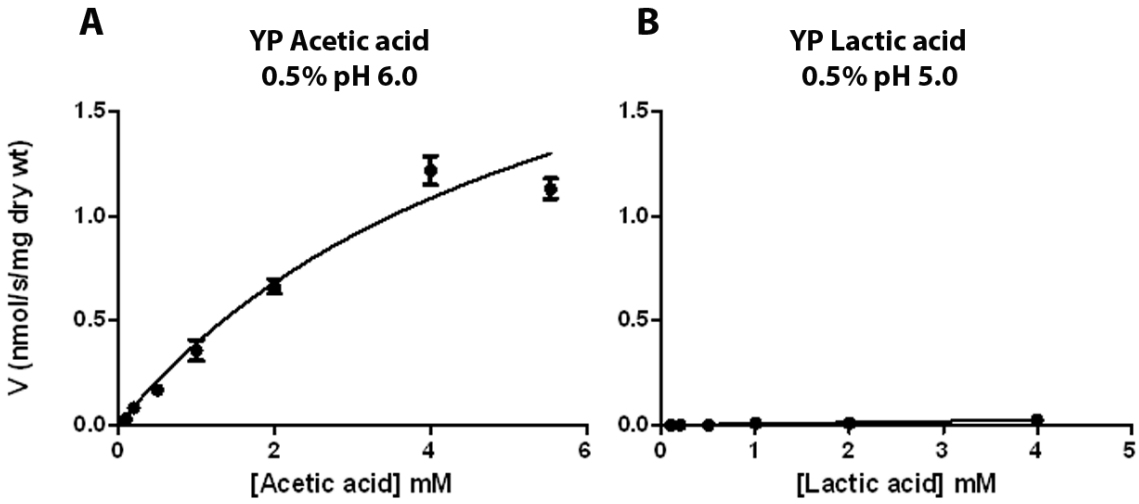

Figure 3 – Transport of acetic and lactic acids in *C. glabrata* ATCC2001. A – Initial uptake rates of radioactive labelled acetic acid, at pH 5.0, as a function of acetic acid concentration, in exponentially growing cells, in YP acetic acid 0.5% pH 6.0. B – Initial uptake rates of radioactive labelled lactic acid at pH 5.0, as a function of lactic acid concentration, in exponentially growing cells, in YP lactic acid 0.5% pH 5.0.

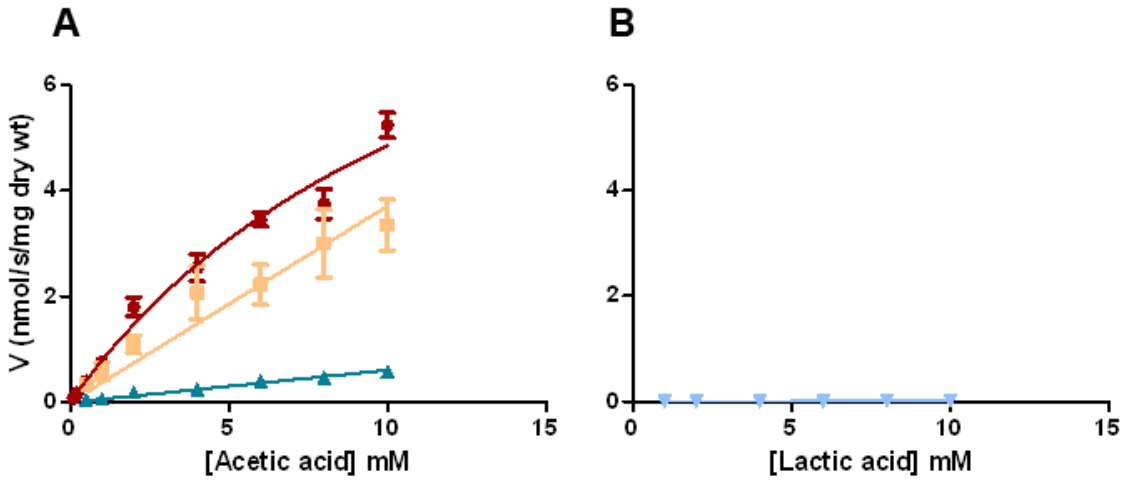

Figure 4 – Transport of labelled acetic and lactic acids in *C. glabrata*. A – Initial uptake rates of labelled acetic acid, as a function of acetic acid concentration in *C. glabrata* strain ATCC2001 (wild-type) acetic acid-grown cells, ●; strain FI1 (*ady2a*) acetic acid-grown cells, ■; strain ATCC2001 (wild-type) glucose-grown cells, ▲. B - Initial uptake rates of labelled lactic acid, as a function of lactic acid concentration *in C. glabrata* ATCC2001 (wild-type) in acetic acid-grown cells, ▼.

*Expression of Cg*ADY2a/b *and Cg*ATO3 *in alternative carbon sources*

C. glabrata cells were grown in different carbon containing media to study the expression of Cg*ADY2a/b*, Cg*ATO3* and Cg*FPS1* by reverse transcription polymerase chain reaction (RT-PCR).

The PCR products obtained, represented in figure 5, indicate that all genes were expressed under the conditions tested. All PCR products had the predicted lengths: 493 bp for the amplification of *CgADY2a*, 576 bp for *CgADY2b*, 638 bp for *CgATO3* and 379 bp for the amplification of *CgACT1* gene (Fig. 5). In order to verify the absence of genomic DNA in the RNA preparations, Superscript RT III/Taq mixture was replaced by Taq polymerase. No product was obtained thereby rulling out the possibility of gDNA contamination. Moreover, no expression was detected when mRNA was omitted from the reaction mixture, indicating the purity of the reagents used.

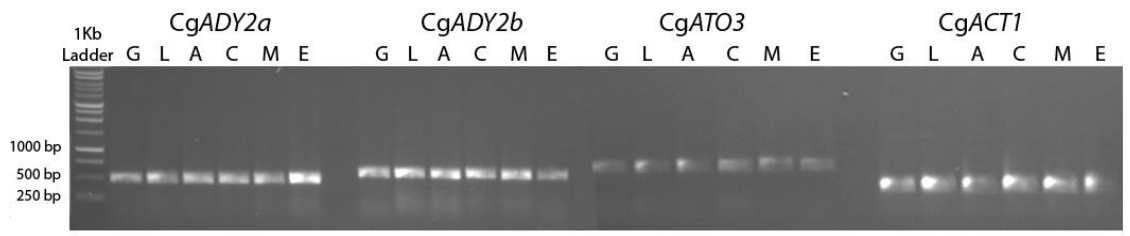

Figure 5 – Expression of *C. glabrata ADY2a*, *ADY2b*, *ATO3* in cells grown in: G- Glucose; L – Lactic acid 0.5% (v/v), pH 5.0; A – Acetic acid 0.5% (v/v), pH 6.0; C –Citric acid 1% (w/v), pH 5.0; M – Malic acid 1% (w/v), pH 5.0; E – Ethanol 1% (v/v). The mRNA was followed by RT-PCR. As a positive control, expression of Cg*ACT1* was monitored. As negative control, no

mRNA was added (data not shown). For genomic DNA contamination control, the SuperScript III RT/Platinum Taq mix was replaced by Taq Polymerase (data not shown).

The RT-PCR method has limitations, when compared to the Real-Time PCR (Mackay *et al.*, 2002), constituting only a semi quantitative or qualitative technique. It allows the detection of even a very low copy number of RNA molecules, due to its high sensitivity. In this manner, we can only infer that all genes are induced in the presence of the carbon sources tested, but the level of that induction remains unknown

Little is known about *C. glabrata* carbon metabolism and glucose repression. Studies performed by Bialkova and co-workers indicated that glucose repression is not observed in *C. glabrata*, although a Snf1 homolog has been identified (Bialkova and Subik, 2006). This could justify the expression of Cg*ADY2a/b* and Cg*ATO3* in the presence of glucose, since in *S. cerevisiae* these homolog genes are repressed in the presence of glucose (Gancedo, 1998; Carlson, 1999). On the other hand, Petter and co-workers demonstrated that Cg*SNF1* could functionally complement Sc*SNF1*, and that its disruption impaired the assimilation of trehalose, indicating that even in an organism with such narrow carbon utilization range, catabolic repression was preserved (Petter and Kwon-Chung, 1996). Moreover, studies performed by Cormack and co-workers indicated that Snf1 was required for growth on non-fermentable carbon sources and on trehalose, implying Snf1's involvement in the relief from glucose catabolite repression in *C. glabrata* (Cormack, B. personal communication).

In summary, our results seem to be in accordance with Bialkova and co-workers' study, where no glucose repression was observed (Bialkova and Subik, 2006). The technique we used allows the detection of even very small amounts of RNA, and due to the multiple amplification rounds, the final amount obtained does not differ significantly from the samples where greater initial expression was present. Thus, it is still possible that there are significantly less copies in the presence of glucose than in the other carbon sources. A quantitative gene expression methodology, such as Real-time PCR or Northern blot analysis should be performed to clarify the obtained results. Moreover, this does not rule out the hypothesis that glucose could be acting post-transcriptionally, affecting protein production and/or activity.

Discussion

The human pathogen *C. glabrata* can thrive in different environments with distinct nutrient availabilities. When glucose is scarce, this yeast must have the ability to efficiently utilize non-fermentable carbon sources to survive within the human host. Previous studies have identified the significance of the glyoxylate function, and hence of lipid and acetate derived acetyl-CoA, in the virulence of a vast range of pathogens (Ramirez and Lorenz, 2007), including *C. glabrata* (Kaur *et al.*, 2007). Recently, Roetzer and co-workers studied the reaction of phagocytosed *C. glabrata* wild-type cells. They evidenced the transient oxidative stress and the carbon starvation this fungus encounters, in the human host, as expected. Additionally, their results indicate that phagocytosed cells present a higher number of peroxisomes, probably to accomplish higher levels of β–oxidation of fatty acids and of the glyoxylate cycle function, supporting previous findings in other pathogens. However, prolonged phagocytosis led to a decrease in peroxisomes numbers, suggesting that peroxisome formation and maintenance can eventually sequester resources essential for the survival of this yeast (Roetzer *et al.*, 2009).

Barnett and co-workers indicated that this yeast could efficiently assimilate glucose, but that growth on trehalose, glycerol, ethanol and DL-lactate was variable between strains. Moreover, they indicated that this yeast species was unable to grow on media supplemented with succinate and citrate (Barnett, 2000). In this study we have shown that *C. glabrata* ATCC2001 background grows in a wide range of temperatures, preferably in the presence of glucose, and that it is capable of assimilating non-fermentable carbon sources such as lactic, acetic, succinic, malic and citric acids, at pH 5.0, and acetic acid at pH 6.0, although with a slower growth rate (Fig. 1 and 2). These findings confirm some of Barnett's results but also show that in fact *C. glabrata* ATCC2001 wild-type strain is able to assimilate mono-, di- and tricarboxylic acids.

We have shown that *C. glabrata* presents a saturable transport mechanism involved in the uptake of acetic acid. This species encodes in its genome two putative orthologs of ScAdy2 (Paiva *et al.*, 2004) and two homologs of ScFps1 (a channel involved in the facilitated diffusion of acetic acid through the plasma membrane) (Mollapour and Piper, 2007). The deletion of the closest homolog of *ScADY2*, Cg*ADY2a*, affected the mediated uptake of acetic acid. The initial uptake rates of acetic acid, presented high standard deviations reinforcing the instable behaviour of the mutant

strain, in this respect. In fact, the growth phenotypes in distinct non-fermentable carbon sources (Fig. 1), were not influenced by the disruption of *CgADY2a*, in comparison to the wild-type strain. It is feasible that CgAdy2b has a role in the uptake of acetic acid, and thus, compensate the disruption of *CgADY2*a. Furthermore, the mutant *Cgfps1 (CAGL0C03267g),* which is the closest homolog of ScFps1, behaved as the wild-type strain in the presence of distinct carbon and energy sources. Since *C. glabrata* has two homologs of ScFps1, it is possible that both genes can contribute to the facilitated diffusion of acetic acid. Clarification of the role of *C. glabrata* ScFps1 homologs should be performed, as previously described for *S. cerevisiae* Fps1, in acetic acid containing media, with concentrations ranging between 80 and 100 mM, at pH 4.5 (if the function of this homolog is similar to ScFps1) (Mollapour and Piper, 2007).

Overall, the growth in carboxylic acids containing media was similar between all the tested strains (wild-type and mutant strains), and was more evident after incubation at 37ºC. When the plates were incubated at 42ºC the ability to grow was generally affected, but especially in acetic acid 0.5% pH 5.0 (Fig. 1). This is probably due to a significant decrease in the diffusion of the undissociated forms of these acids, through the plasma membrane. However, since the growth defect in the presence of acetic acid at pH 5.0 is more pronounced than at pH 6.0, we believe that acetate uptake is not only due to diffusion, but also to the presence of a mediated transport mechanism, involved in the uptake of the dissociated form of the acid, that is affected at higher temperatures. If so, both copies of ScAdy2 homologs in *C. glabrata* can be involved in the uptake of acetate. Furthermore, *in vivo* studies in infection models of systemic candidiasis with *Cgady2a/b* disruption mutants could clarify their significance in *C. glabrata* pathogenicity. Further studies should be carried out in order to elucidate the metabolic pathways involved in the survival of this pathogen within the human host, and to clarify its carbon assimilation.

References

Banerjee, S. N., Emori, T. G., Culver, D. H., Gaynes, R. P., Jarvis, W. R., Horan, T., Edwards, J. R., Tolson, J., Henderson, T. and Martone, W. J. 1991. Secular trends in nosocomial primary bloodstream infections in the United States, 1980-1989. National Nosocomial Infections Surveillance System. *Am J Med.* **91**: 86S-89S.

Barnett, J. A., Payne, R. W. and Yarrow, D. 2000. Yeast, Characteristics and Identification. Third edition. Cambridge University Press.

Bialkova, A. and Subik, J. 2006. Biology of the pathogenic yeast *Candida glabrata*. *Folia Microbiol (Praha).* **51**: 3-20.

Boshoff, H. I. and Barry, C. E. 2005. A low-carb diet for a high-octane pathogen. *Nat Med.* **11**: 599-600.

Carlson, M. 1999. Glucose repression in yeast. *Curr Opin Microbiol.* **2**: 202-207.

De Hertogh, B., Hancy, F., Goffeau, A. and Baret, P. V. 2006. Emergence of species-specific transporters during evolution of the hemiascomycete phylum. *Genetics.* **172**: 771-781.

Dujon, B., Sherman, D., Fischer, G., Durrens, P., Casaregola, S., Lafontaine, I., De Montigny, J., Marck, C., Neuveglise, C., Talla, E., *et al.* 2004. Genome evolution in yeasts. *Nature.* **430**: 35-44.

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

Idnurm, A. and Howlett, B. J. 2002. Isocitrate lyase is essential for pathogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). *Eukaryot Cell.* **1**: 719-724.

Iraqui, I., Garcia-Sanchez, S., Aubert, S., Dromer, F., Ghigo, J. M., d'Enfert, C. and Janbon, G. 2005. The Yak1p kinase controls expression of adhesins and biofilm formation in *Candida glabrata* in a Sir4p-dependent pathway. *Mol Microbiol.* **55**: 1259-1271.

Kao, A. S., Brandt, M. E., Pruitt, W. R., Conn, L. A., Perkins, B. A., Stephens, D. S., Baughman, W. S., Reingold, A. L., Rothrock, G. A., Pfaller, M. A., Pinner, R. W. and Hajjeh, R. A. 1999. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. *Clin Infect Dis.* **29**: 1164-1170.

Kaur, R., Ma, B. and Cormack, B. P. 2007. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. *Proc Natl Acad Sci U S A.* **104**: 7628-7633.

Lorenz, M. C. and Fink, G. R. 2002. Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryot Cell.* **1**: 657-662.

Mackay, I. M., Arden, K. E. and Nitsche, A. 2002. Real-time PCR in virology. *Nucleic Acids Res.* **30**: 1292-1305.

McKinney, J. D., Honer zu Bentrup, K., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr. and Russell, D. G. 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature.* **406**: 735-738.

Messer, S. A., Jones, R. N. and Fritsche, T. R. 2006. International surveillance of *Candida spp*. and *Aspergillus spp.:* report from the SENTRY Antimicrobial Surveillance Program (2003). *J Clin Microbiol.* **44**: 1782-1787.

Mollapour, M. and Piper, P. W. 2007. Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Mol Cell Biol.* **27**: 6446-6456.

Nguyen, M. H., Peacock, J. E., Jr., Morris, A. J., Tanner, D. C., Nguyen, M. L., Snydman, D. R., Wagener, M. M., Rinaldi, M. G. and Yu, V. L. 1996. The changing face of candidemia: emergence of non-*Candida albicans* species and antifungal resistance. *Am J Med.* **100**: 617-623.

Noble, S. M. and Johnson, A. D. 2005. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot Cell.* **4**: 298-309.

Paiva, S., Devaux, F., Barbosa, S., Jacq, C. and Casal, M. 2004. Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. *Yeast.* **21**: 201-210.

Perlroth, J., Choi, B. and Spellberg, B. 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med Mycol.* **45**: 321-346.

Petter, R. and Kwon-Chung, K. J. 1996. Disruption of the *SNF1* gene abolishes trehalose utilization in the pathogenic yeast *Candida glabrata*. *Infect Immun.* **64**: 5269-5273.

Pfaller, M. A. and Diekema, D. J. 2004. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of Candida. *Clin Microbiol Infect.* **10 Suppl 1**: 11-23.

Pfaller, M. A., Jones, R. N., Messer, S. A., Edmond, M. B. and Wenzel, R. P. 1998. National surveillance of nosocomial blood stream infection due to *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program. *Diagn Microbiol Infect Dis.* **31**: 327-332.

Piekarska, K., Mol, E., van den Berg, M., Hardy, G., van den Burg, J., van Roermund, C., MacCallum, D., Odds, F. and Distel, B. 2006. Peroxisomal fatty acid beta-oxidation is not essential for virulence of *Candida albicans*. *Eukaryot Cell.* **5**: 1847-1856.

Ramirez, M. A. and Lorenz, M. C. 2007. Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. *Eukaryot Cell.* **6**: 280-290.

Reuss, O., Vik, A., Kolter, R. and Morschhauser, J. 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene.* **341**: 119-127.

Richter, S. S., Galask, R. P., Messer, S. A., Hollis, R. J., Diekema, D. J. and Pfaller, M. A. 2005. Antifungal susceptibilities of *Candida species* causing vulvovaginitis and epidemiology of recurrent cases. *J Clin Microbiol.* **43**: 2155-2162.

Roetzer, A., Gratz, N., Kovarik, P. and Schuller, C. 2009. Autophagy supports *Candida glabrata* survival during phagocytosis. *Cell Microbiol.*

Shen, J., Guo, W. and Kohler, J. R. 2005. *CaNAT1*, a heterologous dominant selectable marker for transformation of *Candida albicans* and other pathogenic *Candida species*. *Infect Immun.* **73**: 1239-1242.

Sherman, F. 1991. Getting started with yeast. *Methods Enzymol.* **194**: 3-21.

Solomon, P. S., Lee, R. C., Wilson, T. J. and Oliver, R. P. 2004. Pathogenicity of *Stagonospora nodorum* requires malate synthase. *Mol Microbiol.* **53**: 1065-1073.

Vereecke, D., Cornelis, K., Temmerman, W., Jaziri, M., Van Montagu, M., Holsters, M. and Goethals, K. 2002. Chromosomal locus that affects pathogenicity of *Rhodococcus fascians*. *J Bacteriol.* **184**: 1112-1120.

Vieira, N., Casal, M., Johansson, B., Maccallum, D. M., Brown, A. J. and Paiva, S. 2009. Functional specialization and differential regulation of short-chain carboxylic acid transporters in the pathogen *Candida albicans*. *Mol Microbiol.* **76:**1337-54.

Wang, Z. Y., Thornton, C. R., Kershaw, M. J., Debao, L. and Talbot, N. J. 2003. The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*. *Mol Microbiol.* **47**: 1601-1612.

CHAPTER V – An acetotrophic methane producing Archaeon has two homologs of *Saccharomyces cerevisiae* Ady2, acetate transporter

Abstract

The methanogen *Methanosarcina acetivorans* belongs to the Methanosarcineae family, which comprises the most metabolically diverse methanogens, thriving in a broad variety of environments. Its genome was deciphered in 2002 and unravelled an unexpected variety of metabolic and cellular features. *M. acetivorans* is an acetate consuming archaea that encodes in its genome two putative homologs of the *S. cerevisiae* monocarboxylate transporter, Ady2, a YaaH family member. The YaaH family (TC 2.A.96) has homologs in the three domains of life: Bacteria, Archaea and Eukaryota but some of its members have not been functionally characterized yet. Additionally, further roles have been attributed to ScAdy2 and its true function remains unclear. The functional characterization of *M. acetivorans* ScAdy2 homologs could therefore, help to clarify the true function of this protein family, as well as to delineate the phylogeny of the YaaH family members. Heterologous expression of both *M. acetivorans* ScAdy2 homologs was attempted in a *S. cerevisiae* strain. However, this strategy did not contribute to the clarification of the role of these genes, most probably due to translational constrains resultant from crossing genes through distinct domains of life.

Introduction

Initially, prokaryotic organisms constituted one group that included the *Eubacteria* and the *Archaebacteria*, but with the advents of gene sequencing technologies, the differences between their 16S rRNA genes became determinant for their separation in two groups, the Archaea and the Bacteria (Woese *et al.*, 1990).

The Archaea domain possesses a unique evolutionary history, including some of the most ancient organisms on earth. Unfortunately, it remains the most poorly understood domain of life, although it is extremely important to the biosphere. The ability to produce methane through methanogenesis, is unique to this domain and plays an essential role in the global carbon cycle (Schlesinger, 1997), with implications in waste treatment industries, as an alternative fuel resource and as a potent greenhouse gas contributing even more to global warming than CO_2.

All known methanogens are archaeons and obligate anaerobes that can be classified as: hydrotropic, when carbon dioxide is reduced to methane using electrons derived by oxidizing H_2; acetotrophic, when acetate is used as carbon and energy source; and as methylotrpophic, when methylated one carbon compounds are used as carbon and energy sources (Galagan *et al.*, 2002).

The microorganism, *Methanosarcina acetivorans* is a methanogen capable of utilizing acetate, belonging to the Methanosarcineae family. The members of this family are capable of forming complex multicellular structures, during distinct growth phases, and in response to different environmental cues, an ability not shared by the other archaea members (Macario and Conway De Macario, 2001). Additionally, they are extremely versatile methanogens in what respects to metabolic and physiological traits, thriving in a vast range of environments. Most members of other orders of methanogens are only capable of utilizing a single pathway for methanogenesis and no more than two substrates, whereas the *Methanosarcina* species present all the three known metabolic pathways involved in the production of methane, and are capable of utilizing nine methanogenic substrates, including the previously mentioned acetate (Galagan *et al.*, 2002). Members of this species have been identified in several distinct environments, such as, fresh water, deep marine sediments, garden soils, trash dumps, sewage heaps, oil wells, termophilic digesters, and even in the gut of many ungulates, namely, cows and goats, among others, and in the human digestive tract (Zinder, 1993).

In 2002, Galagan and co-workers reported the complete genome sequence of *M. acetivorans* and evidenced its markedly metabolic and physiological diversity. This genome constitutes the largest known archaeal genome with 5,751,492 bp, with approximately 4525 ORFs, encoding an unexpected genetic diversity and redundancy. This probably underlyis its ability to efficiently adapt to a broad range of environments, as well as the potential unanticipated metabolic and cellular abilities (Galagan *et al.*, 2002). *M. mazei* has the second largest known archaeal genome with 4, 096,345 bp. The archaea *M. acetivorans* encodes in its genome two genes homologous to the *S. cerevisiae ADY2* gene, *Ma0103* (NCBI Reference: NP_615077.1) and *Ma4008* (NCBI Reference: NP_618878.1). Sc*ADY2* encodes a monocarboxylate permease, that accepts acetate, propionate or formate and that is present in cells grown in non-fermentable carbon sources (Casal *et al.*, 1996). This permease belongs to the YaaH family and was previously classified, in the transport classification system database, as a possible or

putative membrane transporter, 9.B.33. Additional roles have been attributed to this protein, namely its involvement in ammonia export (Palkova *et al.*, 2002). Very recently, the previous TCDB annotation was updated and now the YaaH family is classified as a real porter family, and Ady2 as an acetate permease required for normal sporulation, 2.A.96.1.4.

The ability of Archaea to grow in extreme habitats has led to the exploration of the molecular mechanism that confers stability to proteins, under high temperature, high pressure or extreme pH values. Moreover, the availability of genetic methods together with its physiological and metabolic diversity makes this methanogen a powerful model organism for the study of Archaeal biology (de Macario *et al.*, 1996; Metcalf *et al.*, 1997; Metcalf *et al.*, 1998; Boccazzi *et al.*, 2000; Zhang *et al.*, 2000). The functional characterization of *M. acetivorans* ScAdy2 homologs, in a heterologous expression system, constitutes the aim of this work.

Material and methods

Yeast strains and growth conditions

The strains used in this study are represented in table 1. Growth was performed at 30 ºC, either in the complex media YPD or in a synthetic minimal media with 0.67% yeast nitrogen base (YNB medium, Difco), supplemented with amino acids to meet auxotrophic requirements. Carbon sources were either 2% glucose, 0.5% acetic acid (pH 6.0) or lactic acid 0.5% (pH 5.0). Cells were always harvested at mid-exponential phase after growth on glucose containing media. For derepression conditions, glucose-grown cells were collected by centrifugation, washed twice in ice-cold deionized water and grown in fresh YNB media supplemented with lactic or acetic acid 0.5% (pH 5.0 or 6.0, respectively). Yeast strains were maintained in solid YPD media and bacteria strains in solid LB media supplemented with ampicilin (100 mg/L).

Table 1 – Strains used in this study.

Strains	Genotype	Reference
S. cerevisiae		
W303-1A	*MATa adeu2, leu2, his3, trp1, ura3*	(Thomas and Rothstein, 1989)
jen1ady2	W303-1A *jen1::HphMx4 ady2::KanMx4*	(Soares-Silva *et al.*, 2007)
NV6	W303-1A *jen1::HphMx4 ady2::KanMx4* ptYEplac 181	This work
NV7	W303-1A *jen1::HphMx4 ady2::KanMx4*	This work

	ptYEplac 181*::Ma0103*	
NV8	W303-1A *jen1::HphMx4 ady2::KanMx4* ptYEplac 181*::Ma4008*	This work
NV9	W303-1A *jen1::HphMx4 ady2::KanMx4* ptYEplac 181*::JEN1*	This work
NV10	W303-1A *jen1::HphMx4 ady2::KanMx4* ptYEplac 181*::ADY2*	This work
NV11	W303-1A *jen1::HphMx4 ady2::KanMx4* p416GPD*::Ma0103*	This work
NV12	W303-1A *jen1::HphMx4 ady2::KanMx4* P416GPD*::Ma4008*	This work
pDS-1	W303-1A *jen1::HphMx4 ady2::KanMx4* p416GPD::*JEN1*	(Soares-Silva *et al.*, 2007)
NV13	W303-1A *jen1::HphMx4 ady2::KanMx4* p416GPD::*ADY2*	This work
p416GPD Ø	W303-1A *jen1::HphMx4 ady2::KanMx4* p416GPD	(Soares-Silva *et al.*, 2007)
E. coli		
XL1Blue	*endA1 gyrA96(nal^R) thi-1 recA1relA1 lac glnV44 F'[:: Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17 (r_K^- m_K^+)*	(Bullock, 1987)

Cloning of M. acetivorans *genes homologs to* S. cerevisiae ADY2

Amplification of the *Ma0103* and *Ma4008* genes was performed with the primers Ma1013 Fwd/Ma0103 Rev and Ma4008 Fwd/Ma4008 Rev (Table 2). A hexa-histidine encoding sequence was included in the reverse primers, in order to produce fusion proteins with a His-tag at the C-terminal. The genes were amplified by PCR using a proofreading DNA Polymerase (Pfu, Invitrogen) and cloned in the pGEM®-T Easy vector (PROMEGA). The DNA sequences of the selected clones were confirmed by sequencing using ABI PRISM310 Genetic Analyzer.

Table 2 – Oligonucleotides used in this study.

Name	Sequence
Ma0103 Fwd	GGGATCCATTAAAATCTCCATAGAATCAAGAATT
Ma0103 Rev	GGAATTCTCAGTGATGGTGATGGTGATGGATAGGCACAACTTTCCTTCCGTA
Ma4008 Fwd	GGGATCCATGAGTGAAGACATTAAAGATGTTATG
Ma4008 Rev	GGAATTCTTAGTGATGGTGATGGTGATGGAGTGGAACTATCTTTTTGCCATA
ptYEplac181JEN1 Fwd	GGGATCCATGTCGTCGTCAATTACAGATGAGAAA
ptYEplac181JEN1 Rev	GAAGCTTTTAGTGATGGTGATGGTGATGAACGGTCTCAATATGCTCCTCATA
ptYEplac181ADY2 Fwd	GGGATCCATGTCTGACAAGGAACAAACGAGCGGA
ptYEplac181ADY2 Rev	GGGATCCTTAGTGATGGTGATGGTGATGAAAGATTACCCTTTCAGTAGATGG

Expression of archaeal ADY2 *homologs in the yeast* S. cerevisiae

The genes were cloned in the expression vector ptYEPlac181, under the control of the PMA1 promoter, using *BamH* I and *EcoR* I restriction enzymes. The vectors were

subsequently used to transform a *S. cerevisiae jen1ady2* strain, which has no mediated transport system for monocarboxylic acids, since it is disrupted on both monocarboxylate proton symporters encoding genes described so far, *JEN1* and *ADY2*. In this manner strains NV6 (control with the empty vector), NV7 (*S. cerevisiae jen1ady2*, ptYEplac181*::Ma0103)* and NV8 (*S. cerevisiae jen1ady2*, ptYEplac181*::Ma4008*) were constructed. Additionally, as further controls, *JEN1* and *ADY2* were cloned in the same expression vector ptYEplac181, using the primers ptYEplac181JEN1 Fwd_/Rev and ptYEplac181ADY2 Fwd_/Rev, respectively (strains NV9 and NV10). Both reverse primers had a hexa-histidine encoding sequence to produce fusion proteins with a His-tag at the C-terminal. Simultaneously, the genes *Ma0103* and *Ma4008* cloned in the pGEM®-T Easy vector were digested with *BamH*I and *Eco*RI and ligated to the expression vector p416GPD (Mumberg *et al.*, 1995), originating strains NV11 and NV12. This expression vector had already been used for the expression of membrane proteins, namely of Jen1 (Soares-Silva *et al.*, 2003). *S. cerevisiae jen1ady2 s*trains containing the empty p416GPD, p416GPD::*JEN1* (pDS-1) and p416GPD::*ADY2* (NV13) were used in this study, as additional controls.

Total membrane extracts and Immunoblotting

NV6, NV7 and NV8 strains were grown in YNB Glucose without leucine, and harvested in the exponential phase for membrane preparation. Total membrane extracts were prepared after derepression in YNB acetic acid, 0.5% (v/v), pH 6.0 (w/o leu), for 6 h. Cells were cooled down on ice, for 30 min, and two sequential centrifugations were performed at 5000 rpm, for 5 min, with disposal of the supernatant. Pellets were weighted, resuspended in cold MBS media (15 ml MBS/10 g cells; Composition: $MgCl_2$ 1 mM, Imidazole 50 mM, Sorbitol 250 mM and PMSF 1mM) and disrupted mechanically. Two centrifugations of 5 min were performed at 3500, another one for 5 min, at 6000 rpm, with sequential recoveries of the supernatant, and a final centrifugation at 13 000 rpm for 40 min. The supernatant was discarded and the pellet resuspended in cold MS (0.5 mg/g cells; Composition: $MgCl_2$ 1 mM, Imidazole 10 mM and PMFS 1 mM). Pellet was divided in aliquots, frozen in liquid nitrogen and stored at -80ºC. Total membranes extracts were then resuspended in sample buffer, and loaded (10, 30 or 60 µg), with no previous incubation (room temperature) or after incubation for 1 or 5 minutes, at either 60ºC or 100ºC, and resolved by SDS-PAGE in 12-13%

acrylamide gels using Tricine buffer. The gels were then stained with Commasie Blue for protein visualization. Total protein extracts were also prepared by the NaOH-TCA lysis technique and the extracts resuspended in sample buffer, heated for 15 min, at 37ºC and resolved by 10% SDS-PAGE, using Tricine buffer and transferred to nitrocellulose membranes. The membranes were probed with monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 (SIGMA) and the signal detected by enhanced chemiluminescence.

Transport assays

Transport assays, using labelled [U-^{14}C] acetic acid and [U-^{14}C] lactic acid (sodium salts; Amersham Biosciences, 4000 dpm/nmol, at pH 5.0), were carried out as described previously (Paiva *et al.*, 2009).

Results

Heterologous expression of Ma0103 *and Ma4008 in the yeast* S. cerevisiae

The acetotrophic methanogen *M. acetivorans* (C2A) had its genome deciphered in 2002 as a result of a joint effort of different scientists. This acetate consuming microorganism encodes in its genome two putative homologs of the *ADY2* gene, with 45% of identity between each other (Fig. 1).

```
 Score =  177 bits (448),  Expect = 2e-49, Method: Compositional matrix adjust.
 Identities = 88/192 (45%), Positives = 128/192 (66%), Gaps = 4/192 (2%)

Ma0103  36   VADVHVKDRTANPSPLGFTGLGLSATLLSLSYIGLYPVDSMIVSMAIFLGGFAQVFAGLM  95
             + DV +KD +ANP+PLG  G G++  LL++   G YP+ ++I+SM IF GG  QV AG+
Ma4008  11   IRDVKIKDLSANPAPLGLMGFGMTTVLLNIHNAGFYPLGAVILSMGIFYGGLGQVIAGIE  70

Ma0103  96   AWKKGSVFGGTAFCAFGLFWFSLAGLILLPAIGWIEGP---EPMSLATYLFFWGVYTFVM  152
              WKKG+ FG TAF ++GLFW +L  ++LLP  G          P   A YLF WG++T  M
Ma4008  71   EWKKGNTFGATAFTSYGLFWLTLVVIVLLPKFGDNFAGLALTPTDFAAYLFLWGLFTLYM  130

Ma0103  153  LIATLKLGSKAIMFIFLTLFVLFILLAIVNATENAGLLVVAGYVGLLLGLSSLYTALGEV  212
              I TLK  ++A+  +FL+L +LF LLA  N   N  ++ +AGY G+ +G S++Y A+G+V
Ma4008  131  FIGTLK-ATRALQVVFLSLTILFFLLAAGNYMGNPAIIKIAGYEGIFVGFSAIYAAMGQV  189

Ma0103  213  LNDAYGRKVVPI  224
             LN+AYG+K+VP+
Ma4008  190  LNEAYGKKIVPL  201
```

Figure 1 – Two sequence alignment of both *M. acetivorans* ScAdy2 predicted homologs, Ma0103 and Ma4008, (http://www.ncbi.nlm.nih.gov/).

A BLASTP homology search performed on the SGD database (http://www.yeastgenome.org/) indicated that Ma0103 is phylogenetically more related to *S. cerevisiae* Ady2, and that Ma4008 is more closely related to *S. cerevisiae* Ato2 (Fig. 2).

SGD BLASTP Ma0103

Sequences producing significant alignments:	Score (bits)	E value
YCR010C ADY2 SGDID:S000000603, Chr III from 133122-132271, revers...	151	3.2e-12
YNR002C ATO2 SGDID:S000005285, Chr XIV from 633859-633011, revers...	145	1.5e-11
YDR384C ATO3 SGDID:S000002792, Chr IV from 1242024-1241197, rever...	122	5.2e-09
YDL210W UGA4 SGDID:S000002369, Chr IV from 84271-85986, Verified ...	64	0.92

SGD BLASTP Ma4008

Sequences producing significant alignments:	Score (bits)	E value
YNR002C ATO2 SGDID:S000005285, Chr XIV from 633859-633011, revers...	267	6.8e-25
YCR010C ADY2 SGDID:S000000603, Chr III from 133122-132271, revers...	263	1.8e-24
YDR384C ATO3 SGDID:S000002792, Chr IV from 1242024-1241197, rever...	189	1.3e-16
YGR189C CRH1 SGDID:S000003421, Chr VII from 878197-876674, revers...	75	0.17

Figure 2 – BLASTP of *M. acetivorans* Ma0103 and Ma4008 in SGD.

A multiple sequence alignment of *S. cerevisiae* Ady2/Ato2/Ato3 and the *M. acetivorans* predicted Ady2 homologs was performed on MUSCLE (Fig. 3) and a phylogenetic tree drawn using Treeview (Fig. 4).

```
ScAto3          --MTSSASSPQDLEKGVNTLENIETLPQQGSIAGVSQGFPNIQEIYS---DRDFITLGSS
ScAto1          MSDKEQTSGNTDLENAPAGYYSSHDNDVNGVAEDERPSHDSLGKIYTGGDNNEYIYIGRQ
ScAto2          MSDREQSSGNTAFEN-PKALDSSEGEFISENNDQSRHSQESICKIYTAGKNNEYIYIGRQ
Ma0103          ----------------------------MKISIESRISVESKTSIKIPEKIRGVILMVEA
Ma4008          -------------------------------------------------MSEDIKDVMNI---
                                                                  .  : :

ScAto3          TYRRRDLLNALDRGDGEEGNCAKYTPHQFANPVPLGLASFSLSCLVLSLINANVRGVTDG
ScAto1          KFLKSDLYQAF--GGTLNPGLAPAPVHKFANPAPLGLSAFALTTFVLSMFNARAQGITVP
ScAto2          KFLRDDLFEAF--GGTLNPGLAPAPVHKFANPAPLGLSGFALTTFVLSMFNARAQGITIP
Ma0103          QEIVADV----------------HVKDRTANPSPLGFTGLGLSATLLSLSYIGL--YPVD
Ma4008          ----RDV----------------KIKDLSANPAPLGLMGFGMTTVLLNIHNAGF--YPLG
                     *:                      *** ***: .:.::  :*.:        .

ScAto3          KWALSLFMFFGGAIELFAGLLCFVIGDTYAMTVFSSFGGFWICY-GYGLTDTDN-LVSGY
ScAto1          NVVVGCAMFYGGLVQLIAGIWEIALENTFGGTALCSYGGFWLSF-AAIYIPWFG-ILEAY
ScAto2          NVVVGCAMFYGGLVQLIAGIWEIALENTFGGTALCSFGGFWLSF-GAIYIPWFG-ILDAY
Ma0103          SMIVSMAIFLGGFAQVFAGLMAWKKGSVFGGTAFCAFGLFWFSLAGLILLPAIG-WIEGP
Ma4008          AVILSMGIFYGGLGQVIAGIEEWKKGNTFGATAFTSYGLFWLTLVVIVLLPKFGDNFAGL
                   :.  :* **  :::**:      ..:. *.: ::* **:          .  . .

ScAto3          TD-PTMLNNVIGFFLAGWTVFTFLMLMCTLK-STWGLFLLLTFLDLTFLLLCIGTFIDNN
ScAto1          EDNESDLNNALGFYLLGWAIFTFGLTVCTMK-STVMFFLLFFLLALTFLLLSIGHFANRL
ScAto2          KDKESDLGNALGFYLLGWALFTFGLSVCTMK-STIMFFALFFLLAVTFLLLSIANFTGEV
Ma0103          E--PMSLAT----YLFFWGVYTFVMLIATLKLGSKAIMFIFLTLFVLFILLAIVNATENA
Ma4008          ALTPTDFAA----YLFLWGLFTLYMFIGTLK-ATRALQVVFLSLTILFFLLAAGNYMGNP
                      :      :*  * ::*: : : *:* .:  :  ::  * : *:**.

ScAto3          NLKMAGGYFGILSSCCGWYSLYCSVVSPSNSYLAFRAHTMPNAP----
ScAto1          GVTRAGGVLGVVVAFIAWYNAYAGVATKQNSYVLARPFPLPSTERVIF
ScAto2          GVTRAGGVLGVIVAFIAWYNAYAGIATRQNSYIMVHPFALPSNDKVFF
Ma0103          GLLVVAGYVGLLLGLSSLYTALGEVLND-----AYGRKVVPI------
Ma4008          AIIKIAGYEGIFVGFSAIYAAMGQVLNE-----AYGKKIVPL------
                 :   .*  *:. .  . *     : .           :*
```

Figure 3 – The sequences were obtained from SGD and from the Broad Institute (http://www.broadinstitute.org/science/projects/fungal-genome-initiative/fungal-genome-initiative). Multiple alignments were carried out at MUSCLE – Multiple Sequence Comparison by Log-Expectation (http://www.ebi.ac.uk/Tools/muscle/index.html).

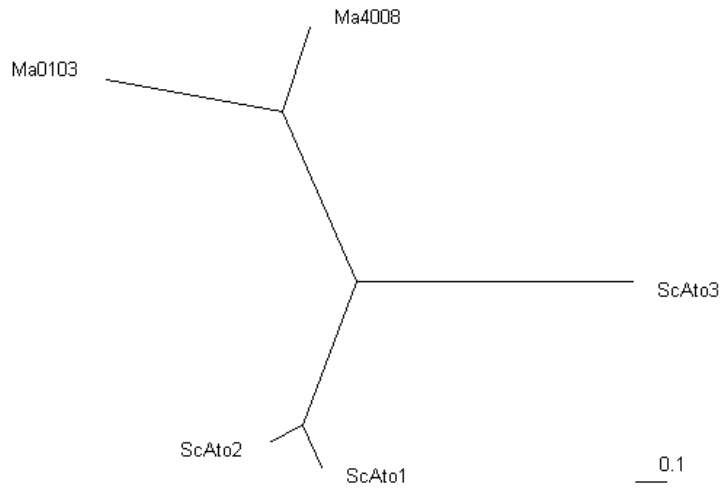

Figure 4 – Phylogenetic tree drawn with ScAdy2 *M. acetivorans* homologs. Tree construction was performed by Phylip suite (Felsenstein, 1989). Evolutionary distances estimations were elaborated by PROTDIST and the clustering was achieved by NEIGHBOUR programs. Finally TREEVIEW was used for the unrooted tree draw.

The heterologous expression of these putative Ady2 homologs was then analysed by SDS-PAGE. The total membrane extracts of *S. cerevisiae jen1ady2* strain, transformed with the expression vector ptYEplac181, under the PMA1 promoter control, ligated either to *Ma0103* or to *Ma4008*, as well as the empty vector, were exposed to different temperatures before loading into the acrylamide gels (12 or 13%) (Fig. 5). These genes should encode proteins of approximately 20-22 KDa, however no significant differences were visible in the protein pattern obtained in all the tested strains, neither after sample incubation at 37ºC for 15 minutes (Fig. 5), nor in all the other conditions tested (data not shown).

Transport of labelled acetic acid in S. cerevisiae jen1ady2 cells *heterologously expressing* Ma0103 *and* Ma4008

Based on the predicted homology between *M. acetivorans* Ma0103 and Ma4008 with *S. cerevisiae* Ady2, the function of both proteins was assessed experimentally. The ability of *S. cerevisiae jen1ady2*, ptYEplac181 Ø (NV6), *S. cerevisiae jen1ady2*, ptYEplac181::Ma0103 (NV7) and *S. cerevisiae jen1ady2*, ptYEplac181::Ma4008 (NV8) strains to transport 1 mM radioactive labelled acetic acid was assessed, after growth in YNB 2% glucose (w/o leu) and derepression in YNB acetic acid 0.5% pH 6.0 (w/o leu) for 3 and 6 hours. The initial uptake rates of labelled acetic acid, shown in figure 6, revealed that no significant differences where obtained in all the tested *S.*

cerevisiae jen1ady2 strains transformed with the putative permease encoding genes (three independent transformants of Ma0103 (NV7); two independent transformants of Ma4008 (NV8)), and the strain transformed with the empty expression vector (NV6).

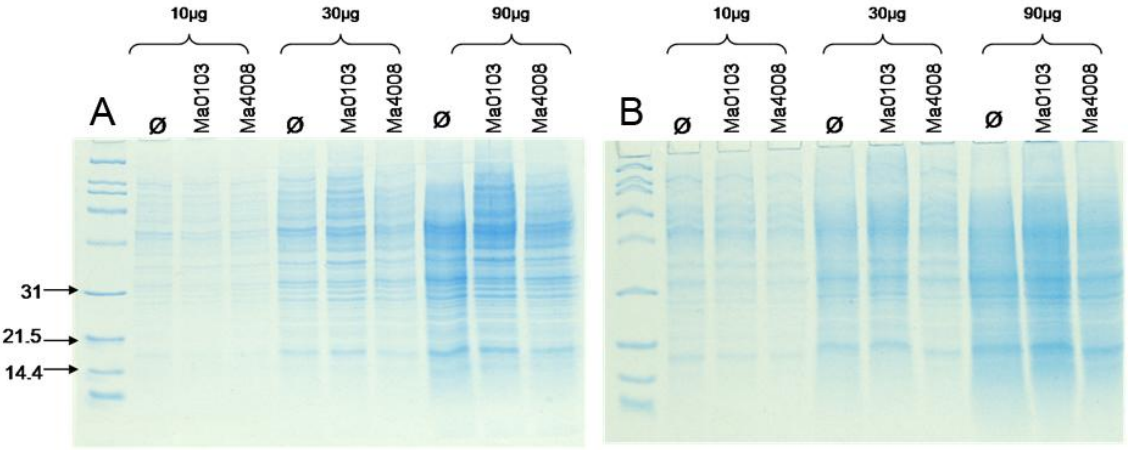

Figure 5 – SDS-PAGE analysis of total membrane extracts prepared from acetic acid derepressed cells, NV6 (*S. cerevisiae jen1ady2*, ptYEplac181 Ø), NV7 (*S. cerevisiae jen1ady2*, ptYEplac181*::Ma0103*) and NV8 (*S. cerevisiae jen1ady2*, ptYEplac181*::Ma4008*). Different protein amounts were loaded in a 12 (A) or 13% (B) acrylamide gels stained using Comassie Blue.

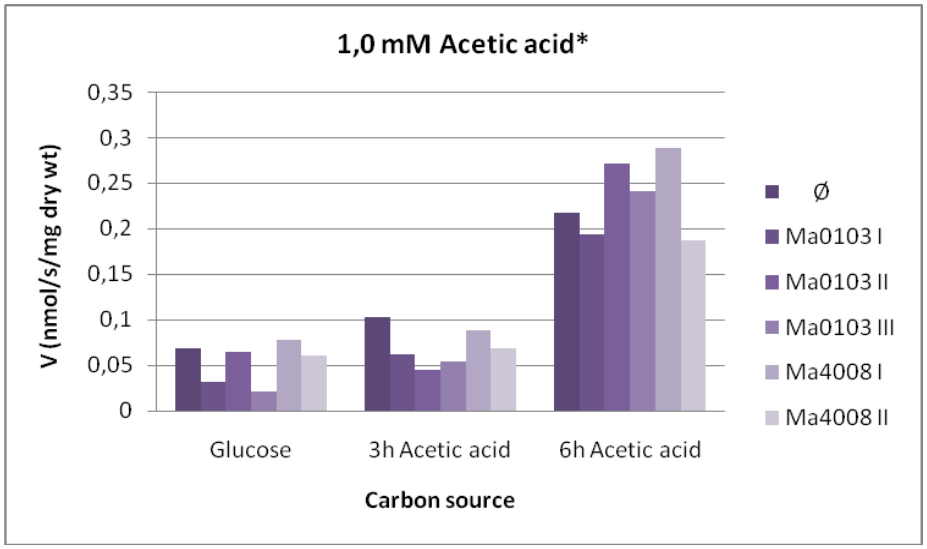

Figure 6 – Time course analyses of the initial uptake rates of 1 mM radioactive labelled acetic acid, pH 5.0. *S. cerevisiae jen1ady2* strains transformed with the empty ptYEplac181 vector or with the ptYEplac181 vector containing the *Ma0103* (3 independent clones) or the *Ma4008* (two independent clones) genes were grown in YNB 2% (w/v) glucose, till mid exponential phase, and derepressed in acetic acid 0.5% (v/v), pH 6.0, for 3 and 6 hours. Samples were collected at the different time points, washed twice with ice-cold deionized water and resuspended in ice-cold deionized water to a final concentration of 25-35 mg dry weight/ml.

The resemblance between the initial uptake rates of labelled 1 mM acetic acid in the *S. cerevisiae* strains bearing the archaeal proteins and the *S. cerevisiae* strain

transformed with the empty plasmid suggested that none of the *M. acetivorans* proteins are involved in the mediated uptake of acetic acid, in the conditions tested. Additionally, the uptake of radioactive labelled lactic acid was followed in the same strains. In figure 7, one can infer that these putative permeases are probably not involved in the mediated uptake of lactic acid and reinforce that Jen1 is the only lactate transporter in *S. cerevisiae*. Nevertheless, it is feasible that *M. acetivorans* proteins experienced different post-translational modifications in *S. cerevisiae*, and/or had an abnormal cellular localization in this baker's yeast, and thus, further assays should be performed to clarify the role of these proteins in *M. acetivorans*.

The *M. acetivorans* putative permeases were cloned the in the yeast expression vector p416GPD, previously used in our group (Vieira *et al.*, 2009), as well as the 6 His tagged versions of *JEN1* and *ADY2* in the yeast expression vector ptYEplac 181, to test an alternative heterologous expression system. However, the results were identical to the ones previously shown (data not shown).

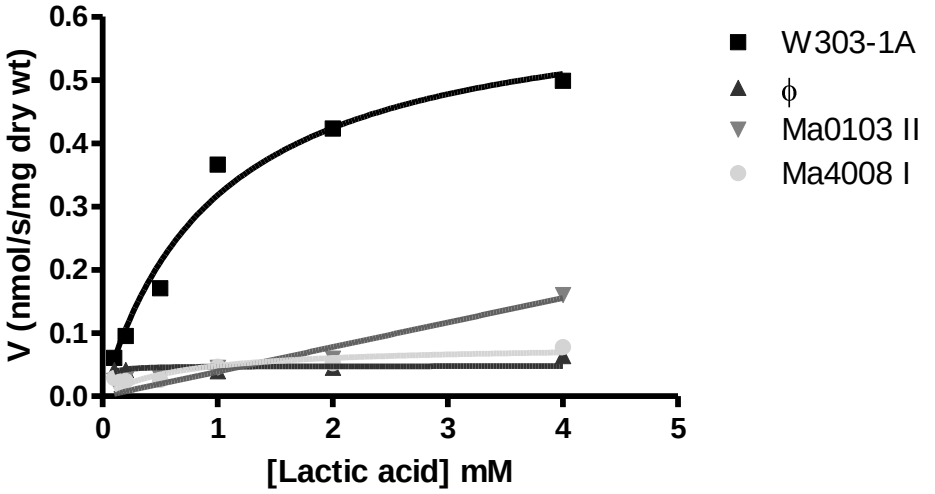

Figure 7 – Initial uptake rates of [U-^{14}C] lactic acid, as a function of lactic acid concentration, after growth of *S. cerevisiae jen1ady2* strains transformed with the ptYEplac181 vector either empty or containing the Ma0103 clone II or the Ma4008 clone I, in YNB 2% glucose, till mid exponential phase, and derepression in YNB lactic acid 0.5% pH 5.0, for 4 hours. As a control, the wild-type *S. cerevisiae* W303-1A is represented. W303-1A (wild-type), ■; Ø (*jen1ady2* ptYEplac181), ▲; NV7 (*jen1ady2* ptYEplac181::Ma0103), ▼; NV8 (*jen1ady2* ptYEplac181::Ma4008), ●.

Western blot analysis of Ma0103 *and Ma4008 heterologously expressed in the yeast* S. cerevisiae

Although no transport activity was found in *S. cerevisiae* strains transformed with the ptYEplac181 vector bearing the archaeal genes, we set up to determine whether the proteins were actually expressed, by Western blot analyses. Based on the previous transport screening assays, total protein extracts were prepared from the clones that evidenced a higher ability to transport 1 mM of radioactive labelled acetic acid (*S. cerevisiae jen1ady2*, ptYEplac181::Ma0103 II and *S. cerevisiae jen1ady2*, ptYEplac181::Ma4008 clone I). The total extracts were then resolved in 12% acrylamide gels and immunobloted against the Ma0103 and Ma4008 His tag. Only the putative permease encoded by *Ma0103* was detected by Western blot analysis indicating that this protein was heterologously expressed in *S. cerevisiae* with the predicted size (Fig. 8).

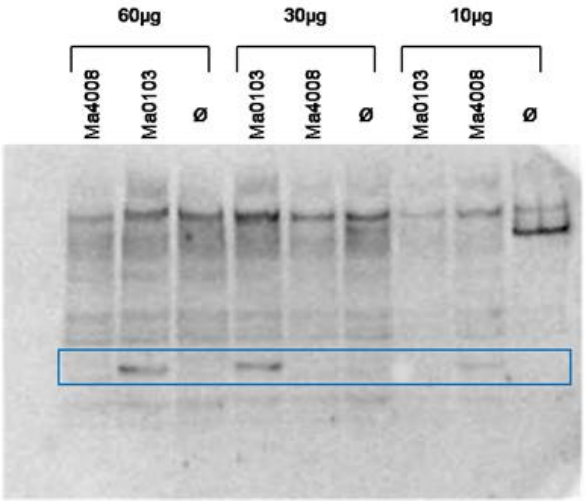

Figure 8 – Heterologous expression analysis of Ma0103 and Ma4008. Mid-exponential *S. cerevisiae jen1ady2* cells transformed with ptYEplac181 Ø (NV6), ptYEplac181*::Ma0103*(NV7) and ptYEplac181*::Ma4008* (NV8) grown in YNB Glucose 2% (w/o leu) were washed twice with ice-cold deionized water and resuspended in fresh YNB acetic acid 0.5%, pH 6.0 (w/o leu) media for 6 hours at 30ºC. Samples were collected after induction to prepare protein extracts that were separated by SDS-PAGE and analysed for Ma0103-HIS and Ma4008-HIS by Western immunoblotting, with a monoclonal HIS antibody.

Discussion

S. cerevisiae monocarboxylate transporter Ady2 belongs to the YaaH family, recently considered in the Transport Classification Database (TC, 2.A.96). This family has homologs in the three domains of life: Bacteria, Archaea and Eukaryota. *S. cerevisiae* Ady2 has been previously described as an acetate transporter (Paiva *et al.*,

2004), however, Palková and co-workers described Ady2/Ato1 as being involved in ammonia export in *S. cerevisiae* colonies (Palkova *et al.*, 2002). In this manner, the functional characterization of Ady2 homologs in other species would definitely contribute to the elucidation of this protein true function and to delineate the phylogeny of these permeases. Therefore, the functional analysis of the archaeal *M. acetivorans* Ady2 homologs would be very important both to elucidate Ady2 function and to understand Archaeal biology.

The methanogen *M. acetivorans* can thrive in a variety of extreme microenvironments and hence, the clarification of the molecular mechanisms underlying protein stability under such diverse conditions would provide new insights into the thermostability of these proteins. *Methanosarcina* species, including *M. acetivorans*, can assimilate a vast range of C-1 compounds and acetate to produce methane, in contrast to the other orders of methanogens (Galagan *et al.*, 2002). In this study we aimed at characterizing the acetotrophic methanogen *M. acetivorans* Ady2 homologs, Ma0103 and Ma4008. Expression of the Ma0103 was accomplished after growth in minimal YNB media supplemented with glucose (without leucine, for plasmid selection) and derepression in YNB acetic acid, pH 6.0 (w/o leu), for 6 h. Nevertheless, transport activity of labelled acetic and lactic acid was not distinct from the negative control. We can not conclude from these results whether Ma0103 is involved or not in the mediated transport of acetate in *M. acetivorans*, since the significant differences between these microorganisms might have impaired the correct expression of these proteins in a heterologous system. Recent findings indicate that although the genetic code of *M. acetivorans* is not different from the standard, the UAG stop codon can be translated into an unusual amino acid, the pyrrolysine (Hao *et al.*, 2002) but also used as a stop codon in a balanced way (Longstaff *et al.*, 2007). In this case, in the yeast *S. cerevisiae* the UAG codon could have been recognised as a stop codon and the protein translation ceased. However, this was not the case, at least for Ma0103, since this protein was detected at the expected size by Western blot analysis. Regarding, Ma4008, a search on the DNA database sequence of the Broad Institute, revealed that no UAG/TAG codons are present in the coding sequence, at least in the predicted reading frame. Furthermore, codon bias between such different organisms can in fact constitute a problem. This issue could be overcome by the *de novo* synthesis of Ma0103 and Ma4008 from an optimized sequence to be expressed in yeast. Additionally,

heterologous expression of both putative permeases, fused with soluble fusion tags, can be attempted in an *E. coli* codon plus strain (*E. coli* BL21-Codon Plus). These *E. coli* cells can supply additional copies of specific tRNA genes, which are rare in these bacteria, such as isoleucine, leucine and arginines, but present in high quantities in many archaea (Kim and Lee, 2006). In the future we hope to overcome these limitations by allowing the correct expression of these proteins in order to elucidate their true function.

References

Boccazzi, P., Zhang, J. K. and Metcalf, W. W. 2000. Generation of dominant selectable markers for resistance to pseudomonic acid by cloning and mutagenesis of the *ileS* gene from the archaeon *Methanosarcina barkeri fusaro*. *J Bacteriol.* **182**: 2611-2618.

Bullock, W. O., Fernandez, J.M. and Short, J.M. 1987. XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with b-galactosidase selection *BioTechniques.* **5**: 376-378.

Casal, M., Cardoso, H. and Leao, C. 1996. Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. *Microbiology.* **142**: 1385-1390.

de Macario, E. C., Guerrini, M., Dugan, C. B. and Macario, A. J. 1996. Integration of foreign DNA in an intergenic region of the archaeon *Methanosarcina mazei* without effect on transcription of adjacent genes. *J Mol Biol.* **262**: 12-20.

Felsenstein, J. 1989. PHYLIP – Phylogeny Inference Package (Version 3.2). *Cladistics* **5**: 5: 164–166.

Galagan, J. E., Nusbaum, C., Roy, A., Endrizzi, M. G., Macdonald, P., FitzHugh, W., Calvo, S., Engels, R., Smirnov, S., Atnoor, D., *et al.* 2002. The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. *Genome Res.* **12**: 532-542.

Hao, B., Gong, W., Ferguson, T. K., James, C. M., Krzycki, J. A. and Chan, M. K. 2002. A new UAG-encoded residue in the structure of a methanogen methyltransferase. *Science.* **296**: 1462-1466.

Kim, S. and Lee, S. B. 2006. Rare codon clusters at 5'-end influence heterologous expression of archaeal gene in *Escherichia coli*. *Protein Expr Purif.* **50**: 49-57.

Longstaff, D. G., Blight, S. K., Zhang, L., Green-Church, K. B. and Krzycki, J. A. 2007. In vivo contextual requirements for UAG translation as pyrrolysine. *Mol Microbiol.* **63**: 229-241.

Macario, A. J. and Conway De Macario, E. 2001. The molecular chaperone system and other anti-stress mechanisms in archaea. *Front Biosci.* **6**: D262-283.

Metcalf, W. W., Zhang, J. K., Apolinario, E., Sowers, K. R. and Wolfe, R. S. 1997. A genetic system for Archaea of the genus *Methanosarcina*: liposome-mediated transformation and construction of shuttle vectors. *Proc Natl Acad Sci U S A.* **94**: 2626-2631.

Metcalf, W. W., Zhang, J. K. and Wolfe, R. S. 1998. An anaerobic, intrachamber incubator for growth of *Methanosarcina* spp. on methanol-containing solid media. *Appl Environ Microbiol.* **64**: 768-770.

Mumberg, D., Muller, R. and Funk, M. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene.* **156**: 119-122.

Paiva, S., Devaux, F., Barbosa, S., Jacq, C. and Casal, M. 2004. Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. *Yeast.* **21**: 201-210.

Paiva, S., Vieira, N., Nondier, I., Haguenauer-Tsapis, R., Casal, M. and Urban-Grimal, D. 2009. Glucose-induced ubiquitylation and endocytosis of the yeast Jen1 transporter: role of lysine 63-linked ubiquitin chains. *J Biol Chem.* **284**: 19228-19236.

Palkova, Z., Devaux, F., Icicova, M., Minarikova, L., Le Crom, S. and Jacq, C. 2002. Ammonia pulses and metabolic oscillations guide yeast colony development. *Mol Biol Cell.* **13**: 3901-3914.

Schlesinger, W. H. 1997. Biogeochemistry: An analysis of global change. Academic Press, San Diego, CA.

Soares-Silva, I., Paiva, S., Diallinas, G. and Casal, M. 2007. The conserved sequence NXX[S/T]HX[S/T]QDXXXT of the lactate/pyruvate:H(+) symporter subfamily defines the function of the substrate translocation pathway. *Mol Membr Biol.* **24**: 464-474.

Soares-Silva, I., Schuller, D., Andrade, R. P., Baltazar, F., Cassio, F. and Casal, M. 2003. Functional expression of the lactate permease Jen1p of *Saccharomyces cerevisiae*. *Biochem J.* **376**: 781-787.

Thomas, B. J. and Rothstein, R. 1989. Elevated recombination rates in transcriptionally active DNA. *Cell.* **56**: 619-630.

Vieira, N., Casal, M., Johansson, B., Maccallum, D. M., Brown, A. J. and Paiva, S. 2009. Functional specialization and differential regulation of short-chain carboxylic acid transporters in the pathogen *Candida albicans*. *Mol Microbiol.* **76:**1337-54.

Woese, C. R., Kandler, O. and Wheelis, M. L. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci U S A.* **87**: 4576-4579.

Zhang, J. K., Pritchett, M. A., Lampe, D. J., Robertson, H. M. and Metcalf, W. W. 2000. In vivo transposon mutagenesis of the methanogenic archaeon *Methanosarcina acetivorans* C2A using a modified version of the insect mariner-family transposable element Himar1. *Proc Natl Acad Sci U S A.* **97**: 9665-9670.

Zinder, S. H. 1993. Physiological ecology of methanogens. In: Methanogenesis (ed. J.G. Ferry), pp. 128–206. Chapman Hall Routledge, New York.

Section II

Carboxylate Permeases mRNA Decay

Introduction

CARBOXYLIC ACID TRANSPORTERS mRNA REGULATION IN S. CEREVISIAE

The global mechanisms of glucose catabolite repression in *S. cerevisiae* are nowadays well elucidated (Gancedo, 1998). In response to glucose, the fate of different transcripts is well regulated within the cell, not only at the transcriptional level but also at the post-transcriptional level. Post-transcriptional regulation is accomplished by means of RNA binding proteins involved in different processes, such as, alternative splicing, alternative polyadenylation, RNA editing, RNA degradation (nuclear and cytoplasmic), nuclear export and storage in P-bodies, among others. In this chapter the pathways of mRNA turnover, as well as the involvement of P-bodies, will be integrated in respect to the carboxylate permeases mRNA expression regulation, pointing to the role of the *DHH1* gene in this regulation in *S. cerevisisae*.

mRNA turnover pathways

The regulation of gene expression is influenced by the control of mRNA turnover. In 1961 Jacob and Monod suggested that the observed small fractions of cellular RNA, with extremely fast synthesis and destruction, constituted the messenger RNAs. These messenger RNAs carry information from genes to ribosomes, and one of their distinguishing features is in fact their rapid turnover. This instability allows the cell to efficiently adapt its protein content to the changing physiological conditions (Jacob and Monod, 1961). The half-lives of mRNAs differ greatly between them and are directly influenced by the immediate physiological needs of the cell. For instance, in yeast, the half-lives of the most stable RNAs are of 90 minutes, or more, whereas unstable RNAs have half-lives of approximately 2 to 3 minutes (Herrick *et al.*, 1990; Wang *et al.*, 2002).

There are two major pathways of mRNA turnover, in eukaryotic cells, and both are initiated by shortening of the poly (A) tail. In yeast, the poly (A) tail needs to be shortened to about 10 nucleotides before mRNAs levels begin to decrease (Decker and Parker, 1993). In the major degradation pathway, this deadenylation primarily leads to the removal of the 5’ cap structure (decapping), thereby exposing the transcript to digestion by a 5’ to 3' exonuclease. The hydrolysis of the 5’ cap is dependent on an

almost complete deadenylation of the poly (A) tail, and therefore this major mRNA turnover pathway is denominated "Deadenylation-dependent decapping pathway" (Hsu and Stevens, 1993; Muhlrad *et al.*, 1994).

The Ccr4 and Pop2 have been identified as components of the major cytoplasmic deadenylase in yeast, being Ccr4 the catalytic subunit of this complex, and Pop2 a putative inducer of the activity of Ccr4, either directly or through additional protein-protein interactions, being required for the interaction of Ccr4 with the Not proteins, which may serve as activators of deadenylation (Tucker *et al.*, 2002).

Decapping of the 5' cap *in vivo* requires two decapping proteins, Dcp1 and Dcp2 (Dunckley and Parker, 1999). The roles of Dcp1 and Dcp2 are not entirely clear. Studies performed in *E. coli* demonstrated that Dcp2 alone presents decapping activity, but the presence of Dcp1 augments that activity. On the other hand, both *dcp1* or *dcp2* mutant strains present a complete block of the decapping activity. (Beelman *et al.*, 1996). It seems like a direct or indirect interaction between both proteins is essential for the decapping activity (Beelman *et al.*, 1996; Dunckley and Parker, 1999). Nowadays, Dcp2 is believed to be the catalytic subunit of the yeast cytoplasmic mRNA-deccaping machinery and Dcp2 its coactivator (Franks and Lykke-Andersen, 2008). Several proteins are thought to enhance the decapping activity of these proteins, although they are not essential for the decapping of stable and non-stable mRNA. For instance, in yeast, Edc1 and Edc2 are two small proteins that bind RNA stimulating the rate of decapping *in vitro* and *in vivo* (Dunckley *et al.*, 2001; Schwartz *et al.*, 2003; Steiger *et al.*, 2003). Edc3 was also shown to specifically affect the function of the decapping enzymes Dcp1 and Dcp2 enhancing mRNA decapping *in vivo* (Kshirsagar and Parker, 2004). Finally, the activity of the Pat1/Lsm1-7 complex and of Dhh1, a DEAD-box RNA helicase, also increases the decapping activity of Dcp1/2 (Bonnerot *et al.*, 2000; Bouveret *et al.*, 2000; Tharun *et al.*, 2000; Coller *et al.*, 2001; Fischer and Weis, 2002). In contrast, proteins involved in translation initiation seem to act as inhibitors of decapping (Steiger *et al.*, 2003).

Digestion by 5' to 3' exonuclease is performed by Xrn1, a divalent cation-dependent processive 5' exonuclease (Stevens, 1980). Mutant *xrn1* strains accumulate decapped mRNAs (Hsu and Stevens, 1993; Muhlrad *et al.*, 1994).

A second minor mRNA decay pathway was identified after mutational silencing of the major pathway. In this second mRNA degradation pathway, RNA is degraded

from the 3' end, after prior deadenylation, by the cytoplasmic exosome (Anderson and Parker, 1998). The 5' cap will then be hydrolyzed by a m^7G-specific pyrophosphatase, the DcpS scavenger decapping enzyme, encoded by *DCS1* in yeast (Wang and Kiledjian, 2001; Liu *et al.*, 2002). The exosome is a complex of polypeptides and 3' exonucleases, localized either in the nucleus or in the cytoplasm (Mitchell *et al.*, 1997). The yeast exosome is formed by 10 subunits, from which 9 form a stable core, and an additional subunit Rrp44, that is more loosely attached. All subunits are shared between the cytoplasmic and nuclear exosome (Allmang *et al.*, 1999; Mitchell and Tollervey, 2000). The purified complex from yeast exhibits only hydrolytic activity, although it contains one proven and several potential phosphorolytic subunits (Meyer *et al.*, 2004). Recombinant Rr41 has phosphorolytic activity, whereas Rr4 and Rr44 present hydrolytic 3' exonuclease activity in the yeast exosome. Sequence similarity indicates that Rr40 is also a hydrolytic enzyme. Most probably, the activity of phosphorolytic subunits is dependent on prior activation, possibly co-factor ligation, and in this manner the hydrolytic activity prevails on purified complexes (Mitchell *et al.*, 1997).

Cytoplasmic exosome interacts with the Ski2-Ski3-Ski8 protein complex, in order to conduct mRNA degradation (Anderson and Parker, 1998; Brown *et al.*, 2000). This complex seems to be recruited to the exosome by the action of the Ski7 protein. Ski2 is a DEAD-box RNA helicase that utilizes ATP hydrolysis to induce RNA-RNA and RNA –protein interactions (Ford *et al.*, 1999).

Individual mRNAs degradation can also be triggered by sequence specific endonucleases or in response to siRNAs and miRNAs (Dodson and Shapiro, 2002; Jinek and Doudna, 2009). At the same time, all eukaryotic cells present mechanisms for the detection and degradation of abnormal mRNAs, using the same nucleases involved in normal mRNA degradation. Unprocessed nuclear pre-mRNAs will either remain in the nucleus or be destroyed in the cytoplasm, probably by the exosome (Moore, 2002). Additionally, mRNAs containing premature stop codons will be recognized and degraded by nonsense mediated decay (NMD). These aberrant transcripts with incorrect stop codons can be degraded by deadenylation-independent decapping, bypassing the deadenylation step, or by accelerated deadenylation and concomitant 3' exonuclease activity (Muhlrad *et al.*, 1994; Takahashi *et al.*, 2003). Nonstop decay (NSD) is another process involved in the degradation of mRNAs without termination codons. These

transcripts will be recognized and degraded by cytoplasmic exosome, through 3' to 5' exonuclease activity (Frischmeyer *et al.*, 2002; van Hoof *et al.*, 2002) (Fig. 1).

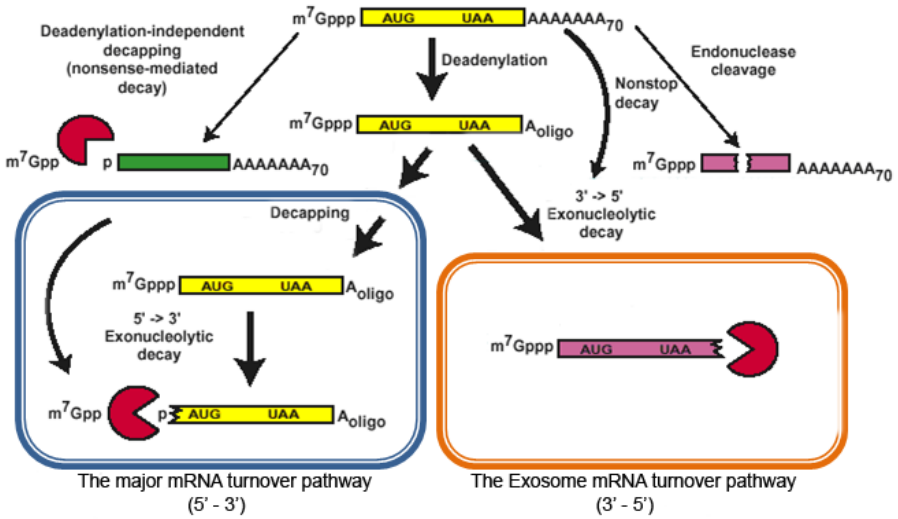

Figure 1 – Schematic representation of the mRNA turnover pathways in eukaryotes, adapted from (Parker and Song, 2004).

P – Bodies, "a place to die a place to sleep"

mRNAs are produced in the nucleus and then transported to the cytoplasm through the nuclear membrane pores, where they will be translated into proteins. In 2003, there were evidences that the untranslated (or inactive) mRNAs could be localized to special cytoplasmic foci within the cell to be stored or destroyed, the processing bodies, or P-bodies (Sheth and Parker, 2003; Wickens and Goldstrohm, 2003). These stored mRNAs could eventually return to translation, moving from the P-bodies to polysomes (Brengues *et al.*, 2005; Balagopal and Parker, 2009).

In *S. cerevisiae*, as mentioned before, the major mRNA decay pathway is dependent on deadenylation and decapping. It was demonstrated by Sheth and Parker that this pathway of mRNA degradation is localized to the P-bodies, where the decapping enzymes Dcp1/Dcp2, the exonuclease 5'-3' Xrn1, the Lsm proteins (small ribonuclear proteins involved in mRNA decay and decapping that connect to mRNA after deadenylation), the Dhh1 (a RNA DeaD-box helicase), and Pat1 (topoisomerase II with pleiotropic roles involved with deadenylation and decapping stimulation), are concentrated (Sheth and Parker, 2003). The decapping pathway is also localized to specific cytoplasmic foci in mammalian cells (Bashkirov *et al.*, 1997; Ingelfinger *et al.*, 2002; van Dijk *et al.*, 2002). A very small number of P-bodies can be seen in *S. cerevisiae* cells, only two to three (Wickens and Goldstrohm, 2003). These processing

bodies have also been identified in several other organisms, such as insect cells, nematodes and mammalian cells, containing also proteins involved in mRNA degradation (Anderson and Kedersha, 2006; Eulalio *et al.*, 2007; Parker and Sheth, 2007).

The movement of mRNAs to the P-bodies should be preceded by deadenylation, since Ccr4, an enzyme that removes the mRNA poly (A) tail is diffused throughout the cytoplasm whereas the decapping enzymes co-localize to P-bodies (Wickens and Goldstrohm, 2003). However, the order of its interaction with the deadenylation and decapping factors, and the trigger for their entry into P-bodies remains unclear.

Teixeira and co-workers tried to dissect P-bodies assembly in *S. cerevisiae* (Teixeira and Parker, 2007). First of all, it seems like mRNA is crucial for their organization and integrity (Teixeira *et al.*, 2005) as well as the highly integrative interactions between the P-bodies components (Hata *et al.*, 1998; Coller *et al.*, 2001; Ho *et al.*, 2002; Fenger-Gron *et al.*, 2005; Gavin *et al.*, 2006; Krogan *et al.*, 2006). Frank and co-workers suggest that P-bodies form by self assembly of mRNPs (a translationally repressed messenger ribonucleoprotein), indicating a direct correlation between the cellular concentration of these mRNPs and P-bodies assembly (Franks and Lykke-Andersen, 2008).

Accumulation of several components of the P-bodies was assessed in deletion mutants lacking one or more of the P-body components. The results obtained indicated that Dcp2 and Pat1 were required for Dcp1 and Lsm1-7 complex recruitment to P-bodies and that none of its components was crucial for its assembly (Teixeira and Parker, 2007). Probably, both Dcp2 and Pat1 aid assembly through the establishment of multiple protein-protein interactions that induce the formation of P-bodies. Additionally, Pat1 seems to enhance mRNA decapping by recruiting Lsm1, and hence the Lsm1-7 complex, important for efficient decapping, to P-bodies (Bouveret *et al.*, 2000; Tharun *et al.*, 2000; Teixeira and Parker, 2007). Moreover, Edc3 and Lsm4, members of two distinct complexes involved in deccaping, were recently identified as promoters of the physical interactions between mRNPs, which are essential for P-bodies assembly. The deletion of specific domains in these proteins led to a complete loss of P-bodies assembly, although mRNA translation and decay were unaffected (Decker *et al.*, 2007; Franks and Lykke-Andersen, 2008; Reijns *et al.*, 2008). Putatively, Pat1 and Dhh1 promote translation repression with simultaneous deccaping enhancement,

enabling assembly of mRNPs into P-bodies through interactions with Edc3 and Lsm4 (Decker *et al.*, 2007). The existence of conserved motifs similar to Lsm4 Q/N-rich region (involved in P-body assembly), in other P-bodies components, indicates that other proteins can direct P-body assembly, like the metazoan Hedls and GW182 (Decker *et al.*, 2007; Franks and Lykke-Andersen, 2008). Hedls interacts with activators of the NMD decay pathway (Fenger-Gron *et al.*, 2005), whereas GW812 is a component of the miRNA pathway that co-localizes to P-bodies in mammalian cells (Eystathioy *et al.*, 2003; Jakymiw *et al.*, 2005; Liu *et al.*, 2005; Rehwinkel *et al.*, 2005). In this manner, different mRNP repression pathways use distinct protein complexes to assemble in P-bodies.

Recently, another group of RNP granules was identified in yeast cells exposed to glucose deprivation. These granules are denominated EGP-bodies and they are functionally analogous to the mammalian stress granules (cytoplasmic ribonucleoprotein granules formed after several stresses that inhibit translation) (Buchan *et al.*, 2008). These EGP-bodies can overlap or be physically separated from P-bodies, aggregating usually, nontranslating mRNAs, translation initiation factors, such as, eIF4E, eIF4G, and Pab1 (a poly (A) binding protein involved in the control of this tail length, that interacts with eIF4G (Amrani *et al.*, 1997; Kessler and Sachs, 1998; Brengues and Parker, 2007; Hoyle *et al.*, 2007; Buchan and Parker, 2009). The content of EGP-bodies can vary according to the stress imposed (Grousl *et al.*, 2009). Sometimes, depending on the experimental conditions, RNA helicases, translation and stability regulators and factors involved in cell signalling can also localize to stress granules (Buchan and Parker, 2009). Most probably, since mRNAs within P-bodies can return to translation (Brengues *et al.*, 2005), mRNPs within these cytoplasmic foci exchange proteins forming additional mRNPs, more competent for translation initiation that accumulate in the EGP-bodies. For instance, during glucose deprivation stress in yeasts, P-bodies are formed initially and, latter on, stress granules appear co-localizing with the pre-existing P-bodies (Hoyle *et al.*, 2007; Buchan *et al.*, 2008). In this manner, EGP-bodies constitute a transition state between mRNA exchange from the P-bodies to polysomes.

In summary, in eukaryotes, movement of mRNAs to P-bodies enables their degradation or storage in specific cytoplasmic foci within the cell, allowing their segregation from translating pools of mRNA, and ensuring that mRNAs destined to

inactivity remain repressed. mRNAs are in this way spatially separated in the cytoplasm between polysomes, EGF-bodies/stress granules and P-bodies (Nover *et al.*, 1983; Anderson and Kedersha, 2002; Brengues *et al.*, 2005; Balagopal and Parker, 2009; Buchan and Parker, 2009).

DHH1, a DEAD-box RNA helicase

DHH1, as previously mentioned, is a RNA helicase included in the subfamily of the DEAD-box helicases (Strahl-Bolsinger and Tanner, 1993). Its function in *S. cerevisiae* is not completely clarified yet. Several homologs of *DHH1* have been identified in *S. pombe*, *STE13* (Maekawa *et al.*, 1994), in *Xenopus laevis*, *XP54* (Ladomery *et al.*, 1997), in *Drosophila melanogaster*, *ME31B* (de Valoir *et al.*, 1991), in mouse, *DDX6* (Akao *et al.*, 1995; Seto *et al.*, 1995), 1995), in humans, *DDX6*/*P54*/*RCK* (encoding for an oncoprotein) (Akao *et al.*, 1995) and in *Caenorhabditis elegans CGH-1* (Navarro *et al.*, 2001).

As mentioned previously, Dhh1 is a cytoplasmatic protein concentrated in specific dynamic "locus", the Processing Bodies, where proteins involved in mRNA decay are localized (Wickens and Goldstrohm, 2003). It interacts selectively with distinct proteins and protein complexes. Researchers have proven its involvement in mRNA transcription, mRNA decay, sporulation, control of cellular morphology, and in the export and translation of mRNA.

The indication of its involvement in mRNA decay and transcription came from two-hybrid analysis and coimmunoprecipitation experiments that revealed its physical interactions with the Ccr4-Pop2-Not deadenylase complex and the decapping enzyme Dcp1 (Hata *et al.*, 1998; Coller *et al.*, 2001).

This Ccr4-Not complex is a regulator of transcription that is involved in the control of the initiation, elongation and degradation of mRNA. Ccr4 is the catalytic subunit involved in the removal of mRNA poly (A) tail. Pop2 most probably enhances Ccr4 catalytic activity by establishing additional protein-protein interactions, namely with the Not proteins (Liu *et al.*, 1998; Bai *et al.*, 1999; Liu *et al.*, 2001; Tucker *et al.*, 2002). There are indications that this complex is necessary to maintain the stable cellular levels of Dhh1 and Pop2, by the N-terminal region of Not1. This contributes to the regulation of mRNA decay, in addition to its role in transcription (C-terminal region

of Not1p is associated with Not2 and Not5, that are involved in the interaction with global transcription factors, *TFIID*) (Maillet and Collart, 2002).

DHH1 also interacts with proteins involved in mRNA decapping, such as the previously mentioned Dcp1 and with the decapping enhancers, Pat1 and Lsm1p. Several observations suggest that Dhh1 might be involved in the mediation of events after deadenylation, promoting in this way, mRNA decay and/or translation repression. Mutants deleted in this gene (*dhh1*), showed a deficient mRNA decay, longer half-live times of several mRNAs and accumulated capped deadenylated transcripts. This suggests that Dhh1 is necessary for an efficient decapping, after deadenylation, interacting with the decapping machinery and increasing its efficacy (Coller *et al.*, 2001).

An additional role was attributed to *DHH1* in mRNA translation. It interacts genetically with Ded1, a DEAD (Asp-Glu-Ala-Asp)-box RNA helicase, dependent on ATP, necessary for the initiation of translation of all mRNAs in yeast (Tseng-Rogenski *et al.*, 2003), and with Pat1 (Coller *et al.*, 2001), a protein that besides its role in deccaping activation can also act as an inhibitor of translation initiation (Wyers *et al.*, 2000; Coller and Parker, 2005). Moreover, overexpression of Caf20, a phosphoprotein of the mRNA cap-binding complex involved in the inhibition of cap-dependent translation is lethal for *dhh1* and *pat1* mutant strains (Coller *et al.*, 2001). This indicates that Dhh1 and Pat1 are affected by decreases in translation, since Caf20 competes with the translation initiation factor, eIF4G for the binding to eIF4E, a cytoplasmic mRNA cap binding protein, involved in cap-dependent mRNA translation (Zanchin and McCarthy, 1995; Altmann *et al.*, 1997; Fortes *et al.*, 2000; Uetz *et al.*, 2000). In this manner, it is possible that Dhh1 plays a role in translation, because overexpression of Caf20 also proved to be lethal in cells disrupted in translation initiation factors (de la Cruz *et al.*, 1997). Finally, another indication of Dhh1 involvement in translation is the physical interaction of Dcp1 with the eIF4F translation initiation complex (Vilela *et al.*, 2000).

Several indications point to a competition between mRNA decapping and translation initiation (Franks and Lykke-Andersen, 2008). Thaurun and co-workers have demonstrated that before decapping can occur, mRNA has to leave translation and assemble into mRNPs that is capable of decapping (Tharun and Parker, 2001). Consequently, a decrease in translation initiation will enhance mRNA decapping rate

and vice versa (Beelman and Parker, 1994; LaGrandeur and Parker, 1999; Muhlrad and Parker, 1999; Schwartz and Parker, 1999; Schwartz and Parker, 2000; Coller and Parker, 2004; Parker and Sheth, 2007). In this manner, it is possible that Dhh1 increases mRNA decay in two manners: by aiding the assembly of decay factors; and by destabilizing the translational initiation complex, after deadenylation, increasing the access of Dcp1p to the cap structure (Coller *et al.*, 2001). Frank and co-workers established a model where, most probably, Dhh1 uses its ATPase activity to release eIF4F complex from the mRNP, or somehow destabilize this eIF4F-mRNA cap complex, and in this manner repress translation with concomitant deccaping stimulation (Franks and Lykke-Andersen, 2008).

In summary, *DHH1* seems to have a pleiotropic role in the cell. Besides its effect in mRNA turnover, it seems like *DHH1* is also involved in several other processes. *dhh1* mutants were unable to grow at 18 ºC and 36 ºC, while a mutant completely blocked in the decapping pathway, *dcp1*, presents a slow growth at these temperatures, but it is viable (Coller *et al.*, 2001). Dhh1 also seems necessary to a proper sporulation in yeasts, like its homolog in *S. pombe*, and it is involved in the control of cellular morphology and cytokinesis. In fact, a homozygotic strain deleted in this gene sporulated very little (Tseng-Rogenski *et al.*, 2003), originated an abnormal cellular morphology, and it was synthetic lethal with a mutation in the *ELM1* (elongation morphology) gene (Moriya and Isono, 1999). It also appears to have an important role in the mRNA export, interacting genetically with Dbp5 (a RNA cytoplasmatic helicase, dependent on ATP, of the DEAD-Box family, involved in mRNA export from the nucleus) (Snay-Hodge *et al.*, 1998; Tseng *et al.*, 1998; Tseng-Rogenski *et al.*, 2003). Additionally, the study of deletion mutants revealed that apoptotic markers were significantly evident in strains lacking genes involved in mRNA decapping, like *DCP1*, *DCP2*, whereas a lower effect was observed in strains lacking the gene *DHH1*. One reason for this is, possibly, the fact that in this last mutant, the increased mRNA stability could be counteracted by a less efficient translation of the accumulated mRNAs, which in turn would result in less severe apoptotic phenotypes. However, this was the first time that a possible connection between mRNA stability and apoptosis was established in yeast, pointing to mRNA decapping as the crucial step responsible for the observed apoptotic phenotypes (Mazzoni *et al.*, 2003).

In this context, one can predict that Dhh1 might act as a multifunctional protein, moving between the cytoplasm and the nucleus, performing several functions in these compartments, integrating mRNA export, translation and turnover pathways**.**

References

Akao, Y., Marukawa, O., Morikawa, H., Nakao, K., Kamei, M., Hachiya, T. and Tsujimoto, Y. 1995. The rck/p54 candidate proto-oncogene product is a 54-kilodalton D-E-A-D box protein differentially expressed in human and mouse tissues. *Cancer Res.* **55**: 3444-3449.

Allmang, C., Petfalski, E., Podtelejnikov, A., Mann, M., Tollervey, D. and Mitchell, P. 1999. The yeast exosome and human PM-Scl are related complexes of 3' --> 5' exonucleases. *Genes Dev.* **13**: 2148-2158.

Altmann, M., Schmitz, N., Berset, C. and Trachsel, H. 1997. A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. *EMBO J.* **16**: 1114-1121.

Amrani, N., Minet, M., Le Gouar, M., Lacroute, F. and Wyers, F. 1997. Yeast Pab1 interacts with Rna15 and participates in the control of the poly(A) tail length in vitro. *Mol Cell Biol.* **17**: 3694-3701.

Anderson, J. S. and Parker, R. P. 1998. The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the *SKI2* DEVH box protein and 3' to 5' exonucleases of the exosome complex. *EMBO J.* **17**: 1497-1506.

Anderson, P. and Kedersha, N. 2002. Stressful initiations. *J Cell Sci.* **115**: 3227-3234.

Anderson, P. and Kedersha, N. 2006. RNA granules. *J Cell Biol.* **172**: 803-808.

Bai, Y., Salvadore, C., Chiang, Y. C., Collart, M. A., Liu, H. Y. and Denis, C. L. 1999. The *CCR4* and *CAF1* proteins of the *CCR4-NOT* complex are physically and functionally separated from *NOT2*, *NOT4*, and *NOT5*. *Mol Cell Biol.* **19**: 6642-6651.

Balagopal, V. and Parker, R. 2009. Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. *Curr Opin Cell Biol.* **21**: 403-408.

Bashkirov, V. I., Scherthan, H., Solinger, J. A., Buerstedde, J. M. and Heyer, W. D. 1997. A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. *J Cell Biol.* **136**: 761-773.

Beelman, C. A. and Parker, R. 1994. Differential effects of translational inhibition in cis and in trans on the decay of the unstable yeast *MFA2* mRNA. *J Biol Chem.* **269**: 9687-9692.

Beelman, C. A., Stevens, A., Caponigro, G., LaGrandeur, T. E., Hatfield, L., Fortner, D. M. and Parker, R. 1996. An essential component of the decapping enzyme required for normal rates of mRNA turnover. *Nature.* **382**: 642-646.

Bonnerot, C., Boeck, R. and Lapeyre, B. 2000. The two proteins Pat1p (Mrt1p) and Spb8p interact in vivo, are required for mRNA decay, and are functionally linked to Pab1p. *Mol Cell Biol.* **20**: 5939-5946.

Bouveret, E., Rigaut, G., Shevchenko, A., Wilm, M. and Seraphin, B. 2000. A Sm-like protein complex that participates in mRNA degradation. *EMBO J.* **19**: 1661-1671.

Brengues, M. and Parker, R. 2007. Accumulation of polyadenylated mRNA, Pab1p, eIF4E, and eIF4G with P-bodies in *Saccharomyces cerevisiae*. *Mol Biol Cell.* **18**: 2592-2602.

Brengues, M., Teixeira, D. and Parker, R. 2005. Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. *Science.* **310**: 486-489.

Brown, J. T., Bai, X. and Johnson, A. W. 2000. The yeast antiviral proteins Ski2p, Ski3p, and Ski8p exist as a complex in vivo. *RNA.* **6**: 449-457.

Buchan, J. R., Muhlrad, D. and Parker, R. 2008. P bodies promote stress granule assembly in *Saccharomyces cerevisiae*. *J Cell Biol.* **183**: 441-455.

Buchan, J. R. and Parker, R. 2009. Eukaryotic stress granules: the ins and outs of translation. *Mol Cell.* **36**: 932-941.

Coller, J. and Parker, R. 2004. Eukaryotic mRNA decapping. *Annu Rev Biochem.* **73**: 861-890.

Coller, J. and Parker, R. 2005. General translational repression by activators of mRNA decapping. *Cell.* **122**: 875-886.

Coller, J. M., Tucker, M., Sheth, U., Valencia-Sanchez, M. A. and Parker, R. 2001. The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. *RNA.* **7**: 1717-1727.

de la Cruz, J., Iost, I., Kressler, D. and Linder, P. 1997. The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A.* **94**: 5201-5206.

de Valoir, T., Tucker, M. A., Belikoff, E. J., Camp, L. A., Bolduc, C. and Beckingham, K. 1991. A second maternally expressed Drosophila gene encodes a putative RNA helicase of the "DEAD box" family. *Proc Natl Acad Sci U S A.* **88**: 2113-2117.

Decker, C. J. and Parker, R. 1993. A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. *Genes Dev.* **7**: 1632-1643.

Decker, C. J., Teixeira, D. and Parker, R. 2007. Edc3p and a glutamine/asparagine-rich domain of Lsm4p function in processing body assembly in *Saccharomyces cerevisiae*. *J Cell Biol.* **179**: 437-449.

Dodson, R. E. and Shapiro, D. J. 2002. Regulation of pathways of mRNA destabilization and stabilization. *Prog Nucleic Acid Res Mol Biol.* **72**: 129-164.

Dunckley, T. and Parker, R. 1999. The *DCP2* protein is required for mRNA decapping in *Saccharomyces cerevisiae* and contains a functional MutT motif. *EMBO J.* **18**: 5411-5422.

Dunckley, T., Tucker, M. and Parker, R. 2001. Two related proteins, Edc1p and Edc2p, stimulate mRNA decapping in *Saccharomyces cerevisiae*. *Genetics.* **157**: 27-37.

Eulalio, A., Behm-Ansmant, I. and Izaurralde, E. 2007. P bodies: at the crossroads of post-transcriptional pathways. *Nat Rev Mol Cell Biol.* **8**: 9-22.

Eystathioy, T., Jakymiw, A., Chan, E. K., Seraphin, B., Cougot, N. and Fritzler, M. J. 2003. The *GW182* protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies. *RNA.* **9**: 1171-1173.

Fenger-Gron, M., Fillman, C., Norrild, B. and Lykke-Andersen, J. 2005. Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping. *Mol Cell.* **20**: 905-915.

Fischer, N. and Weis, K. 2002. The DEAD box protein Dhh1 stimulates the decapping enzyme Dcp1. *EMBO J.* **21**: 2788-2797.

Ford, L. P., Watson, J., Keene, J. D. and Wilusz, J. 1999. ELAV proteins stabilize deadenylated intermediates in a novel in vitro mRNA deadenylation/degradation system. *Genes Dev.* **13**: 188-201.

Fortes, P., Inada, T., Preiss, T., Hentze, M. W., Mattaj, I. W. and Sachs, A. B. 2000. The yeast nuclear cap binding complex can interact with translation factor eIF4G and mediate translation initiation. *Mol Cell.* **6**: 191-196.

Franks, T. M. and Lykke-Andersen, J. 2008. The control of mRNA decapping and P-body formation. *Mol Cell.* **32**: 605-615.

Frischmeyer, P. A., van Hoof, A., O'Donnell, K., Guerrerio, A. L., Parker, R. and Dietz, H. C. 2002. An mRNA surveillance mechanism that eliminates transcripts lacking termination codons. *Science.* **295**: 2258-2261.

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

Gavin, A. C., Aloy, P., Grandi, P., Krause, R., Boesche, M., Marzioch, M., Rau, C., Jensen, L. J., Bastuck, S., Dumpelfeld, B., *et al.* 2006. Proteome survey reveals modularity of the yeast cell machinery. *Nature.* **440**: 631-636.

Grousl, T., Ivanov, P., Frydlova, I., Vasicova, P., Janda, F., Vojtova, J., Malinska, K., Malcova, I., Novakova, L., Janoskova, D., Valasek, L. and Hasek, J. 2009. Robust heat shock induces eIF2alpha-phosphorylation-independent assembly of stress granules containing eIF3 and 40S ribosomal subunits in budding yeast, *Saccharomyces cerevisiae*. *J Cell Sci.* **122**: 2078-2088.

Hata, H., Mitsui, H., Liu, H., Bai, Y., Denis, C. L., Shimizu, Y. and Sakai, A. 1998. Dhh1p, a putative RNA helicase, associates with the general transcription factors Pop2p and Ccr4p from *Saccharomyces cerevisiae*. *Genetics.* **148**: 571-579.

Herrick, D., Parker, R. and Jacobson, A. 1990. Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae*. *Mol Cell Biol.* **10**: 2269-2284.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P., Bennett, K., Boutilier, K., *et al.* 2002. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature.* **415**: 180-183.

Hoyle, N. P., Castelli, L. M., Campbell, S. G., Holmes, L. E. and Ashe, M. P. 2007. Stress-dependent relocalization of translationally primed mRNPs to cytoplasmic granules that are kinetically and spatially distinct from P-bodies. *J Cell Biol.* **179**: 65-74.

Hsu, C. L. and Stevens, A. 1993. Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. *Mol Cell Biol.* **13**: 4826-4835.

Ingelfinger, D., Arndt-Jovin, D. J., Luhrmann, R. and Achsel, T. 2002. The human LSm1-7 proteins colocalize with the mRNA-degrading enzymes Dcp1/2 and Xrnl in distinct cytoplasmic foci. *RNA.* **8**: 1489-1501.

Jacob, F. and Monod, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. *J Mol Biol.* **3**: 318-356.

Jakymiw, A., Lian, S., Eystathioy, T., Li, S., Satoh, M., Hamel, J. C., Fritzler, M. J. and Chan, E. K. 2005. Disruption of GW bodies impairs mammalian RNA interference. *Nat Cell Biol.* **7**: 1267-1274.

Jinek, M. and Doudna, J. A. 2009. A three-dimensional view of the molecular machinery of RNA interference. *Nature.* **457**: 405-412.

Kessler, S. H. and Sachs, A. B. 1998. RNA recognition motif 2 of yeast Pab1p is required for its functional interaction with eukaryotic translation initiation factor 4G. *Mol Cell Biol.* **18**: 51-57.

Krogan, N. J., Cagney, G., Yu, H., Zhong, G., Guo, X., Ignatchenko, A., Li, J., Pu, S., Datta, N., Tikuisis, A. P., *et al.* 2006. Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature.* **440**: 637-643.

Kshirsagar, M. and Parker, R. 2004. Identification of Edc3p as an enhancer of mRNA decapping in *Saccharomyces cerevisiae*. *Genetics.* **166**: 729-739.

Ladomery, M., Wade, E. and Sommerville, J. 1997. *Xp54*, the Xenopus homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. *Nucleic Acids Res.* **25**: 965-973.

LaGrandeur, T. and Parker, R. 1999. The cis acting sequences responsible for the differential decay of the unstable *MFA2* and stable *PGK1* transcripts in yeast include the context of the translational start codon. *RNA.* **5**: 420-433.

Liu, H., Rodgers, N. D., Jiao, X. and Kiledjian, M. 2002. The scavenger mRNA decapping enzyme DcpS is a member of the HIT family of pyrophosphatases. *EMBO J.* **21**: 4699-4708.

Liu, H. Y., Badarinarayana, V., Audino, D. C., Rappsilber, J., Mann, M. and Denis, C. L. 1998. The *NOT* proteins are part of the *CCR4* transcriptional complex and affect gene expression both positively and negatively. *EMBO J.* **17**: 1096-1106.

Liu, H. Y., Chiang, Y. C., Pan, J., Chen, J., Salvadore, C., Audino, D. C., Badarinarayana, V., Palaniswamy, V., Anderson, B. and Denis, C. L. 2001. Characterization of *CAF4* and *CAF16* reveals a functional connection between the *CCR4-NOT* complex and a subset of SRB proteins of the RNA polymerase II holoenzyme. *J Biol Chem.* **276**: 7541-7548.

Liu, J., Rivas, F. V., Wohlschlegel, J., Yates, J. R., 3rd, Parker, R. and Hannon, G. J. 2005. A role for the P-body component *GW182* in microRNA function. *Nat Cell Biol.* **7**: 1261-1266.

Maekawa, H., Nakagawa, T., Uno, Y., Kitamura, K. and Shimoda, C. 1994. The *ste13+* gene encoding a putative RNA helicase is essential for nitrogen starvation-induced G1 arrest and initiation of sexual development in the fission yeast *Schizosaccharomyces pombe*. *Mol Gen Genet.* **244**: 456-464.

Maillet, L. and Collart, M. A. 2002. Interaction between Not1p, a component of the Ccr4-not complex, a global regulator of transcription, and Dhh1p, a putative RNA helicase. *J Biol Chem.* **277**: 2835-2842.

Mazzoni, C., Mancini, P., Verdone, L., Madeo, F., Serafini, A., Herker, E. and Falcone, C. 2003. A truncated form of KlLsm4p and the absence of factors involved in mRNA decapping trigger apoptosis in yeast. *Mol Biol Cell.* **14**: 721-729.

Meyer, S., Temme, C. and Wahle, E. 2004. Messenger RNA turnover in eukaryotes: pathways and enzymes. *Crit Rev Biochem Mol Biol.* **39**: 197-216.

Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M. and Tollervey, D. 1997. The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'-->5' exoribonucleases. *Cell.* **91**: 457-466.

Mitchell, P. and Tollervey, D. 2000. Musing on the structural organization of the exosome complex. *Nat Struct Biol.* **7**: 843-846.

Moore, M. J. 2002. Nuclear RNA turnover. *Cell.* **108**: 431-434.

Moriya, H. and Isono, K. 1999. Analysis of genetic interactions between *DHH1*, *SSD1* and *ELM1* indicates their involvement in cellular morphology determination in *Saccharomyces cerevisiae*. *Yeast.* **15**: 481-496.

Muhlrad, D., Decker, C. J. and Parker, R. 1994. Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5'-->3' digestion of the transcript. *Genes Dev.* **8**: 855-866.

Muhlrad, D. and Parker, R. 1999. Recognition of yeast mRNAs as "nonsense containing" leads to both inhibition of mRNA translation and mRNA degradation: implications for the control of mRNA decapping. *Mol Biol Cell.* **10**: 3971-3978.

Navarro, R. E., Shim, E. Y., Kohara, Y., Singson, A. and Blackwell, T. K. 2001. cgh-1, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in *C. elegans*. *Development.* **128**: 3221-3232.

Nover, L., Scharf, K. D. and Neumann, D. 1983. Formation of cytoplasmic heat shock granules in tomato cell cultures and leaves. *Mol Cell Biol.* **3**: 1648-1655.

Parker, R. and Sheth, U. 2007. P bodies and the control of mRNA translation and degradation. *Mol Cell.* **25**: 635-646.

Rehwinkel, J., Behm-Ansmant, I., Gatfield, D. and Izaurralde, E. 2005. A crucial role for *GW182* and the *DCP1*:*DCP2* decapping complex in miRNA-mediated gene silencing. *RNA.* **11**: 1640-1647.

Reijns, M. A., Alexander, R. D., Spiller, M. P. and Beggs, J. D. 2008. A role for Q/N-rich aggregation-prone regions in P-body localization. *J Cell Sci.* **121**: 2463-2472.

Schwartz, D., Decker, C. J. and Parker, R. 2003. The enhancer of decapping proteins, Edc1p and Edc2p, bind RNA and stimulate the activity of the decapping enzyme. *RNA.* **9**: 239-251.

Schwartz, D. C. and Parker, R. 1999. Mutations in translation initiation factors lead to increased rates of deadenylation and decapping of mRNAs in *Saccharomyces cerevisiae*. *Mol Cell Biol.* **19**: 5247-5256.

Schwartz, D. C. and Parker, R. 2000. mRNA decapping in yeast requires dissociation of the cap binding protein, eukaryotic translation initiation factor 4E. *Mol Cell Biol.* **20**: 7933-7942.

Seto, M., Yamamoto, K., Takahashi, T. and Ueda, R. 1995. Cloning and expression of a murine cDNA homologous to the human *RCK*/*P54*, a lymphoma-linked chromosomal translocation junction gene on 11q23. *Gene.* **166**: 293-296.

Sheth, U. and Parker, R. 2003. Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science.* **300**: 805-808.

Snay-Hodge, C. A., Colot, H. V., Goldstein, A. L. and Cole, C. N. 1998. Dbp5p/Rat8p is a yeast nuclear pore-associated DEAD-box protein essential for RNA export. *EMBO J.* **17**: 2663-2676.

Steiger, M., Carr-Schmid, A., Schwartz, D. C., Kiledjian, M. and Parker, R. 2003. Analysis of recombinant yeast decapping enzyme. *RNA.* **9**: 231-238.

Stevens, A. 1980. Purification and characterization of a *Saccharomyces cerevisiae* exoribonuclease which yields 5'-mononucleotides by a 5' leads to 3' mode of hydrolysis. *J Biol Chem.* **255**: 3080-3085.

Strahl-Bolsinger, S. and Tanner, W. 1993. A yeast gene encoding a putative RNA helicase of the "DEAD"-box family. *Yeast.* **9**: 429-432.

Takahashi, S., Araki, Y., Sakuno, T. and Katada, T. 2003. Interaction between Ski7p and Upf1p is required for nonsense-mediated 3'-to-5' mRNA decay in yeast. *EMBO J.* **22**: 3951-3959.

Teixeira, D. and Parker, R. 2007. Analysis of P-body assembly in *Saccharomyces cerevisiae*. *Mol Biol Cell.* **18**: 2274-2287.

Teixeira, D., Sheth, U., Valencia-Sanchez, M. A., Brengues, M. and Parker, R. 2005. Processing bodies require RNA for assembly and contain nontranslating mRNAs. *RNA.* **11**: 371-382.

Tharun, S., He, W., Mayes, A. E., Lennertz, P., Beggs, J. D. and Parker, R. 2000. Yeast Sm-like proteins function in mRNA decapping and decay. *Nature.* **404**: 515-518.

Tharun, S. and Parker, R. 2001. Targeting an mRNA for decapping: displacement of translation factors and association of the Lsm1p-7p complex on deadenylated yeast mRNAs. *Mol Cell.* **8**: 1075-1083.

Tseng-Rogenski, S. S., Chong, J. L., Thomas, C. B., Enomoto, S., Berman, J. and Chang, T. H. 2003. Functional conservation of Dhh1p, a cytoplasmic DExD/H-box protein present in large complexes. *Nucleic Acids Res.* **31**: 4995-5002.

Tseng, S. S., Weaver, P. L., Liu, Y., Hitomi, M., Tartakoff, A. M. and Chang, T. H. 1998. Dbp5p, a cytosolic RNA helicase, is required for poly(A)+ RNA export. *EMBO J.* **17**: 2651-2662.

Tucker, M., Staples, R. R., Valencia-Sanchez, M. A., Muhlrad, D. and Parker, R. 2002. Ccr4p is the catalytic subunit of a Ccr4p/Pop2p/Notp mRNA deadenylase complex in *Saccharomyces cerevisiae*. *EMBO J.* **21**: 1427-1436.

Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, *et al.* 2000. A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. *Nature.* **403**: 623-627.

van Dijk, E., Cougot, N., Meyer, S., Babajko, S., Wahle, E. and Seraphin, B. 2002. Human Dcp2: a catalytically active mRNA decapping enzyme located in specific cytoplasmic structures. *EMBO J.* **21**: 6915-6924.

van Hoof, A., Frischmeyer, P. A., Dietz, H. C. and Parker, R. 2002. Exosome-mediated recognition and degradation of mRNAs lacking a termination codon. *Science.* **295**: 2262-2264.

Vilela, C., Velasco, C., Ptushkina, M. and McCarthy, J. E. 2000. The eukaryotic mRNA decapping protein Dcp1 interacts physically and functionally with the eIF4F translation initiation complex. *EMBO J.* **19**: 4372-4382.

Wang, Y., Liu, C. L., Storey, J. D., Tibshirani, R. J., Herschlag, D. and Brown, P. O. 2002. Precision and functional specificity in mRNA decay. *Proc Natl Acad Sci U S A.* **99**: 5860-5865.

Wang, Z. and Kiledjian, M. 2001. Functional link between the mammalian exosome and mRNA decapping. *Cell.* **107**: 751-762.

Wickens, M. and Goldstrohm, A. 2003. Molecular biology. A place to die, a place to sleep. *Science.* **300**: 753-755.

Wyers, F., Minet, M., Dufour, M. E., Vo, L. T. and Lacroute, F. 2000. Deletion of the PAT1 gene affects translation initiation and suppresses a *PAB1* gene deletion in yeast. *Mol Cell Biol.* **20**: 3538-3549.

Zanchin, N. I. and McCarthy, J. E. 1995. Characterization of the in vivo phosphorylation sites of the mRNA.cap-binding complex proteins eukaryotic initiation factor-4E and p20 in *Saccharomyces cerevisiae*. *J Biol Chem.* **270**: 26505-26510.

CHAPTER VI – Role of the *DHH1* gene in the regulation of monocarboxylic acids transporters expression in *Saccharomyces cerevisiae*

The work presented in this chapter is in preparation for submission:
Neide Vieira, Fréderic Devaux, Sónia Barbosa, Xavier Darzacq, Sandra Paiva and Margarida Casal (2010) *Role of the* DHH1 *gene in the regulation of monocarboxylic acids transporters expression in* Saccharomyces cerevisiae.

.

Abstract

Previous experiments, carried out by our group, revealed that the gene *DHH1* complemented the phenotype of a *Saccharomyces cerevisiae* mutant (Ace8), affected in the expression of the genes coding for monocarboxylic-acids transporters, *JEN1* and *ADY2* (Paiva *et al.*, 1999). Northern blot analysis showed that, in contrast to what was observed for the wild type strain, there was expression of *JEN1* in a *dhh1* mutant, when formic or propionic acids were used, as sole carbon and energy sources. *DHH1* gene is known to interact with the decapping activator Dcp1 and with the deadenylase complex. This led to the hypothesis that the reason for *JEN1* mRNA presence in the *dhh1* strain is due to a defective decapping machinery, delaying or inhibiting degradation of these mRNAs. Results will be presented regarding the study of those mRNAs decay in the wild type and in the mutant strain, which reinforce our hypothesis. The protein expression and localization of Jen1p in the *dhh1* mutant was studied using a GFP tag. In cells of the mutant strain, derepressed in formic acid, no signal of fluorescence for Jen1::GFP was observed. In these conditions, no measurable activity for the acetate and lactate carrier was found. These results reinforce the role of *DHH1* as a regulator of the expression of *JEN1* in *S. cerevisiae*. Co-localization of *JEN1* mRNA in P-bodies was also assessed by Fluorescent in situ hybridization (FISH) experiments.

Introduction

The uptake of carboxylic acids, across the plasma membrane, plays a crucial role in the metabolism of yeast cells and in the pH-stasis (Casal *et al.*, 2008). *Saccharomyces cerevisiae* is able to use non-fermentable carbon sources, such as short-chain carboxylic acids, as the only carbon and energy source, aerobically. Previous physiological studies, carried out in this baker's yeast, identified two distinct monocarboxylate proton symporters, strongly repressed by glucose, with different specificities and regulation. When *S. cerevisiae* cells are grown in lactic or pyruvic acids, a permease involved in the uptake of lactate-pyruvate-acetate-proprionate was identified (Cassio *et al.*, 1987; Casal *et al.*, 1995), encoded by *JEN1*(Casal *et al.*, 1999). Cells grown in non-fermentable carbon sources, such as acetic or ethanol, present another permease shared by acetate-proprionate and formate (Casal *et al.*, 1996; Makuc *et al.*, 2001).

In a previous work, and in an early attempt to identify the acetate transporter,

classical genetic studies were carried out. The strain *S. cerevisiae* W303-1A was subjected to UV mutagenesis, in order to obtain mutants unable to utilise acetic acid, but not affected on the capacity to grow in ethanol, as the sole carbon and energy source (Paiva *et al.*, 1999). According to this strategy, it was hypothesised that mutants specifically affected in monocarboxylate permease(s) activity could be found. A mutant clone, exhibiting growth on ethanol, but unable to grow in a medium with acetic acid, as the sole carbon and energy source, was isolated (Ace8 strain) (Paiva *et al.*, 1999). This mutant was strongly affected in the activity for the acetate carrier, but it revealed not to be affected on the transport system of lactate (Jen1). The transformation of the Ace8 mutant to leucine prototrophy, with a genomic library from *S. cerevisiae*, allowed the selection of transformants with the recovered ability to grow on acetic acid, as the sole carbon and energy source. The sequencing of the plasmids that conferred the transformants the ability to recover growth on acetic acid, identified four distinct ORFs. Two of them were questionable ORFs, one encoded *STE7*, a ser/thr/tyr protein kinase of MAP kinase family and the other encoded *DHH1*. The phenotypic analysis of the strains deleted in these ORFs proved that only the deletion of *DHH1* presented the same phenotype as the mutant Ace8, in what respects the growth on acetic acid, as the sole carbon and energy source. However, due to its structural features, *DHH1* could not be assigned as a putative candidate for an acetate transporter in *S. cerevisiae* (Paiva, 2002 PhD thesis). Transport assays performed on acetic acid derepressed wild-type and *dhh1* mutant cells, confirmed that Dhh1 was not involved, at least directly, in the transport of this short-chain carboxylic acid across the plasma membrane, since its disruption had no effect on the initial uptake rates of radioactive labelled acetic acid, in comparison to the wild-type strain (Paiva, 2002 PhD thesis). Latter, *ADY2* was identified as the acetate permease encoding gene in *S. cerevisiae* (Paiva *et al.*, 2004).

The role of Dhh1 on carboxylic acids metabolism remained, in this manner, intriguing and unclear. *DHH1* is a RNA helicase included in the subfamily of the DEAD-box helicases (Strahl-Bolsinger and Tanner, 1993), with a pleiotropic role within the yeast cell. Several homologs have been described in a broad range of organisms, namely in: *S. pombe*, *STE13* (Maekawa *et al.*, 1994), in *Xenopus laevis*, *XP54* (Ladomery *et al.*, 1997), in *Drosophila melanogaster*, *ME31B* (de Valoir *et al.*, 1991), in mouse, *DDX6* (Akao *et al.*, 1995; Seto *et al.*, 1995), 1995), in humans, *DDX6*/*P54*/*RCK* (encoding for an oncoprotein) (Akao *et al.*, 1995) and in

Caenorhabditis elegans CGH-1 (Navarro *et al.*, 2001). *DHH1* encodes a cytoplasmatic protein concentrated in specific dynamic "locus", the Processing Bodies. P-bodies have been observed in yeasts, insect cells, nematodes and mammalian cells containing proteins mainly involved in mRNA decay, including the deccaping enzymes (Dcp1/Dcp2), as well as general activators of deccaping, like Dhh1, Pat1, Lsm1-7 complex, Edc3 and the 5'-3' exonuclease Xrb1(Wickens and Goldstrohm, 2003; Anderson and Kedersha, 2006; Eulalio *et al.*, 2007; Parker and Sheth, 2007). Additional roles have been attributed to P-bodies besides their role in mRNA decay, namely on mRNA storage and translation repression (Brengues *et al.*, 2005; Coller and Parker, 2005; Balagopal and Parker, 2009), miRNA-mediated repression (Liu *et al.*, 2005; Pillai *et al.*, 2005), nonsense-mediated decay (Unterholzner and Izaurralde, 2004; Sheth and Parker, 2006) and viral packing (Beliakova-Bethell *et al.*, 2006). In this way, mRNAs targeted for degradation, in a constitutive manner or under stress conditions, will be mainly localized in P-bodies where they can also be stored for further translation. Dhh1 plays a fundamental role in mRNA decay and transcription, physically interacting with the Ccr4-Pop2-Not deadenylase complex, decapping enzyme activator Dcp1 and the deccaping enhancers Pat1 and Lsm1 (Hata *et al.*, 1998; Coller *et al.*, 2001; Franks and Lykke-Andersen, 2008). Mutants deleted in this gene, *dhh1*, showed a deficient mRNA decay, longer half-live times of several mRNAs and accumulated capped deadenylated transcripts, indicating that Dhh1 is crucial for an efficient decapping, after deadenylation, interacting with the decapping machinery and increasing its efficacy (Coller *et al.*, 2001). Moreover, additional roles have been attributed to *DHH1*, namely in mRNA translation (Wyers *et al.*, 2000; Tseng-Rogenski *et al.*, 2003; Coller and Parker, 2005). Based on the reported competition between mRNA deccaping and translation initiation (Brengues *et al.*, 2005; Franks and Lykke-Andersen, 2008), probably, Dhh1 uses its ATPase activity to release eIF4F complex from the mRNP, or somehow destabilize this eIF4F-mRNA cap complex, and in this manner repress translation with concomitant deccaping stimulation (Franks and Lykke-Andersen, 2008). Furthermore, *DHH1* is also involved in mRNA export, interacting genetically with Dbp5 (a RNA cytoplasmatic helicase, dependent on ATP, of the DEAD-Box family, involved in mRNA export from the nucleus) (Snay-Hodge *et al.*, 1998; Tseng *et al.*, 1998; Tseng-Rogenski *et al.*, 2003). In this context, we reasoned that Dhh1 might act as a multifunctional protein, moving between the cytoplasm and the nucleus,

integrating mRNA export, translation and turnover pathways, and hence, capable of affecting *JEN1* and *ADY2* expression, according to growth conditions.

In order to elucidate the involvement of this Dead-box RNA helicase with monocarboxylate permeases and unravel its role in the regulation of non-fermentable carbon sources utilization in *S. cerevisiae,* Northern blot analysis were performed in the wild-type and the *dhh1* mutant strains, after derepression in different non-fermentable carbon sources. Co-localization of *JEN1* mRNA in P-bodies was assessed by Fluorescent in situ hybridization (FISH) experiments, which enabled the detection of single mRNA molecules. Furthermore, analyses of the wild-type and mutant cells transcriptome evidenced the involvement of this RNA helicase in alternative carbon metabolism.

Materials and Methods

Yeast strains, plasmids and growth conditions

S. cerevisiae strains used in this work are listed in table 1 and the plasmids in table 2. The cultures were maintained on plates of yeast extract (1%, w/v), peptone (1%, w/v), glucose (2%, w/v) and agar (2%, w/v). Yeast cells were grown in YNB glucose 2.0 % (w/v), supplemented with adequate requirements for prototrophic growth. Carbon sources were glucose (2%, w/v), lactic acid (0.5%, v/v, pH 5.0), acetic acid (0.5%,v/v, pH 6.0), formic acid (0.5%, w/v, pH 5.0) and propionic acid (0.5%, v/v, pH 5.0). Solid media were prepared adding agar (2%, w/v) to the respective liquid media. Growth was carried out at 30ºC, both in solid or liquid media. The cells were also directly grown in rich media, YP lactic acid 0.5% pH 5.0, or YP acetic acid 0.5% pH 6.0. YNB glucose-containing media was used for growth of yeast cells under repression conditions. For derepression conditions glucose-grown cells were harvested during the exponential phase of growth, centrifuged, washed twice in ice-cold deionised water and cultivated into fresh YNB medium supplemented with a carbon source of choice.

Table 1 – *S. cerevisiae* strains used in this work.

Strains	Genotype	Reference
W303-1A	MATa ade2 leu2 his3 trp1 ura3	(Thomas and Rothstein, 1989)
ACE 147	W303-1A; *dhh1*Δ::KAN^r	Paiva S., Unpublished
BLC 491-U2	MATa ura3-52 *JEN1::GFP::KAN*^{r}	(Paiva *et al.*, 2002)
NV1	ACE 147; *dhh1*Δ::Hph^r	This work
NV2	NV1; *JEN1::GFP::KAN*^{r}	This work

Table 2 – Plasmids used in this study.

Plasmids	Source or references
pT12	(Casal *et al.*, 1999)
pPDA1	Andrade, R. (This work)
pAG32	(Goldstein and McCusker, 1999)

Strains construction

The mutant strain, *dhh1* (ACE 147), carrying a *dhh1::kanMX4* locus, was transformed with the hygromycin resistance gene *HphMX4* resulting in a marker switch producing the *dhh1::HphMx4 locus* (strain NV1) (Goldstein and McCusker, 1999). The *S. cerevisiae* strain BLC 491-U2 (Table 1), was used to amplify the genetic chimaera, *JEN1::GFP::KANr*, using the primers W303-1A forward and W303.1A reverse (Table 3). The *dhh1::HphMx4* strain was subsequently transformed with the 2.8 Kb *JEN1::GFP::KANr* PCR product resulting in strain NV2. Transformed cells were grown in YPD media, for 4 h, and spread on YPD plates, containing 200 mg/L of Geneticin (G418 from Invitrogen) and 300 mg/L of Hygromycin (Hygromycin B from Invitrogen). The obtained transformants were confirmed by analytical PCR, with primers A1 and GFP rev (Table 3) (Kruckeberg *et al.*, 1999). Cloning and PCR amplification analyses were performed as described previously (Sambrook, 1989).

Table 3 – Primers used in this work

Primers	Sequence
W303-1A forward	GATTTGTCCTCTCCTGTTATGAAG
W303-1A reverse	ATCTTGCTAGTGTTAACGGCTGTTA
A1	GGCCTATCCAAGGATGCTGTC
GFP_rev	AACATCACCATCTAATTCAAC

Transport assay

Cells were incubated under derepression conditions, in the presence of acetic, lactic and formic acids. Cells were harvested during the exponential phase (OD_{640nm} = 0.5), by centrifugation, washed twice and resuspended in ice-cold deionized water to a final concentration of 25-35 mg dry weight/ml. Initial uptake rates of labelled D,L-[U-^{14}C] lactic acid and [U-^{14}C] acetic acid (sodium salts, Amersham Biosciences) were measured, as described previously. A computer assisted non-linear regression analysis program (GraphPad software, San Diego, CA, USA) was used, in order to determine the best-fitting transport kinetics to the experimental data and to estimate the kinetic parameters. All the experiments were performed in triplicate, and the data presented represent average values.

Microscopy

S. cerevisiae living cells were examined with a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

RNA analysis

Total RNA was isolated using the standard hot acidic phenol protocol. In a 1.5% (w/v) agarose/Mops/formaldehyde gel, samples of 20 μg RNA were electrophorised and blotted onto a Hybond- N+ membrane (Ausubel and JA, 1998). An internal fragment of approximately 1000 bp was amplified, from the pT12 (Table 2), ^{32}P-labelled and used as a *JEN1* probe. As internal control RNA of *PDA1* was also used. For mRNA half-life times (t ½ mRNA) determination, inhibition of transcription was accomplished by the addition of 1,10-phenantroline (0.1 mg.ml^{-1}) {R. Parker, 1991 #107. Transcription half-live times were determined by applying, a non-linear regression equation, to the values fitting the initial slope of mRNA decay semi-log plot, and calculating the value correspondent to the time point where 50% of the initial mRNA levels were present. The reported half-live times represent a mean value obtained from different experiments.

Microarray analysis

Detailed protocols are described at http://www.transcriptome.ens.fr/sgdb/protocols/. The *S. cerevisiae* microarrays used are fully described in Array express (www.ebi.ac.uk/microarray-as/aer/entry; accession number A-MEXP-337). The microarray experiments were conducted as previously described {Fardeau, 2007 #711}. Raw data were normalized using global lowess followed by print-tip median methods, with background removal, as implemented in Goulphar (Lemoine *et al.*, 2006). Experiments were carried out 2 times, with dye swapping. The clustering data of figure 6 were represented using Treeview (Eisen and Brown, 1999).

Fluorescent in situ hybridization

Wild-type cells were grown till mid-exponential phase on medium containing glucose, and derepressed, for 4h, in lactic or formic acids, as sole carbon and energy sources, and

treated, as described in a previous study (Garcia *et al.*, 2007a). The combined hybridization of five antisense oligonucleotides was used to enhance the visualization of *JEN1* mRNA. Typically, each oligonucleotide was designed such that it contained 50 to 55 nucleotides, with five aminoallyl thymidines and a coherent Tm value. After synthesis by Eurogentec, the oligonucleotides were directly labelled with CY3 or CY5 fluorochromes and hybridized to fixed cells, as previously described (Garcia *et al.*, 2007b).

Results

Expression profile of JEN1

W303-1A and *dhh1* cells were grown in different carbon and energy sources, in an effort to clarify the mechanisms involved in the expression regulation of *JEN1* by *DHH1*. The expression pattern was studied by Northern blot analysis. An internal fragment of 844 bp, obtained by the digestion of the pT12 plasmid (Table 2), with the restriction enzymes *Nco*I and *Pst*I was utilized as the *JEN1* probe. Different expression profile of *JEN1* could be identified in the mutant *dhh1* in comparison to the wild-type strain (Fig. 1). In the *DHH1* deletion strain it was possible to observe expression of *JEN1* in the presence of formic or propionic acids, as sole carbon and energy sources, in contrast to what was found in the wild-type strain. In the other tested carbon sources, the expression profile of *JEN1* was similar between the wild-type and the *dhh1* mutant strain. These results show that *DHH1* regulates the expression of *JEN1* at the mRNA level, pointing to an involvement of this RNA helicase in the regulation of monocarboxylic acids utilization, in *S. cerevisiae*.

Decay of JEN1 *mRNA in the* S. cerevisiae dhh1 *strain*

The decay of Jen1 permease was analysed in the *S. cerevisiae* wild-type strain and in a *dhh1* genetic background. A pulse of 1,10-phenanthroline (0.1 mg/ml) was added to YP lactic acid-grown cells, both with and without 2% (w/v) glucose addition. Glucose-triggered *JEN1* mRNA decay was significantly slower in the *dhh1* strain, both in the presence of glucose and 1,10-phenantroline or just of 1,10-phenantroline (Fig. 2). These results suggest that in fact *DHH1* gene is involved in the turnover of *JEN1* mRNA in *S. cerevisiae*. To clarify these observations *JEN1* mRNA was quantified, using as an internal control the quantity of the constitutive gene, *PDA1* mRNA, and the

half-live times (t ½ mRNA) were calculated (see material and methods). In fact the half-live times in the *dhh1* mutant strain are significantly longer when compared to the ones found for the wild-type strain (Table 4). Most probably, the disruption of *DHH1* impairs the access of Dcp1/Dcp2 to the 5´cap structure of *JEN1* mRNA, leading to an accumulation of these mRNAs.

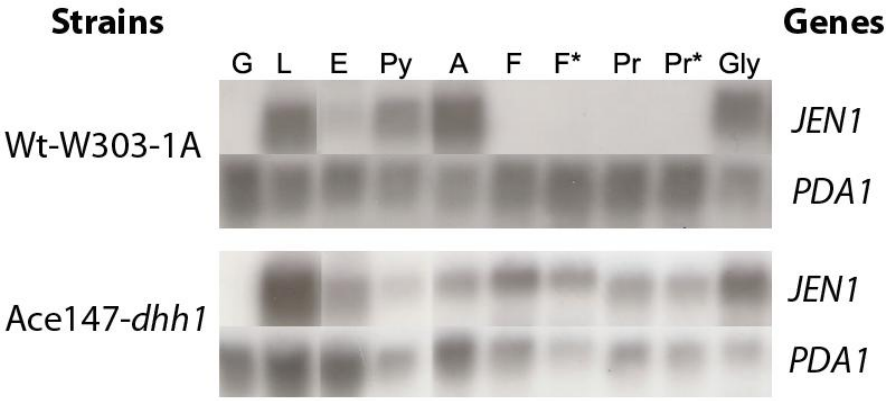

Figure 1 – Transcription analyses of *JEN1* in *S. cerevisiae* W303-1A and *dhh1* cells. Total RNA was isolated from YNB Glucose 2%-grown cells, collected at mid exponential phase, and after induction for 4 or 6 hours in different non-fermentable carbon sources: G – glucose; L – lactic acid (4 hours); E – ethanol (4 hours); Py – pyruvic acid (4 hours); 5 – Acetic acid (4 hours); F – formic acid (4 hours); F* – formic acid (6 hours); Pr – propionic acid (4 hours); Pr* – propionic acid (6 hours); Gly – glycerol (4 hours). An internal *JEN1* fragment was used as probe. *PDA1* was used as a reference, for relatively constant transcription.

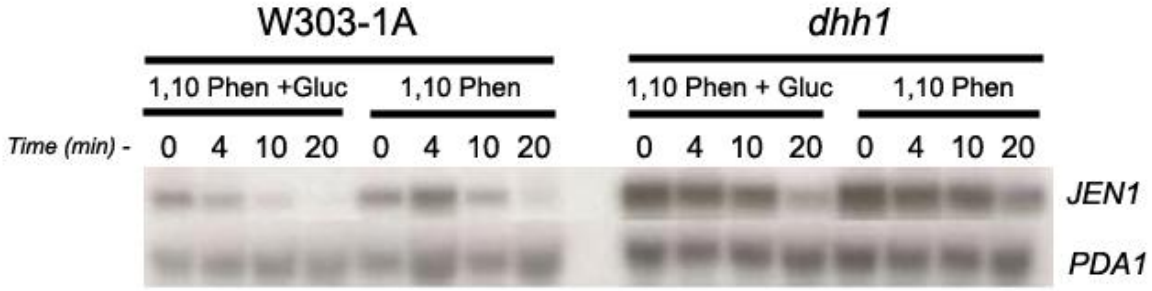

Figure 2 – Effect of glucose and 1,10-phenantroline in *S. cerevisiae JEN1* mRNA expression profile, during different periods of time. *JEN1* mRNA expression was followed by Northern blot analysis. An internal fragment of *JEN1* with 844 bp was used as a probe, and as an internal control for RNA quantity, an internal fragment of the constitutive gene *PDA1*, with 800 bp, was selected. Time zero corresponds to YP lactic acid-grown W303-1A and *dhh1* cells, collected immediately before the addition of a pulse of 1,10-phenantroline (0.1 mg/ml) and glucose (2.0 % w/v), or collected before a pulse of only 1,10-phenantroline. The mRNA levels were monitored by Northern blot, at different times, after the pulse.

Table 4 – Half-live times of the *JEN1* mRNA in the wild-type and in the mutant *dhh1* strain, either in the presence of glucose + 1,10-phenantroline, or in the presence of only 1,10-phenantroline.

	JEN1 mRNA ½ life (min)	
***S. cerevisiae* strains**	1,10-Phenantroline	1,10-Phenantroline + Glucose
W303-1A	8.8	5
dhh1	27	17

Activity of monocarboxylic acids transporters in the S. cerevisiae dhh1 *strain*

Regarding the mRNA expression of *JEN1* in formic acid derepressed cells, the uptake of radiolabelled lactate and acetate was followed, in order to determine whether *JEN1* mRNA was being translated to a functional protein. Initial uptake rates of labelled lactic and acetic acid (pH 5.0, acid concentrations between 0.03 and 2.00 mM and 0.04 and 2.00 mM, respectively) were measured in the *dhh1* and wild-type cells, derepressed in YNB formic acid 0.5%, pH 5.0, for 4 hours. A computer-assisted non linear regression analysis to the experimental data, pointed to a diffusion of lactate and acetate across the plasma membrane, for both wild-type and mutant cells (Fig. 3). In this manner, as expected, no Jen1 or Ady2 transporter activity was observed in the wild-type cells, after derepression in formic acid (Fig. 3), a condition where no mRNA expression was found for any of the genes (Fig. 1). Moreover, after derepression in formic acid the *JEN1* mRNA detected in the *dhh1* strain was not translated, at least into a functional protein.

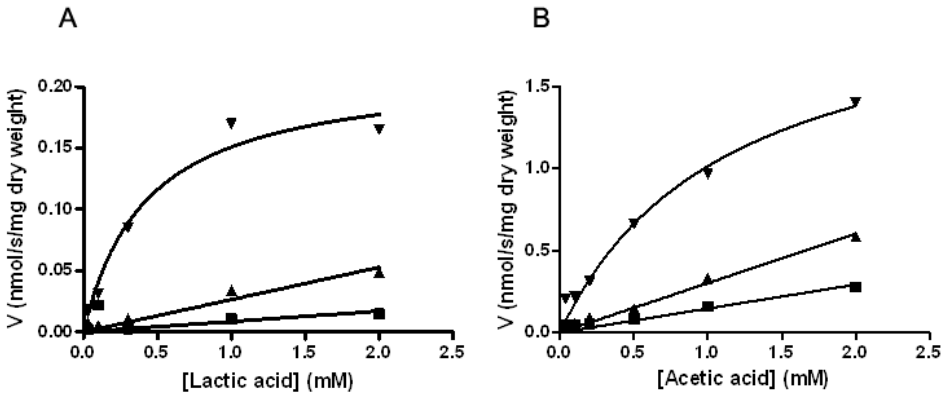

Figure 3 – Initial uptake rates of labelled monocarboxylic acids in *S. cerevisiae* W303-1A (■) and *dhh1* (▲) YNB Formic acid-derepressed cells, as a function of the acid concentration. A: Labelled lactic acid; B: Labelled acetic acid. (▼) Symbol represents the initial uptake rates of labelled lactic acid (A) and of labelled acetic acid (B), in lactic or acetic acid-induced cells, respectively.

Expression of Jen1::GFP fusion in the S. cerevisiae *mutant strain,* dhh1

Cells of the BLC 491-U2 (control) and the *dhh1* (NV2, Table 1) strains, harboring the Jen1::GFP chimaera, were derepressed in YNB lactic acid for 4 hours. Cells were harvested and equal volumes of cell suspension were resuspended in low-melt agarose (1.0%, w/v), and observed by epifluorescence microscopy. In both cases the fluorescence was, clearly, localized to the plasma membrane, as previously described for the wild-type strain (Paiva *et al.*, 2002) (Fig. 4). The *dhh1* mutant had a similar fluorescence level in comparison to the wild type strain, hence, not interfering with the protein expression of *JEN1,* in this induction condition. In turn, after derepression in formic acid, as sole carbon and energy source for 4 and 6 hours, there was no fluorescence of Jen1::GFP in the wild type (as expected) and in the *dhh1* strain, implying that the previously detected *JEN1* mRNA wasn't translated at all, in the mutant strain. These findings reinforce the hypothesis that the observed mRNA expression profile results from the degradation machinery deficiency, pointing to a possible entrapment of *JEN1* mRNAs in P-bodies, after derepression in formic acid.

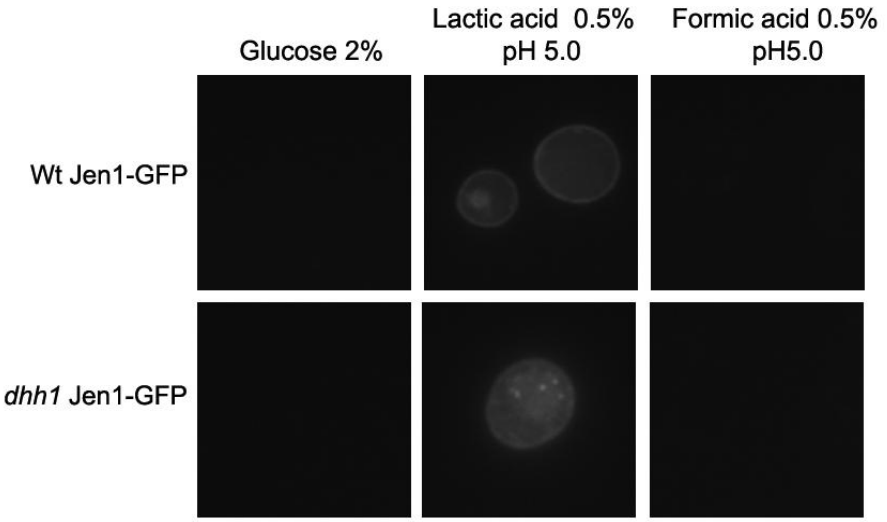

Figure 4 – Subcellular localization of *JEN1::GFP* in *S. cerevisiae* living cells. Wild-type, BLC 491-U2, and NV2 (*dhh1* mutant) cells harboring Jen1::GFP were used to follow Jen1 expression after growth in YNB glucose, and derepression for 4 hours in YNB lactic 0.5% pH 5.0 or YNB formic acid 0.5% pH 5.0

FISH analyses of the sub-cellular localisation of JEN1 mRNA

Based on the results above, we explored the possibility, that *JEN1* mRNA would be trapped in the P-bodies in formic acid, in the absence of *DHH1*. Roy Parker's laboratory sent us yeast strains in which P-bodies are labelled by GFP fused to either Dcp1 or Dcp2. Those cells were grown in the presence of glucose, and derepressed in

lactic or formic acids, as sole carbon and energy sources, as mentioned above. FISH experiments were conducted, in order to detect individual *JEN1* mRNA molecules and possibly study the co-localisation of these mRNA and GFP labelled P bodies. To test our ability to detect *JEN1* mRNA in the *dhh1* mutant strain, we first compared glucose and lactic acid-grown cells. As expected, no significant signal was detected from glucose cultures (Fig. 5A), while many intense spots were present in lactic acid-grown cells (Fig. 5B). Unfortunately, several problems occurred which make our experiments non-conclusive, at the moment. First, few spots of mRNA were detected in formic acid in comparison with lactic acid (Fig. 5C), as expected from our Northern blot analyses (Fig. 1). However, the same amounts of spots were present in the wild type and the *dhh1* mutant (data not shown). We realized that, in our last experiments, numerous artefactual signals were detected, even on cell-free field on the slides (Fig. 5C). This may explain that a reliable number of spots cannot be defined in formate, a growth condition in which *JEN1* expression is very low. We are still trying to solve this problem. Secondly, the GFP signal was completely bleached during the FISH procedure. We will then have to combine FISH with immunostaining, using fluorescent anti-GFP antibodies, to solve this problem

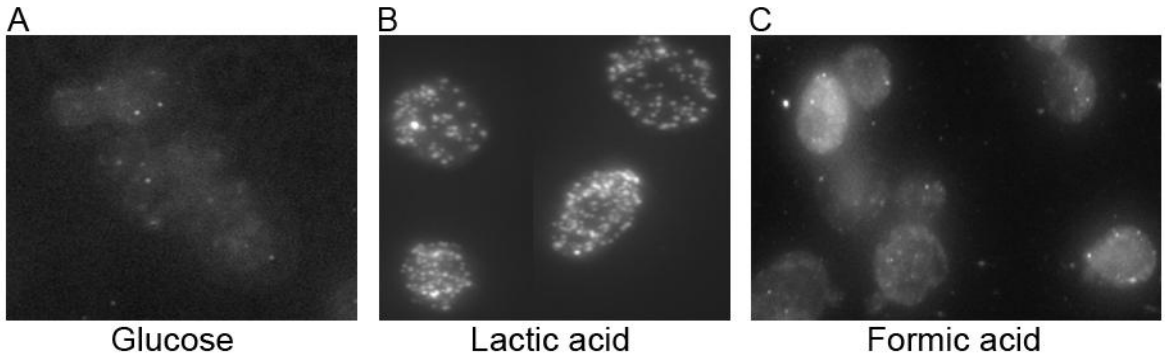

Figure 5 – Characterization of Jen1 localization *in vivo* by Fluorescent in situ hybridization (FISH). *S. cerevisiae dhh1* mutant cells were grown in glucose, and derepressed for 4h in lactic acid pH 5.0, or formic acid pH 5.0, containing media. *JEN1* mRNA molecules were detected by the combined hybridization of five antisense oligonucleotides.

Genome-wide analyses of yeast adaptation to formic acid

Based on the results presented above, DNA microarray analyses of the transcriptome of yeast wild-type and *dhh1* mutant cells, shifted from glucose to formic acid 0.5%, pH 5.0, was performed. About 1000 genes were detected with a 2 fold RNA change, in at least one of the tested conditions. Among these, the expression of about 400 genes increased in the mutant compared with the wild type strain. This emphasized

the large and complex role of Dhh1 in gene expression regulation. In glucose-grown cells the genes whose expression increased in the mutant, were basically involved in carbon metabolism (the proteasome complex, mitochondria, carbohydrate metabolism and energy pathways). Approximately 200 RNAs involved in carbohydrate metabolism, heat shock response, oxidative phosphorylation and redox homeostasis accumulated in formic acid, but not in glucose (Fig. 6). Some genes subjected to glucose repression were derepressed in the mutant strain, both in glucose and in formic acid (*ADR1*, *GPG1*, *ACS1*), while others were derepressed only in formic acid (*JEN1*, *ADY2*, *CAT8*, *ACH1*, *SCF1*). *ADR1/CAT8* and *ACS1/ACH1* cases are particularly demonstrative since they are supposed to be similarly regulated but behaved differently in our experiments. Another aspect was that many genes encoding proteins involved in decapping or P-bodies, namely Dcs1, Edc1, Edc2, accumulated in the *dhh1* mutant, reinforcing its role in mRNA decay.

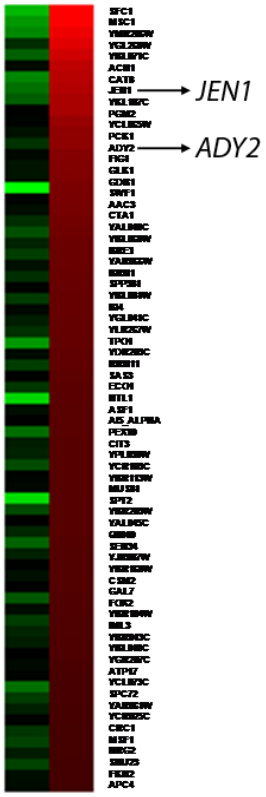

Figure 6 – *DHH1* deletion affected the global carbon response. Treeview software (Eisen and Brown, 1999) was used to represent the ratios of expression (see colour scale) of genes of the formate response that differed between W303-1A and *dhh1*, after a shift from glucose to formate during 4 hours. Here are represented 71 mRNAs that accumulated in the *dhh1* mutant, only after derepression on formic acid.

Discussion

Jen1 is localized at the plasma membrane of *S. cerevisiae* cells and it is involved in the transport of lactic, pyruvic, acetic and propionic acids. This permease is induced in the presence of non-fermentable carbon and energy sources, like lactic and pyruvic acids and it is repressed by glucose (Casal *et al.*, 1999). The dual expression phenotype observed after derepression in formic and propionic acids, between the wild-type and the mutant strains, implies an involvement of Dhh1 in the regulation of *JEN1* expression in this baker's yeast. Moreover, the disruption of this RNA helicase impaired growth on acetic acid, although it had no effect on the mediated uptake rates of the dissociated form of this acid. The *JEN1* mRNA was shown not to be translated into functional protein, since no active transport of lactic or acetic acid, and also no fluorescence of the Jen1 GFP tagged permease was detected in the plasma membrane of *S. cerevisiae*, after derepression in formic acid 0.5% (w/v), pH 5.0. An intriguing possibility is that *DHH1* gene is involved in these mRNAs turnover, and its disruption permits the visualization of both mRNAs in the derepression conditions mentioned above. The involvement with mRNA turnover is reinforced by the fact that mRNAs half-live times are significantly longer in the *dhh1* mutant strain, and by *microarray analysis* data, where approximately 400 genes had a 2 fold increase. The fact that *JEN1* mRNA accumulates in formic acid in the mutant strain, but is not translated, suggests that the mRNAs are stabilized and probably trapped in P-Bodies. Unfortunately, our FISH experiments did not release any conclusion at this point. Still, the model that we can draw of *JEN1* regulation is the following. In glucose, *JEN1* is transcriptionally silent, as described previously. Its transcription is induced, and results in translationally active mRNAs, in lactic acid. In formic acid, the glucose transcriptional repression is also released, but *JEN1* mRNAs are rapidly degraded, possibly by being targeted to P-bodies. This post-transcriptional regulation is clearly under the control of Dhh1. Our microarray experiments suggest that other important genes, like the transcription factor encoding *CAT8* gene, may encounter similar regulation. These genome-wide experiments also revealed an unexpected complexity of glucose catabolic repression. Indeed, among the genes known to be repressed in glucose, some have an increased expression in the absence of Dhh1, suggesting that their glucose-driven negative regulation mainly occurs through post-transcriptional processes, while others, like

JEN1, are not sensitive to the deletion of *DHH1* in the presence of glucose, suggesting that they are mainly repressed at the transcriptional level.

References

Akao, Y., Marukawa, O., Morikawa, H., Nakao, K., Kamei, M., Hachiya, T. and Tsujimoto, Y. 1995. The rck/p54 candidate proto-oncogene product is a 54-kilodalton D-E-A-D box protein differentially expressed in human and mouse tissues. *Cancer Res.* **55**: 3444-3449.

Anderson, P. and Kedersha, N. 2006. RNA granules. *J Cell Biol.* **172**: 803-808.

Ausubel, F., Brent R, Kingston D, Moore D, Seidman JG, Smith and JA, S. K., (1998) In: Current protocols in molecular biology. In. wiley (ed). New York, pp. 4.9.1-4.9.11.

Balagopal, V. and Parker, R. 2009. Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. *Curr Opin Cell Biol.* **21**: 403-408.

Beliakova-Bethell, N., Beckham, C., Giddings, T. H., Jr., Winey, M., Parker, R. and Sandmeyer, S. 2006. Virus-like particles of the Ty3 retrotransposon assemble in association with P-body components. *RNA.* **12**: 94-101.

Brengues, M., Teixeira, D. and Parker, R. 2005. Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. *Science.* **310**: 486-489.

Casal, M., Blazquez, M. A., Gamo, F. J., Gancedo, C. and Leao, C. 1995. Lack of lactate-proton symport activity in pck1 mutants of *Saccharomyces cerevisiae*. *FEMS Microbiol Lett.* **128**: 279-282.

Casal, M., Cardoso, H. and Leao, C. 1996. Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. *Microbiology.* **142**: 1385-1390.

Casal, M., Paiva, S., Andrade, R. P., Gancedo, C. and Leao, C. 1999. The lactate-proton symport of *Saccharomyces cerevisiae* is encoded by *JEN1*. *J Bacteriol.* **181**: 2620-2623.

Casal, M., Paiva, S., Queiros, O. and Soares-Silva, I. 2008. Transport of carboxylic acids in yeasts. *FEMS Microbiol Rev.* **32**: 974-994.

Cassio, F., Leao, C. and van Uden, N. 1987. Transport of lactate and other short-chain monocarboxylates in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* **53**: 509-513.

Coller, J. and Parker, R. 2005. General translational repression by activators of mRNA decapping. *Cell.* **122**: 875-886.

Coller, J. M., Tucker, M., Sheth, U., Valencia-Sanchez, M. A. and Parker, R. 2001. The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. *RNA.* **7**: 1717-1727.

de Valoir, T., Tucker, M. A., Belikoff, E. J., Camp, L. A., Bolduc, C. and Beckingham, K. 1991. A second maternally expressed *Drosophila* gene encodes a putative RNA helicase of the "DEAD box" family. *Proc Natl Acad Sci U S A.* **88**: 2113-2117.

Eisen, M. B. and Brown, P. O. 1999. DNA arrays for analysis of gene expression. *Methods Enzymol.* **303**: 179-205.

Eulalio, A., Behm-Ansmant, I. and Izaurralde, E. 2007. P bodies: at the crossroads of post-transcriptional pathways. *Nat Rev Mol Cell Biol.* **8**: 9-22.

Fardeau, V., Lelandais, G., Oldfield, A., Salin, H., Lemoine, S., Garcia, M., Tanty, V., Le Crom, S., Jacq, C. and Devaux, F. 2007. The central role of *PDR1* in the foundation of yeast drug resistance. *J Biol Chem.* **282**: 5063-5074.

Franks, T. M. and Lykke-Andersen, J. 2008. The control of mRNA decapping and P-body formation. *Mol Cell.* **32**: 605-615.

Garcia, M., Darzacq, X., Delaveau, T., Jourdren, L., Singer, R. H. and Jacq, C. 2007a. Mitochondria-associated yeast mRNAs and the biogenesis of molecular complexes. *Mol Biol Cell.* **18**: 362-368.

Garcia, M., Darzacq, X., Devaux, F., Singer, R. H. and Jacq, C. 2007b. Yeast mitochondrial transcriptomics. *Methods Mol Biol.* **372**: 505-528.

Goldstein, A. L. and McCusker, J. H. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast.* **15**: 1541-1553.

Hata, H., Mitsui, H., Liu, H., Bai, Y., Denis, C. L., Shimizu, Y. and Sakai, A. 1998. Dhh1p, a putative RNA helicase, associates with the general transcription factors Pop2p and Ccr4p from *Saccharomyces cerevisiae*. *Genetics.* **148**: 571-579.

Kruckeberg, A. L., Ye, L., Berden, J. A. and van Dam, K. 1999. Functional expression, quantification and cellular localization of the Hxt2 hexose transporter of *Saccharomyces cerevisiae* tagged with the green fluorescent protein. *Biochem J.* **339**: 299-307.

Ladomery, M., Wade, E. and Sommerville, J. 1997. Xp54, the Xenopus homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. *Nucleic Acids Res.* **25**: 965-973.

Lemoine, S., Combes, F., Servant, N. and Le Crom, S. 2006. Goulphar: rapid access and expertise for standard two-color microarray normalization methods. *BMC Bioinformatics.* **7**: 467.

Liu, J., Rivas, F. V., Wohlschlegel, J., Yates, J. R., 3rd, Parker, R. and Hannon, G. J. 2005. A role for the P-body component *GW182* in microRNA function. *Nat Cell Biol.* **7**: 1261-1266.

Maekawa, H., Nakagawa, T., Uno, Y., Kitamura, K. and Shimoda, C. 1994. The *ste13+* gene encoding a putative RNA helicase is essential for nitrogen starvation-induced G1 arrest and initiation of sexual development in the fission yeast *Schizosaccharomyces pombe*. *Mol Gen Genet.* **244**: 456-464.

Makuc, J., Paiva, S., Schauen, M., Kramer, R., Andre, B., Casal, M., Leao, C. and Boles, E. 2001. The putative monocarboxylate permeases of the yeast *Saccharomyces cerevisiae* do not transport monocarboxylic acids across the plasma membrane. *Yeast.* **18**: 1131-1143.

Navarro, R. E., Shim, E. Y., Kohara, Y., Singson, A. and Blackwell, T. K. 2001. cgh-1, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in *C. elegans*. *Development.* **128**: 3221-3232.

Paiva, S., Althoff, S., Casal, M. and Leao, C. 1999. Transport of acetate in mutants of *Saccharomyces cerevisiae* defective in monocarboxylate permeases. *FEMS Microbiol Lett.* **170**: 301-306.

Paiva, S., Devaux, F., Barbosa, S., Jacq, C. and Casal, M. 2004. Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. *Yeast.* **21**: 201-210.

Paiva, S., Kruckeberg, A. L. and Casal, M. 2002. Utilization of green fluorescent protein as a marker for studying the expression and turnover of the monocarboxylate permease Jen1p of *Saccharomyces cerevisiae*. *Biochem J.* **363**: 737-744.

Parker, R. and Sheth, U. 2007. P bodies and the control of mRNA translation and degradation. *Mol Cell.* **25**: 635-646.

Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E. and Filipowicz, W. 2005. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science.* **309**: 1573-1576.

R. Parker, D. H., S.W. Peltz, A. Jacobson, (1991) Measurement of mRNA decay rates in *Saccharomyces cerevisiae*. In: C. Guthrie, G.R. Fink (Eds.), Guide to Yeast Genetics and molecular Biology, Methods in exymology. London: Academic Press, pp. 415-423.

Sambrook, J., Fritsch, E.F., and Maniatis, T., (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY

Seto, M., Yamamoto, K., Takahashi, T. and Ueda, R. 1995. Cloning and expression of a murine cDNA homologous to the human *RCK/P54*, a lymphoma-linked chromosomal translocation junction gene on 11q23. *Gene.* **166**: 293-296.

Sheth, U. and Parker, R. 2006. Targeting of aberrant mRNAs to cytoplasmic processing bodies. *Cell.* **125**: 1095-1109.

Snay-Hodge, C. A., Colot, H. V., Goldstein, A. L. and Cole, C. N. 1998. Dbp5p/Rat8p is a yeast nuclear pore-associated DEAD-box protein essential for RNA export. *EMBO J.* **17**: 2663-2676.

Strahl-Bolsinger, S. and Tanner, W. 1993. A yeast gene encoding a putative RNA helicase of the "DEAD"-box family. *Yeast.* **9**: 429-432.

Thomas, B. J. and Rothstein, R. 1989. Elevated recombination rates in transcriptionally active DNA. *Cell.* **56**: 619-630.

Tseng-Rogenski, S. S., Chong, J. L., Thomas, C. B., Enomoto, S., Berman, J. and Chang, T. H. 2003. Functional conservation of Dhh1p, a cytoplasmic DExD/H-box protein present in large complexes. *Nucleic Acids Res.* **31**: 4995-5002.

Tseng, S. S., Weaver, P. L., Liu, Y., Hitomi, M., Tartakoff, A. M. and Chang, T. H. 1998. Dbp5p, a cytosolic RNA helicase, is required for poly(A)+ RNA export. *EMBO J.* **17**: 2651-2662.

Unterholzner, L. and Izaurralde, E. 2004. *SMG7* acts as a molecular link between mRNA surveillance and mRNA decay. *Mol Cell.* **16**: 587-596.

Wickens, M. and Goldstrohm, A. 2003. Molecular biology. A place to die, a place to sleep. *Science.* **300**: 753-755.

Wyers, F., Minet, M., Dufour, M. E., Vo, L. T. and Lacroute, F. 2000. Deletion of the PAT1 gene affects translation initiation and suppresses a *PAB1* gene deletion in yeast. *Mol Cell Biol.* **20**: 3538-3549.

Acknowledgements

This study was supported by the Portuguese grant POCI/BIA-BCM/57812/2004 (Eixo 2, Medida 2.3, QCAIII – FEDER). N. V. received FCT PhD fellowship (SFRH/BD/23503/2005).

Section III

Traffic and turnover of proteins

CHAPTER VII – Glucose-induced Ubiquitylation and Endocytosis of the Yeast Jen1 Transporter. Role of Lysine 63-linked ubiquitin chains

The work presented in this chapter has been published:
Sandra Paiva, Neide Vieira, Isabelle Nondier, Rosine Haguenauer-Tsapis, Margarida Casal,and Danièle Urban-Grimal, (2009) *Glucose-induced Ubiquitylation and Endocytosis of the Yeast Jen1 Transporter*. J. Biol. Chem. 284(29):19228-36.

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/05/11/M109.008318.DC1.html

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 29, pp. 19228–19236, July 17, 2009

Glucose-induced Ubiquitylation and Endocytosis of the Yeast Jen1 Transporter

ROLE OF LYSINE 63-LINKED UBIQUITIN CHAINS[*S]

Received for publication, April 15, 2009 Published, JBC Papers in Press, May 11, 2009, DOI 10.1074/jbc.M109.008318

Sandra Paiva[‡1], **Neide Vieira**[‡2], **Isabelle Nondier**[§], **Rosine Haguenauer-Tsapis**[§3], **Margarida Casal**[‡], **and Danièle Urban-Grimal**[§]

From the [‡]*Department of Biology, Molecular and Environmental Biology Centre, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal and the* [§]*Institut Jacques Monod, CNRS-Université Paris Diderot-Paris 7, 75013 Paris, France*

Protein ubiquitylation is essential for many events linked to intracellular protein trafficking. Despite the significance of this process, the molecular mechanisms that govern the regulation of ubiquitylation remain largely unknown. Plasma membrane transporters are subjected to tightly regulated endocytosis, and ubiquitylation is a key signal at several stages of the endocytic pathway. The yeast monocarboxylate transporter Jen1 displays glucose-regulated endocytosis. We show here that casein kinase 1-dependent phosphorylation and HECT-ubiquitin ligase Rsp5-dependent ubiquitylation are required for Jen1 endocytosis. Ubiquitylation and endocytosis of Jen1 are induced within minutes in response to glucose addition. Jen1 is modified at the cell surface by oligo-ubiquitylation with ubiquitin-Lys^{63} linked chain(s), and Jen1-Lys^{338} is one of the target residues. Ubiquitin-Lys^{63}-linked chain(s) are also required directly or indirectly to sort Jen1 into multivesicular bodies. Jen1 is one of the few examples for which ubiquitin-Lys^{63}-linked chain(s) was shown to be required for correct trafficking at two stages of endocytosis: endocytic internalization and sorting at multivesicular bodies.

Ubiquitylation is one of the most prevalent protein post-translational modifications in eukaryotes. In addition to its role in promoting proteasomal degradation of target proteins, ubiquitylation has been shown to regulate multiple processes, including DNA repair, signaling, and intracellular trafficking. Ubiquitylation serves as a key signal mediating the internalization of plasma membrane receptors and transporters, followed by their intracellular transport and subsequent recycling or lysosomal/vacuolar degradation (1, 2). In *Saccharomyces cerevisiae*, transporters usually display both constitutive and accelerated endocytosis regulated by factors such as excess substrate, changes in nutrient availability, and stress conditions. Ubiquitylation of these cell surface proteins acts as a signal triggering their internalization (1). A single essential E3[4] ubiquitin ligase, Rsp5, has been implicated in the internalization of most, if not all, endocytosed proteins (3). Rsp5 is the unique member in *S. cerevisiae* of the HECT (homologous to E6AP COOH terminus)-ubiquitin ligases of the Nedd4/Rsp5 family (4). In a few cases, Rsp5-dependent cell surface ubiquitylation was shown to involve PY-containing adapters that bind to Rsp5 (5–7). Rsp5-mediated ubiquitylation is also required for sorting into multivesicular bodies (MVBs) of endosomal membrane proteins that come from either the plasma membrane (through endocytosis) or the Golgi (through vacuolar protein sorting (VPS) pathway) (8). Although much progress has been made in elucidating the mechanistic basis of various steps in protein trafficking, the precise requirement for a specific type and length of Ub chains at various stages of the endocytic pathway remains to be addressed.

The ubiquitin profile needed for proper internalization has been established for some yeast membrane proteins (1). The α-factor receptor Ste2 was described as undergoing monoubiquitylation on several lysines (multimonoubiquitylation). The a-factor receptor, Ste3p; the general transporter of amino acids, Gap1; the zinc transporter, Ztr1; and the uracil transporter, Fur4, have been shown to be modified by short chains of two to three ubiquitins, each attached to one, two, or more target lysine residues (oligo-ubiquitylation). Among them, Fur4 and Gap1 were the only transporters demonstrated to undergo plasma membrane oligo-ubiquitylation with ubiquitin residues linked via ubiquitin-Lys^{63} (9, 10). In addition, the two siderophore transporters Arn1 and Sit1 were also shown to undergo Lys^{63}-linked cell surface ubiquitylation (11, 12). Whether these four transporters are representative of a larger class of plasma membrane substrates remains to be determined. Little is known about the type of ubiquitylation involved and/or required for sorting to MVBs. Some MVB cargoes appear to undergo monoubiquitylation (8), whereas Sna3, an MVB cargo of unknown function, undergoes Lys^{63}-linked ubiquitylation (13). Lys^{63}-linked ubiquitin chains were also recently

* Experiments performed in the laboratory of R. H.-T. were supported by CNRS-University Paris Diderot Paris 7, by Association pour la Recherche sur le Cancer Grant 3298, a grant from the Ministère de l'Enseignement et de la Recherche and the Action Concertée Incitative (BCMS; contrat 183), and a grant from the European Network VI Framework (RUBICON; contract 018683). Experiments performed in the laboratory of M. C. were supported by Portuguese Grant POCI/BIA-BCM/57812/2004 (Eixo 2, Medida 2.3, QCAIII-FEDER).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

[1] To whom correspondence may be addressed. Tel.: 351-253604310 (ext. 1514); Fax: 351-253678980; E-mail: spaiva@bio.uminho.pt.

[2] Recipient of Fundação para a Ciência e a Tecnologia Ph.D. Fellowship SFRH/BD/23503/2005.

[3] To whom correspondence may be addressed. Tel.: 33-1-57-27-80-52; Fax: 33-1-57-27-80-63; E-mail: haguenauer.rosine@ijm.univ-paris-diderot.fr.

[4] The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; MVB, multivesicular body; GFP, green fluorescent protein; NTA, nitrilotriacetic acid; VPS, vacuolar protein sorting; Ub, ubiquitin; HA, hemagglutinin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/05/11/M109.008318.DC1.html

TABLE 1
Strains and plasmids used in this study

	Genotype	Reference/Source
Strain		
W303-1A*jen1*Δ	*MATa ura3-52 trp1-1 leu2-3-112 his3-11 ade2-1 can1-100 jen1::KanMX4*	Ref. 17
BLC 493	*MATa his4 leu2 ura3 ar1-1 end3-1 JEN1-GFP-KanMX4*	Ref. 23
27061b	*MATa ura3 trp1*	Ref. 27
MOB52	*MATa ura3 trp1 end3::KanMX4*	Ref. 42
27064b	*MATa ura3 trp1 npi1*	Ref. 27
SP1	*MATa ura3 trp1 JEN1-6His-3HA-loxP-KanMX4-loxP*	This study
SP2	*MATa ura3 trp1 npi1 JEN1-6His-3HA-loxP-KanMX4-loxP*	This study
SP5	*MATa his3 leu2 ura3-52 JEN1-6His-3HA-loxP-KanMX4-loxP*	This study
SP6	*MATa his3 leu2 ura3-52 Δyck1 yck2-1ts JEN1-6His-3HA-loxP-KanMX4-loxP*	This study
SP7	*MATa ura3-52 trp1-1 leu2-3-112 his3-11 ade2-1 can1-100 JEN1-6His-3HA-loxP-KanMX4-loxP*	This study
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
BY4741*end3*Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 end3::KanMX4*	Euroscarf
BY4741*npi1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 npi1*	R. H.-T. laboratory
SUB280	*MATa lys2-801 leu2-3 112 ura3-52 his3-Δ200 trp1-1 ubi1-Δ1::TRP1 ubi2-Δ2:ura3 ubi3-Δub-2 ubi4-Δ2::LEU2* (pUB39 Ub, LYS2) (pUB100, HIS3)	Ref. 40
SUB413	*MATa lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1(am) ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-ΔUb-2, ubi4-Δ2::LEU2* (pUB39 UbK63R, LYS2) (pUB100, HIS3)	Ref. 40
Plasmid		
pSP2	*CEN, URA3, pJEN1-JEN1-GFP*	This study
pSP3	*CEN, URA3, pJEN1-JEN-K9R-GFP*	This study
pSP4	*CEN, URA3 pJEN1-JEN-K338R-GFP*	This study
pJEN-GFP-6HIS	*CEN, URA3, pGAL-JEN1-GFP-6×HIS*	This study

reported to be required, directly or indirectly, for MVB sorting of the siderophore transporter, Sit1, when trafficking through the VPS pathway in the absence of its external substrate (11). In agreement with the possibility that additional membrane-bound proteins might undergo Lys^{63}-linked ubiquitylation, a proteomic study aiming to uncover ubiquitylated yeast proteins showed that Lys^{63}-ubiquitin chains are far more abundant than previously thought (14).

The transport of monocarboxylates, such as lactate and pyruvate, as well as ketone bodies across the plasma membrane is essential for the metabolism of cells of various organisms. A family of monocarboxylate transporters has been reported that includes mainly mammalian members (15). In *S. cerevisiae*, two monocarboxylate-proton symporters have been described, Jen1 and Ady2 (16, 17). These transporters exhibit differences in their mechanisms of regulation and specificity. Jen1 is a lactate-pyruvate-acetate-propionate transporter induced in lactic or pyruvic acid-grown cells (18). Ady2, which accepts acetate, propionate, or formate, is present in cells grown in non-fermentable carbon sources (19). Jen1 has unique regulatory characteristics and has been extensively studied. It was the first secondary porter of *S. cerevisiae* characterized by heterologous expression in *Pichia pastoris* at both the cell and the membrane vesicle levels (20). The addition of glucose to lactic acid-grown cells very rapidly triggers loss of Jen1 activity and repression of *JEN1* gene expression (21, 22). Newly synthesized Jen1-GFP fusion protein is sorted to the plasma membrane in an active and stable form, and loss of Jen1-GFP activity upon glucose addition is the result of its endocytosis followed by vacuolar degradation (23). Data from large scale analyses based on mass spectrometry approaches led to the detection of two sites of ubiquitylation for Jen1, one located in the N terminus of the protein and the second in the central loop (14), and several sites of phosphorylation in the N terminus, central loop, and C terminus of the protein (14, 24). In the present study, we aimed at further characterizing the internalization step of endocytosis of the transporter Jen1 and the potential role of the phosphorylation and ubiquitylation events required for its correct endocytic trafficking.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—Complex medium with 1% yeast extract and 1% peptone (YP medium) or a synthetic minimal medium with 0.67% yeast nitrogen base (Difco), supplemented with amino acids to meet auxotrophic requirements (YNB medium), was used for submerged culture at 30 °C. Carbon sources were either 2% glucose, 2% raffinose, 2% galactose, or lactic acid (0.5%, pH 5.0). Cells were always harvested during exponential growth phase. Glucose (2%)-containing medium was used for growth of cells encoding *JEN1* from its own promoter under repression conditions. For derepression conditions, glucose-grown cells were collected by centrifugation, washed twice in ice-cold deionized water, and grown in fresh medium with lactic acid (0.5%, pH 5.0). Raffinose-containing medium was used for growth of cells encoding *JEN1* from the *GAL* promoter, under non-induction conditions. For induction conditions, 2% galactose was added to raffinose-grown cells.

Strains and Plasmid Constructions—The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cells were transformed as described in Ref. 25. The *JEN1* gene was C-terminally tagged on the chromosome with a His_6 tag and three consecutive hemagglutinin (HA) epitope tags by integration of a *Kan*MX4 PCR product obtained from plasmid pU6H-3HA, following the strategy described in Ref. 26. The PCR product was used to transform strains 27064b and LRB346, containing deletions or mutations for *RSP5*/*NPI1* (27) and *YCK1 YCK2* (28), respectively, strains 27061b and LBR351, the corresponding wild-types, and strain W303-1A (29). With the transformants obtained, a colony PCR was performed to confirm correct integration. In this way, we constructed strains SP1, SP2, SP5, SP6, and SP7, harboring JEN1-3HA in the chromosome. For construction of the p*JEN1-JEN1-GFP* fusion expression plasmid, 1 kb of the promoter region immediately upstream of *JEN1* (excluding the start codon) was amplified with a primer introducing homology with the plasmid p416-*GPD* (30) and another with 56 bp homologous to the 5′ part of the *JEN1* coding sequence. Plasmid p416-*GPD-JEN1* (20), containing the *JEN1*

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/05/11/M109.008318.DC1.html

coding sequence, under the control of the *GPD* promoter, was digested with the enzymes SacI and BamHI, removing the promoter. The linearized plasmid was co-transformed with the *JEN1* promoter PCR product described above into the *S. cerevisiae* W303-1A *jen1*Δ mutant strain. The resulting plasmid pSP1, obtained by gap repair, expresses the *JEN1* gene under control of the native promoter. Correct clones were identified by colony PCR. In a similar fashion, plasmid pSP2 was obtained by gap repair between EcoRI-linearized pSP1 and a PCR product containing the *GFP* open reading frame. The PCR product was obtained from the vector pFA6a-*GFP-S65T-kan*MX6 (31) using primers introducing regions of homology to the 3′ of *JEN1* and the *CYC1* terminator of p416-*GPD*. Correct clones were identified by colony PCR. The pSP2 plasmid expresses a C-terminal Jen1-GFP fusion protein under the control of the native *JEN1* promoter. Mutations that substitute *JEN1* lysine codons for arginine were introduced in the plasmid pSP2, using site-directed mutagenesis as described (32). The resulting plasmids pSP3 to -5 express the resulting mutant alleles of *JEN1* (Table 1). All of the mutations were confirmed by DNA sequencing. For construction of the *JEN1-GFP-6HIS* fusion expression plasmid, a PCR product containing the *GFP* coding sequence fused in the 3′-end with a *6HIS* coding sequence, was digested with BamHI and XhoI and cloned into the plasmid p416-*GAL* (33) digested with the same enzymes; this resulted in the plasmid p416-*GFP-6HIS*. The *JEN1* coding sequence, excluding the stop codon, was amplified by PCR and digested with BamHI and EcoRI. The fragment obtained was cloned into p416-*GFP-6HIS* digested with the same enzymes, resulting in plasmid p*JEN1-GFP-6HIS*.

Transport Assays—Transport assays, using labeled [U-^{14}C]lactic acid (sodium salt; Amersham Biosciences) (4000 dpm/nmol), at pH 5.0, were carried out as described (23).

Fluorescence Microscopy—Cells grown to exponential growth phase in YNB medium were concentrated by a factor of 10 by centrifugation. The cells were viewed immediately, without fixation, under a fluorescence microscope (type BY61; Olympus, Tokyo, Japan), and images were captured with a digital camera.

Cell Extracts and Immunoblotting—Total protein extracts were prepared by the NaOH-TCA lysis technique (34). Lysates were prepared from cells harvested by centrifugation at 4 °C in the presence of 10 mM sodium azide. The cells were washed once and resuspended in cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl plus a mixture of EDTA-free protease inhibitors: Complete from Roche Applied Science and 25 mM freshly prepared *N*-ethylmaleimide to prevent artifactual deubiquitylation). They were then disrupted in a "One Shot" Cell Disrupter (Constant Systems LDT, Daventry, UK) at a maximum pressure of 2.7 kilobars. The disrupted cells were centrifuged twice (3000 × *g* for 3 min at 4 °C) to remove unbroken cells. Proteins from either total extracts or lysates were resuspended in sample buffer, heated at 37 °C, and resolved by SDS-PAGE in 10% acrylamide gels using Tricine buffer and transferred to nitrocellulose membranes. The membranes were probed with monoclonal antibodies anti-HA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-GFP antiserum (Roche Applied Science), anti-phosphoglycerol kinase (Molecular Probes), or anti-Ub (clone P4D1)-HRP conjugate (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G was used as the secondary antibody (Sigma) and was detected by enhanced chemiluminescence.

His$_6$-tagged Jen1-GFP Purification—His$_6$-tagged Jen1-GFP purification experiments were performed essentially as previously described (35), except that Ni^{2+}-NTA resin was used in batch rather than in column. 6–7 × 10^8 cells were harvested, and lysate was prepared. The lysate was subjected to centrifugation at 13,000 × *g* for 30 min to generate the supernatant and pellet fractions. The pellet was resuspended in 300 μl of buffer A (lysis buffer supplemented with 5 mM imidazole, 0.1% SDS, and 1% Triton X-100). The suspension was incubated on ice for 30 min and then diluted by adding 300 μl of buffer B (lysis buffer supplemented with 5 mM imidazole and 1% Triton X-100) and centrifuged for 10 min at 13,000 × *g* to remove the remaining insoluble material. The supernatant was added to 200 μl of Ni^{2+}-NTA Superflow resin (Qiagen Inc., Hilden, Germany) and incubated with mixing for 1 h at 4 °C. The unbound fraction was collected, and the resin was washed three times with 200 μl of buffer B. Jen1-GFP-6His was eluted three times with 200 μl of elution buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 200 mM imidazole). Aliquots of different fractions were prepared for Western blot analysis.

RESULTS

The Casein Kinase I Activity Is Required for Jen1 Turnover—The ubiquitylation and internalization of the Fur4 and Ste2 membrane proteins depend on their prior phosphorylation at several Ser residues, directly or indirectly by the casein kinase 1 isoforms Yck1/Yck2 (36–38). The potential state of phosphorylation of Jen1-HA on extracts of whole cells grown on lactic acid was initially tested (Fig. 1*A*). The electrophoretic pattern of Jen1-HA displayed several bands with different mobilities on an immunoblot. Treatment with alkaline phosphatase increased the electrophoretic mobility of the slower running bands, suggesting that Jen1-HA is constitutively phosphorylated. A mutant strain lacking the *YCK1* gene and carrying a temperature-sensitive allele of the *YCK2* gene, *yck2-2* (hereafter referred to as *yck-ts*), was used to investigate the potential role of Yck activity in controlling Jen1-HA phosphorylation and internalization. The transporter activity was followed after glucose addition to lactic acid-induced wild-type and *yck-ts* cells, grown at 24 °C and shifted to 37 °C, for 30 min (Fig. 1*B*). The addition of glucose caused a sharp decrease in lactic acid uptake in wild-type cells incubated at 37 °C with an approximate half-life time of 25 min. The decrease in lactic acid uptake was less severe in *yck-ts* cells shifted to the restrictive temperature (approximate half-life time of 60 min). The relative protection against loss of transport activity indicated that the defect in Yck activity stabilized the transporter at the plasma membrane. Extracts from cells withdrawn at various times after glucose addition were analyzed by immunoblotting (Fig. 1*C*). Significant protection against degradation was observed in *yck-ts* cells; therefore, the glucose-induced internalization and subsequent degradation of the transporter are dependent on Jen1 phosphorylation, which directly or indirectly requires Yck kinase activity.

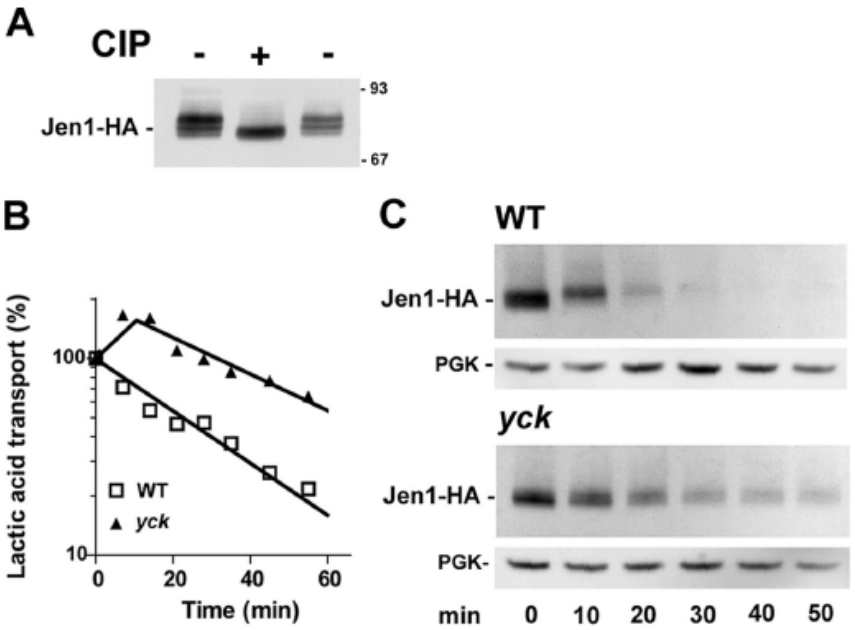

FIGURE 1. **Endocytosis of Jen1-HA at restrictive temperature in Casein Kinase I (*yck*-ts) mutant cells.** *A*, protein extracts from SP7 cells harboring chromosomally encoded Jen1-HA and induced for the production of Jen1-HA in lactic acid for 4 h were incubated at 37 °C for 1 h in the presence (+) or absence (−) of 50 units of calf intestinal phosphatase (*CIP*). The samples were then separated by SDS-PAGE and analyzed for Jen1-HA by Western immunoblotting with an anti-HA antibody. The sizes of molecular weight markers are indicated. *B*, parental SP5 (*YCK*) and SP6 (*yck*) cells harboring chromosomally encoded Jen1-HA were induced for the production of Jen1-HA in lactic acid for 3.5 h at permissive temperature (23 °C), followed by 30 min at restrictive temperature (37 °C). Lactic acid uptake was measured at the times indicated after the addition of glucose (2%). Results are percentages of initial activities. □, wild type; ▲, *yck* cells. *C*, protein extracts were prepared at the same time points and analyzed for Jen1-HA by Western immunoblotting with an anti-HA antibody. Blots were reprobed with an anti-phosphoglycerol kinase (*PGK*) antibody to provide loading controls.

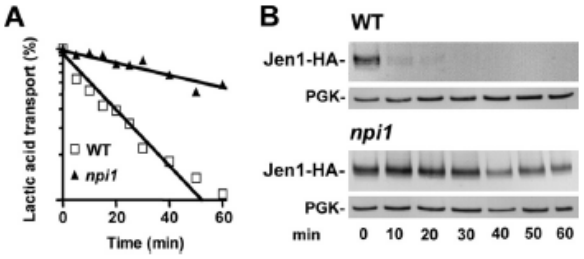

FIGURE 2. **Endocytosis of Jen1-HA in *rsp5/npi1* mutant cells.** Parental SP1 (*WT*) and SP2 (*rsp5/npi1*) cells harboring chromosomally encoded Jen1-HA were induced for 4 h in lactic acid before glucose addition. *A*, lactic acid uptake was measured at the times indicated after the addition of glucose (2%). The results are percentages of initial activities. □, wild-type; ▲, *rsp5/npi* cells. *B*, protein extracts were prepared at the same time points and analyzed by Western immunoblotting with an anti-HA antibody. The blots were reprobed with an anti-phosphoglycerol kinase (*PGK*) antibody to provide loading controls.

The Ubiquitin Ligase Rsp5 Is Required for Jen1 Turnover—The ubiquitin ligase Rsp5 is involved in the internalization step of endocytosis of numerous transporters (3). Since Rsp5 is essential for cell viability, we investigated its potential role on the turnover of Jen1 in a hypomorphic allele of *rsp5*, *npi1* (39). The fully viable *rsp5/npi1* mutant strain displays low levels of *RSP5* expression, allowing production of less than one-tenth the amount of Rsp5 present in wild-type cells (35, 39). Jen1 transport activity (Fig. 2*A*) and stability (Fig. 2*B*) were followed after a pulse of glucose to lactic acid-induced cells. In wild-type cells, both Jen1 activity and the Jen1 immunodetection signal decreased rapidly, reflecting internalization and subsequent vacuolar degradation of the transporter. In contrast, Jen1 activity and the Jen1 signal were greatly stabilized in *rsp5/npi1* cells (relative protection against loss of transport activity, 3-fold). Thus, the protection against degradation of Jen1 in *rsp5/npi1* cells is mainly due to stabilization of Jen1 at the cell surface.

Cell Surface Rsp5-dependent Oligo-ubiquitylation of Jen1—The involvement of Rsp5 in destabilizing Jen1 suggested that ubiquitylation of the transporter is required for transporter degradation. Parental *rsp5/npi1* together with *end3*Δ cells impaired in the internalization step of endocytosis were transformed with plasmid pJEN1-GFP-6HIS, induced in galactose for the production of Jen1-GFP-6His, and then treated with glucose to trigger endocytosis of the protein. First, the localization of the protein was followed by fluorescence microscopy before and after the addition of glucose for 30 min (Fig. 3*A*). Fluorescence was detected both at the plasma membrane and in the vacuolar lumen in WT cells before the addition of glucose. In contrast, Jen1-GFP-6His only stained the plasma membrane of *end3*Δ cells, and the vacuolar signal was hardly detectable. This suggests that constitutive endocytosis rather than direct, premature sorting of Jen1-GFP to the VPS pathway might be the cause of the vacuolar staining in WT cells. Jen1-GFP-6HIS stained essentially the plasma membrane of *rsp5/npi1* cells, and the vacuolar fluorescent signal was less bright when compared with WT cells, suggesting that some constitutive endocytosis may occur in these mutant cells. The fluorescent signal stained the vacuolar lumen of parental cells 30 min after glucose addition, whereas fluorescence was only detectable at the cell surface of *rsp5/npi1* and *end3*Δ cells.

We used the *end3*Δ mutant strain to uncover possible ubiquitylation of Jen1 triggered by glucose addition. WT and *end3*Δ cells expressing pJEN1-GFP-6HIS were induced in galactose and then treated with glucose to trigger endocytosis of the protein. Fig. 3*B* shows immunoblots of cell extracts revealed with anti-GFP antibody. Two to three more slowly migrating bands of immunoreactive material were observed above the main Jen1-GFP signal of *end3*Δ cell extracts. These bands were hardly visible at time 0 of the experiment (*i.e.* before glucose addition). This ladder of bands was far less clear in WT cells, in which internalization was not blocked. An immunoblot of *end3*Δ cell extracts in which chromosomally encoded Jen1-GFP was induced in lactic acid and treated with glucose to

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/05/11/M109.008318.DC1.html

Ub-Lys^{63}-dependent Endocytosis of Jen1

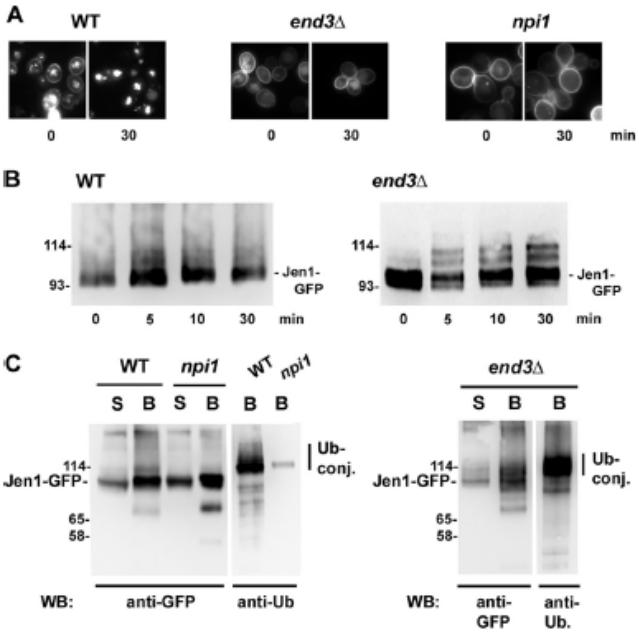

FIGURE 3. **Ubiquitylation of Jen1-GFP-6His in *end3*Δ and *rsp5/npi1* mutant cells.** Parental 27061b (*WT*), *rsp5/npi1*, and *end3*Δ cells were transformed with p*JEN1-GFP-6HIS*. The cells were induced for the production of Jen1-GFP-6His in galactose for 2 h (30 °C). *A*, microscopy images of Jen1-GFP-6His in living cells before and after the addition of 2% glucose for 30 min. *B*, protein extracts from induced parental and *end3*Δ cells were prepared before and at the indicated times after the addition of 2% glucose and were then analyzed by Western immunoblotting with an anti-GFP antibody. *C*, lysates of cells incubated for 10 min with glucose were fractionated, as described under "Experimental Procedures." All experiments were conducted in identical conditions of growth and cell fractionation. Solubilized membranes were incubated with Ni^{2+}-NTA beads. The bound fraction corresponding to His_6-tagged ubiquitylated proteins were eluted by buffer containing 200 mM imidazole. Aliquots of solubilized membranes and bound fractions were resolved by electrophoresis and analyzed by Western immunoblotting (*WB*) with anti-GFP and anti-Ub antibodies. Note that this particular anti-Ub antibody hardly recognizes mono- and diubiquitins. *S*, solubilized membrane fractions; *B*, purified His_6-tagged proteins. The sizes of molecular weight markers are indicated.

induce endocytosis was also performed (Fig. S1). Immunoreactive material displayed the same profile with two to three more slowly migrating bands above the main Jen1-GFP signal. Hence, the post-translational modifications of Jen1 were identical with or without an additional His_6 tag and with different levels of expression. Strikingly, in both experiments, Jen1 modifications occurred within minutes after the glucose addition.

A biochemical characterization of the Jen1-GFP-6His more slowly migrating species was attempted. Aliquots of WT, *rsp5/npi1*, and *end3*Δ cells were withdrawn 10 min after glucose addition (*i.e.* under conditions where a high proportion of Jen1-GFP was still present at the plasma membrane), and the corresponding protein extracts were subjected to cell fractionation (Fig. 3*C*). Membrane-enriched fractions were solubilized and incubated with Ni^{2+}-NTA beads. Aliquots from the solubilized pellet together with corresponding aliquots of bound material were resolved by electrophoresis and subjected to Western blotting to identify the immunoreactive transporter. Jen1-GFP-6His was specifically retained on nickel beads, as visible with anti-GFP antibodies. The slower migrating bands were detected with anti-ubiquitin antibodies in WT cells, indicating that they correspond to Jen1-GFP-6His conjugated with ubiquitin. The slowly migrating bands detected with anti-ubiquitin antibodies were far less abundant in the Ni^{2+}-NTA-retained fractions of *rsp5/npi1* cells, showing that Rsp5 is involved in ubiquitylation of the transporter. The slowly migrating bands detected with anti-ubiquitin antibodies were also present in the Ni^{2+}-NTA-retained fractions of *end3*Δ cells, showing that ubiquitylation of the transporter occurs at the plasma membrane. These experiments indicate that the Jen1 transporter is a target for ubiquitylation by Rsp5 at the cell surface. The profile of ubiquitin conjugates corresponding to Jen1-GFP purified on nickel beads appeared more like a smear (Fig. 3*C*), suggesting that Jen1 ubiquitylation may correspond to the conjugation of more ubiquitin species than the two to three Ub bands visualized directly on cell extracts (Fig. 3*B*).

Lys to Arg Substitution at Lys^{338} Extends the Half-life of Jen1—The hydrophilic parts of Jen1 harbor numerous lysines that could be potential targets for ubiquitylation of the protein. We focused our analysis on Lys^{9} and Lys^{338}, which were identified by a proteomic approach, as target sites for ubiquitylation (14). Lys^{9} is located in the N-terminal hydrophilic part of the transporter, whereas Lys^{338} is located in its third predicted cytoplasmic loop. To define whether ubiquitylation of these two lysines plays a role in Jen1 endocytosis, they were replaced by conservative but non-ubiquitylatable arginine residues. The corresponding variant proteins were tested for their cellular trafficking and stability (Fig. 4). Ectopic Jen1-GFP and the corresponding Lys to Arg variants were expressed under the *JEN1* promoter. The variant transporters were expressed in lactic acid-induced cells, and endocytosis was triggered by glucose addition. At time 0, fluorescence was mainly detected at the plasma membrane, in all types of cells, showing that all of the variant proteins were correctly targeted to the plasma membrane (Fig. 4*C*). Following a pulse of glucose, plasma membrane fluorescence corresponding to wild-type Jen1-GFP disappeared progressively, and intracellular dots were detected. These dots, heterogeneous in size, might correspond to early and late endosomes. Some staining of the vacuole was also observed. After 60 min, the fluorescence was only detected in the vacuolar lumen. Trafficking of the protein from the cell surface to the vacuole appeared more efficient for the K9R variant, since staining was exclusively detected in the vacuole 20 min after glucose addition. In contrast, the K338R variant still stained the plasma membrane 80 min after glucose addition.

To follow more quantitatively the fate of the Lys to Arg variants relative to wild-type Jen1-GFP, their stability was tested at the plasma membrane by monitoring Jen1 transport activity after the addition of glucose to lactic acid-induced cells. Glucose treatment caused a drop in lactic acid uptake in wild-type cells, with a half-life time of roughly 70 min (Fig. 4*A*). Transporter immunoreactivity declined in parallel to the drop in transporter activity (Fig. 4*B*). The same drop in transport activity was observed with the K9R variant transporter. However, the amount of K9R variant immunoreactive species appeared to decline more rapidly than the amount of the wild-type species, confirming the fluorescent observations. In contrast, the decrease in transport activity was far less severe in cells producing the K338R variant, with a half-life time higher than 120 min. In agreement, the K338R variant transporter was more slowly

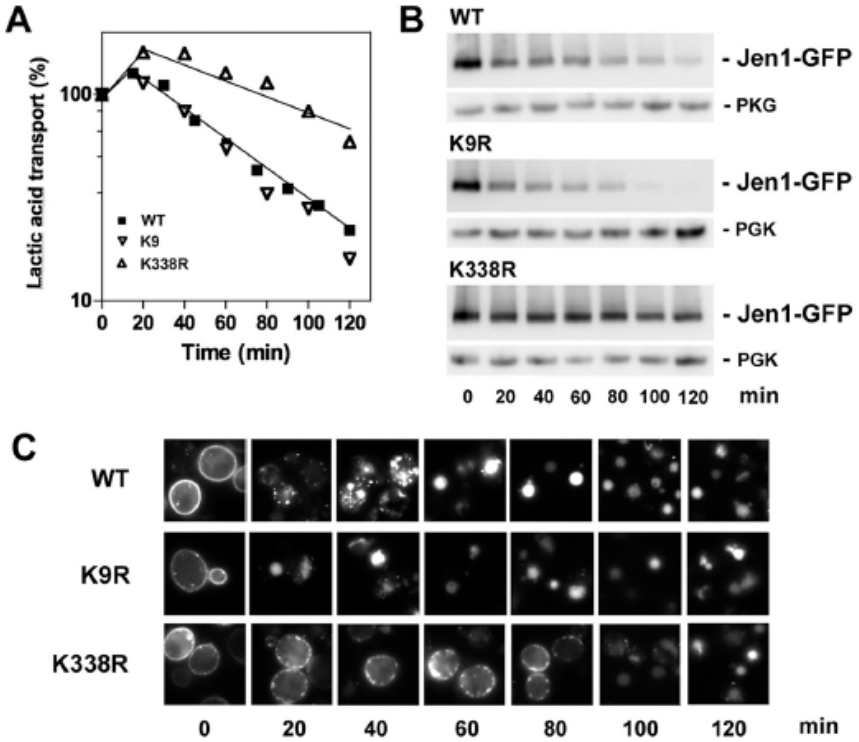

FIGURE 4. **Effect of Lys to Arg mutations on the stability of Jen1-GFP.** W303-1A *jen1*Δ cells transformed with either pSP2 (*JEN1-GFP*), pSP3 (*JEN1-K9R-GFP*), or pSP4 (*JEN1-K338R-GFP*) were induced for the production of Jen1-GFP in lactic acid for 4 h at 30 °C. *A*, acid lactic uptake was measured at the times indicated after the addition of 2% glucose. The results are percentages of initial activities. ■, Jen1-GFP; ▽, Jen1-K9R-GFP; △, Jen1-K338R-GFP; *B*, protein extracts collected at the same time points were analyzed by Western immunoblotting with an anti-GFP antibody. The blots were reprobed with an anti-phosphoglycerol kinase (*PGK*) antibody to provide loading controls. *C*, microscopy images of Jen1-GFP in living cells at the time points indicated after the addition of 2% glucose. *WT*, wild type (Jen1-GFP).

degraded than the wild-type protein. The relative protection (2-fold) against loss of transport activity (Fig. 4*A*) and the relative stabilization of the K338R variant protein at the plasma membrane (Fig. 4, *B* and *C*) suggested that Lys^{388} is a major target site for ubiquitylation of Jen1 at the cell surface. In contrast, the absence of stabilization observed when Lys^{9} was substituted for Arg (Fig. 4*A*) is unexpected and may argue against Lys^{9} being a target for ubiquitylation at the plasma membrane. However, it may be possible that other Lys residue(s) of Jen1 may be ubiquitylated in place of Lys^{9} when the latter is mutated. Surprisingly, the internalized K9R variant is more quickly targeted to the vacuole for degradation than the WT protein (Fig. 4, *B* and *C*). It might indicate that, for unknown reasons, Jen1-K9R-GFP is a more efficient substrate for sorting of the protein into the MVB than Jen1-GFP or is unable to undergo putative recycling from endosomes to the plasma membrane.

We monitored the ubiquitylation status of the Jen1-GFP variants produced in *end3*Δ cells after ubiquitylation was triggered by glucose addition. Immunoblots of all of the corresponding lysates displayed a similar ladder of ubiquitin-permease conjugates with three minor bands with slower mobilities than the main transporter signal (data not shown). This result most probably suggests that Jen1 may be ubiquitylated on more than one lysine and is in agreement with the partial stabilization observed at the cell surface for the K338R variant.

These overall data indicate that Jen1-Lys^{338} is probably one of the targets for cell surface ubiquitylation, involved in Jen1 internalization, in agreement with proteomic data (14). The situation appears more complex for Jen1-Lys^{9}.

Ubiquitin Lys^{63} Is Involved in Trafficking of Jen1—We analyzed the localization and ubiquitin status of Jen1-GFP-6His during endocytosis in a collection of SUB strains, deleted in the four natural ubiquitin genes and expressing different ubiquitin Lys to Arg variants, as their sole source of ubiquitin. It was previously shown that all ubiquitin Lys residues can be used for the formation of ubiquitin chains in yeast (14) and that the unique lysine in ubiquitin required for viability is Lys^{48}, which is the favorite lysine involved in the formation of chains used for proteasome degradation (40, 41). Jen1-GFP was correctly internalized and sorted to the vacuolar lumen over time, after glucose addition to galactose-induced SUB280 cells producing wild-type ubiquitin (Fig. 5*A*). Jen1-GFP was also internalized and sorted to the vacuolar lumen in cells producing K6R, K11R, K27R, K29R, and K33R variants of ubiquitin (data not shown). In contrast, Jen1-GFP was stabilized at the plasma membrane after glucose addition to SUB413 cells, producing the Ub-K63R variant. An immunoblot of cell extracts prepared at different times after glucose addition confirmed that Jen1-GFP was protected from degradation in SUB413 cells (Fig. 5*B*). We therefore examined the ubiquitylation pattern of Jen1-GFP. Lysates from SUB280 and SUB413 cells producing Jen1-GFP-6His were subjected to cell fractionation just after glucose addition (*i.e.* under conditions where a high proportion of Jen1-GFP was still present at the plasma membrane). Solubilized membrane-bound Jen1-GFP-6His was retained on Ni^{2+}-NTA beads, as visualized with an anti-GFP antibody (Fig. 5*C*). In wild-type cells, up to four bands of higher molecular weight than the main unmodified Jen1-GFP species were visible. In SUB413 cells, almost all of these species could also be detected, with high enrichment of a species that probably corresponds to monoubiquitylated Jen1-GFP.

We then analyzed the localization and ubiquitin status of Jen1-GFP expressed under its own promoter during endocytosis in SUB280 and SUB413 (Fig. 5*D*). Jen1-GFP was stabilized at the plasma membrane after glucose addition to SUB413 cells. Immunoreactive material in SUB413 cell lysates also displayed high enrichment of a species that probably corresponds to monoubiquitylated Jen1-GFP.

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/05/11/M109.008318.DC1.html

Ub-Lys^{63}-dependent Endocytosis of Jen1

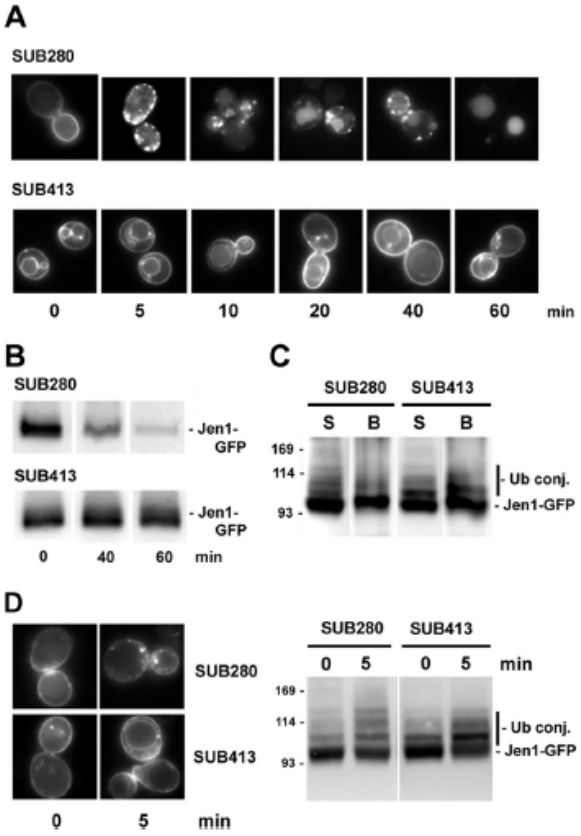

FIGURE 5. **Ubiquitylation and stability of Jen1-GFP in SUB280 and SUB413 cells.** SUB280 and SUB413 cells producing WT or Ub-K63R ubiquitin, respectively, were transformed with p*JEN1-GFP-6HIS*. Cells were induced for the production of Jen1-GFP-6His in galactose for 2 h at 30 °C. *A*, microscopy images of Jen1-GFP-6His in living cells at the time points indicated after the addition of 2% glucose. *B*, protein extracts collected at the indicated times were analyzed by Western immunoblotting with an anti-GFP antibody. *C*, lysates of 5-min glucose-incubated cells were fractionated, as described under "Experimental Procedures." All experiments were conducted in identical conditions of growth and cell fractionation. Solubilized membranes were incubated with Ni^{2+}-NTA beads. The bound fraction corresponding to His_6-tagged ubiquitylated proteins was eluted by 200 mM imidazole-containing buffer. Aliquots of solubilized membranes and bound fractions were resolved by electrophoresis and analyzed by Western immunoblotting with an anti-GFP antibody. *S*, solubilized membrane fractions; *B*, purified His_6-tagged proteins. *D*, SUB280 and SUB413 cells were transformed with pSP2 (*JEN1-GFP*). Cells were induced for the production of Jen1-GFP in lactic acid for 4 h at 30 °C before the addition of glucose for 5 min. *Left*, microscopy images of Jen1-GFP in living cells at the time points indicated after the addition of 2% glucose. *Right*, protein lysates collected at the indicated times were analyzed by Western immunoblotting with an anti-GFP antibody. The sizes of molecular weight markers are indicated.

These observations could suggest that Jen1-GFP carries one main target lysine and that it is modified by a Lys^{63}-linked ubiquitin chain. These results are in agreement with the fact that partial stabilization of Jen1-K338R was observed, and they also show that Jen1-GFP is probably modified on several target lysines that could be monoubiquitylated in SUB413 cells. Importantly, endocytosis was not entirely blocked in SUB413 cells, and some Jen1-GFP also stained the vacuolar rim even at time 0. This is the typical localization observed for membrane proteins coming from the Golgi apparatus, when MVB sorting is inhibited as a result of either defective ubiquitylation before or at the MVB (11, 42) or defective MVB sorting machinery (*i.e.* in *vps* class E mutants) (43). The deficiency in vacuolar delivery of Jen1-GFP in SUB413 cells could result either from impaired ubiquitylation of Jen1 itself or from a deficiency in MVB sorting machineries. Whatever the case, this indicates that formation of Ub-Lys^{63}-linked chains plays an important role for MVB sorting of Jen1-GFP.

DISCUSSION

In the present report, we provide critical data demonstrating rapid glucose-triggered ubiquitylation and subsequent endocytosis of Jen1 and the involvement of casein kinase 1 in this process. We also demonstrate that the HECT-ubiquitin ligase Rsp5 modifies Jen1 at the cell surface by oligo-ubiquitylation. Additionally, Jen1-Lys^{338} was identified as one of the targets for ubiquitylation, confirming the data obtained by a proteomic approach. Results concerning the other potential target, Jen1-Lys^{9}, are more enigmatic. Finally, our data suggest that Lys^{63}-linked ubiquitin chains are required for Jen1 endocytic internalization and directly or indirectly for Jen1 sorting at the MVB.

Most plasma membrane receptors and transporters display regulated ubiquitylation and internalization in yeast. For instance, Gap1, Fur4, Tat2, and Ctr1 ubiquitylation and internalization are triggered by ammonium, high uracil, high tryptophan, and high copper concentration, respectively (44, 45). The presence of Jen1 at the cell surface is tightly controlled by glucose. Jen1 is one of the few documented examples in which ubiquitylation and endocytosis of a transporter are induced within minutes, as a response to a metabolic change. It is tempting to hypothesize that phosphorylation events may play a role in these processes. It was previously shown that yeast casein kinase 1 phosphorylation of Ste2 (triggered by its ligand, α-factor) and of Fur4 drive their subsequent ubiquitylation and internalization (36–38). We show here that phosphorylation of Jen1 is also required prior to its internalization. The yeast casein kinase 1 isoforms, Yck1/Yck2, two plasma membrane anchored proteins (46), are involved in this process.

Rsp5-mediated ubiquitylation and subsequent internalization appears to be a general feature of the vast majority of plasma membrane proteins in yeast. Ubiquitin-dependent endocytosis also occurs in mammalian cells, mediated both by Nedd4/Nedd4-like ligases and by RING finger ligases (1, 2). This has raised questions concerning substrate recognition and mode of ubiquitylation. All members of the Nedd4 family, including Rsp5, display three to four WW modules that interact with PY motifs. But no Rsp5 plasma membrane endocytic substrates carry such PY motifs. However, Bul1 and Bul2, two PY-containing proteins, regulate endocytosis of a subset of plasma membrane transporters (7, 45, 47). Very recently, PY-containing arrestin-like proteins were also shown to play the role of Rsp5-substrate adaptors at the cell surface (5, 6). Whether glucose-triggering phosphorylation of Jen1 may result in a conformational change rendering certain Lys residues directly accessible for ubiquitylation or whether such a conformational change may trigger the interaction of Rsp5 with Jen1 via an arrestin-like adaptor that remains to be discovered still needs to be further investigated.

Jen1 harbors numerous cytoplasmic lysines. We and others previously suggested that Lys residues lying in (D/E)XK(S/T) motifs are probably primary targets for ubiquitylation of plasma

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/05/11/M109.008318.DC1.html

membrane proteins, at least in yeast (1, 48). A proteomic approach identified two ubiquitylation targets for Jen1, Lys^{9} and Lys^{388} (14). These two lysine-surrounding sequences are DEK^{9}IS and DAVK^{388}AN. We demonstrate here that at least Jen1-Lys^{388} must be a target for ubiquitylation at the plasma membrane, even if the sequence surrounding Lys^{388} does not exactly fit the established consensus. In contrast, the sequence around Lys^{9} does fit, but the results obtained for the K9R variant protein were unexpected. The rapid destabilization of the internalized Jen1-K9R variant could not be easily explained. One possibility is that the amount of Jen1 observed at the plasma membrane could result from its endocytosis, followed by its recycling to the cell surface, since plasma membrane redistribution of endosomal targeted transporters was described in the case of the *vps* class E mutants (49, 50) and corresponds to the physiological fate of some transporters in WT cells (51). In that case, the instability of the internalized K9R variant, upon glucose addition, would be due to an unexpected increase in internalization into MVBs or to a defect in recycling. This would allow its rapid clearance from the plasma membrane into the vacuole for degradation that could mask a potential stabilization at the plasma membrane linked to the absence of the Lys^{9} ubquitylation site. This hypothesis raises new questions that will constitute the basis of future investigations.

Little is known so far about the ubiquitylation features associated with the internalization step of endocytosis. Ubiquitylation of yeast plasma membrane proteins, when defined, occurs via conjugation of ubiquitin monomers (multimonoubiquitylation) or of short chains of ubiquitin (oligo-ubiquitylation) (1). Fur4 and Gap1 transporters are modified by Lys^{63}-linked oligo-ubiquitylation for efficient endocytosis (9, 10). It was recently shown that the siderophore transporters Arn1 and Sit1 are also modified with Ub-Lys^{63}-linked ubiquitin chains at the plasma membrane (11, 12). The same holds true in mammalian cells, where a number of plasma membrane transporters and receptors were shown to undergo modification by Lys^{63}-linked ubiquitin chains, a process carried out by Nedd4 in the case of the dopamine DAT transporter (2). Our data indicate that Ub-Lys^{63} is also essential for internalization of Jen1 and that Jen1 is most probably modified by oligo-ubiquitylation. But whether Jen1 is modified at the cell surface by Ub-Lys^{63}-linked ubiquitin chains remains to be further documented. However, this modification is likely to occur given both our data and the recent report that Rsp5 preferentially assembles this type of ubiquitin chains *in vitro* and *in vivo* (52, 53).

Far less is known about the type of ubiquitylation associated with and required for MVB sorting. Ubiquitin fused in frame to lysineless MVB cargoes trafficking through the VPS pathway is sufficient for correct MVB sorting of these cargoes (35, 54, 55). However, Sna3 is modified with a unique and long Ub-Lys^{63}-linked ubiquitin chain (13). These proteins are all substrates of Rsp5 at the Golgi/MVB level (13, 35, 54, 55). The precise identification of the ubiquitylation status of other proteins at the level of MVB is still poorly documented. Nevertheless, it was reported that the Sit1 transporter diverted from the Golgi to the VPS pathway for premature degradation in the absence of its substrate displays deficient MVB sorting in cells unable to form Ub-Lys^{63}-linked ubiquitin chains (11). Similarly, Jen1 coming from the plasma membrane was recovered at the vacuolar rim instead of the vacuolar lumen of cells producing Ub-K63R as sole source of ubiquitin. This implies that Lys^{63}-linked ubiquitin chains are needed directly (ubiquitylation of the cargo) or indirectly (ubiquitylation of the MVB sorting machinery) or both, for proper Jen1 sorting at the MVB. A requirement for this type of ubiquitin chains at MVB fits neatly with the knowledge that the mammalian deubiquitylating enzyme AMSH, which specifically disassembles Ub-Lys^{63} chains, associates with Vps class E proteins (56, 57) and the observation that the ubiquitin binding domain of the Vps class E protein Hrs/Vps27 displays specific or increased affinity for Lys^{63}-linked ubiquitin chains (58). If the requirement for MVB sorting depends on direct modification of cargo by Ub-Lys^{63}-linked chains, it would imply that transporters undergo a step of deubiquitylation (allowing potential recycling to the cell surface), followed by appropriate Rsp5-dependent reubiquitylation for proper entry of endocytic cargoes into the MVB. Whether such a process exists is currently speculative. Further studies on transporter trafficking, including Jen1, will certainly clarify this hypothesis.

Acknowledgments—We thank members of the R. H.-T. laboratory for comments on the manuscript and B. Johansson and A. L. Haenni for editorial assistance. We are grateful to Z. Erpapazoglou for sharing unpublished results and N. Buisson for providing the BY4741npi1 strain.

Note in Proof—While this manuscript was in revision, it was reported that MVB sorting of Gap1 and CPS1 also depends on Lys^{63}-linked ubiquitin (Lauwers, E., Jacob, C., and André, B. (2009) J. Cell Biol. **185,** 493–502).

REFERENCES

1. Dupré, S., Urban-Grimal, D., and Haguenauer-Tsapis, R. (2004) *Biochim. Biophys. Acta* **1695,** 89–111
2. Miranda, M., and Sorkin, A. (2007) *Mol. Interv.* **7,** 157–167
3. Horák, J. (2003) *Biochim. Biophys. Acta* **1614,** 139–155
4. Rotin, D., Staub, O., and Haguenauer-Tsapis, R. (2000) *J. Membr. Biol.* **176,** 1–17
5. Lin, C. H., MacGurn, J. A., Chu, T., Stefan, C. J., and Emr, S. D. (2008) *Cell* **135,** 714–725
6. Nikko, E., Sullivan, J. A., and Pelham, H. R. (2008) *EMBO Rep.* **9,** 1216–1221
7. Soetens, O., De Craene, J. O., and Andre, B. (2001) *J. Biol. Chem.* **276,** 43949–43957
8. Katzmann, D. J., Odorizzi, G., and Emr, S. D. (2002) *Nat. Rev. Mol. Cell Biol.* **3,** 893–905
9. Galan, J. M., and Haguenauer-Tsapis, R. (1997) *EMBO J.* **16,** 5847–5854
10. Springael, J. Y., Galan, J. M., Haguenauer-Tsapis, R., and André, B. (1999) *J. Cell Sci.* **112,** 1375–1383
11. Erpapazoglou, Z., Froissard, M., Nondier, I., Lesuisse, E., Haguenauer-Tsapis, R., and Belgareh-Touzé, N. (2008) *Traffic* **9,** 1372–1391
12. Kim, Y., Deng, Y., and Philpott, C. C. (2007) *Mol. Biol. Cell* **18,** 1790–1802
13. Stawiecka-Mirota, M., Pokrzywa, W., Morvan, J., Zoladek, T., Haguenauer-Tsapis, R., Urban-Grimal, D., and Morsomme, P. (2007) *Traffic* **8,** 1280–1296
14. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003) *Nat. Biotechnol.* **21,** 921–926
15. Casal, M., Paiva, S., Queirós, O., and Soares-Silva, I. (2008) *FEMS Microbiol. Rev.* **32,** 974–994

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2009/05/11/M109.008318.DC1.html

16. Casal, M., Paiva, S., Andrade, R. P., Gancedo, C., and Leão, C. (1999) *J. Bacteriol.* **181,** 2620–2623
17. Paiva, S., Devaux, F., Barbosa, S., Jacq, C., and Casal, M. (2004) *Yeast* **21,** 201–210
18. Cássio, F., Leão, C., and van Uden, N. (1987) *Appl. Environ. Microbiol.* **53,** 509–513
19. Casal, M., Cardoso, H., and Leão, C. (1996) *Microbiology* **142,** 1385–1390
20. Soares-Silva, I., Schuller, D., Andrade, R. P., Baltazar, F., Cássio, F., and Casal, M. (2003) *Biochem. J.* **376,** 781–787
21. Andrade, R. P., and Casal, M. (2001) *Fungal Genet. Biol.* **32,** 105–111
22. Andrade, R. P., Kötter, P., Entian, K. D., and Casal, M. (2005) *Biochem. Biophys. Res. Commun.* **332,** 254–262
23. Paiva, S., Kruckeberg, A. L., and Casal, M. (2002) *Biochem. J.* **363,** 737–744
24. Reinders, J., Wagner, K., Zahedi, R. P., Stojanovski, D., Eyrich, B., van der Laan, M., Rehling, P., Sickmann, A., Pfanner, N., and Meisinger, C. (2007) *Mol. Cell Proteomics* **6,** 1896–1906
25. Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20,** 1425
26. De Antoni, A., and Gallwitz, D. (2000) *Gene* **246,** 179–185
27. Galan, J. M., Moreau, V., Andre, B., Volland, C., and Haguenauer-Tsapis, R. (1996) *J. Biol. Chem.* **271,** 10946–10952
28. Robinson, L. C., Menold, M. M., Garrett, S., and Culbertson, M. R. (1993) *Mol. Cell. Biol.* **13,** 2870–2881
29. Thomas, B. J., and Rothstein, R. (1989) *Genetics* **123,** 725–738
30. Mumberg, D., Müller, R., and Funk, M. (1995) *Gene* **156,** 119–122
31. Wach, A., Brachat, A., Pöhlmann, R., and Philippsen, P. (1994) *Yeast* **10,** 1793–1808
32. Ansaldi, M., Lepelletier, M., and Méjean, V. (1996) *Anal. Biochem.* **234,** 110–111
33. Mumberg, D., Müller, R., and Funk, M. (1994) *Nucleic Acids Res.* **22,** 5767–5768
34. Volland, C., Urban-Grimal, D., Géraud, G., and Haguenauer-Tsapis, R. (1994) *J. Biol. Chem.* **269,** 9833–9841
35. Morvan, J., Froissard, M., Haguenauer-Tsapis, R., and Urban-Grimal, D. (2004) *Traffic* **5,** 383–392
36. Hicke, L., Zanolari, B., and Riezman, H. (1998) *J. Cell Biol.* **141,** 349–358
37. Marchal, C., Haguenauer-Tsapis, R., and Urban-Grimal, D. (1998) *Mol. Cell. Biol.* **18,** 314–321
38. Marchal, C., Haguenauer-Tsapis, R., and Urban-Grimal, D. (2000) *J. Biol. Chem.* **275,** 23608–23614
39. Hein, C., Springael, J. Y., Volland, C., Haguenauer-Tsapis, R., and André, B. (1995) *Mol. Microbiol.* **18,** 77–87
40. Spence, J., Sadis, S., Haas, A. L., and Finley, D. (1995) *Mol. Cell. Biol.* **15,** 1265–1273
41. Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D., and Peng, J. (2009) *Cell* **137,** 133–145
42. Blondel, M. O., Morvan, J., Dupré, S., Urban-Grimal, D., Haguenauer-Tsapis, R., and Volland, C. (2004) *Mol. Biol. Cell* **15,** 883–895
43. Katzmann, D. J., Babst, M., and Emr, S. D. (2001) *Cell* **106,** 145–155
44. Haguenauer-Tsapis, R., and André, B. (2004) *Top. Curr. Genet.* **9,** 273–323
45. Liu, J., Sitaram, A., and Burd, C. G. (2007) *Traffic* **8,** 1375–1384
46. Vancura, A., Sessler, A., Leichus, B., and Kuret, J. (1994) *J. Biol. Chem.* **269,** 19271–19278
47. Umebayashi, K., and Nakano, A. (2003) *J. Cell Biol.* **161,** 1117–1131
48. Catic, A., Collins, C., Church, G. M., and Ploegh, H. L. (2004) *Bioinformatics* **20,** 3302–3307
49. Bugnicourt, A., Froissard, M., Sereti, K., Ulrich, H. D., Haguenauer-Tsapis, R., and Galan, J. M. (2004) *Mol. Biol. Cell* **15,** 4203–4214
50. Rubio-Texeira, M., and Kaiser, C. A. (2006) *Mol. Biol. Cell* **17,** 3031–3050
51. Strochlic, T. I., Setty, T. G., Sitaram, A., and Burd, C. G. (2007) *J. Cell Biol.* **177,** 115–125
52. Kee, Y., Lyon, N., and Huibregtse, J. M. (2005) *EMBO J.* **24,** 2414–2424
53. Kee, Y., Muñoz, W., Lyon, N., and Huibregtse, J. M. (2006) *J. Biol. Chem.* **281,** 36724–36731
54. Dunn, R., Klos, D. A., Adler, A. S., and Hicke, L. (2004) *J. Cell Biol.* **165,** 135–144
55. Katzmann, D. J., Sarkar, S., Chu, T., Audhya, A., and Emr, S. D. (2004) *Mol. Biol. Cell* **15,** 468–480
56. Clague, M. J., and Urbé, S. (2006) *Trends Cell Biol.* **16,** 551–559
57. McCullough, J., Clague, M. J., and Urbé, S. (2004) *J. Cell Biol.* **166,** 487–492
58. Barriere, H., Nemes, C., Du, K., and Lukacs, G. L. (2007) *Mol. Biol. Cell* **18,** 3952–3965

CHAPTER VIII – Art4 arrestin-like protein mediates Rsp5 dependent ubiquitylation in glucose induced endocytosis of the Jen1 transporter in yeast

The work presented in this chapter is in preparation for submission:
Neide Vieira, Sébastien Léon, Jéssica Gomes, Carina Cunha, Rosine Haguenauer-Tsapis, Margarida Casal and Sandra Paiva (2010).

Abstract

The intracellular trafficking of plasma membrane proteins, such as receptors and transporters, in eukaryotic cells, is a highly regulated process. Ubiquitylation of cell surface transporters acts as a signal triggering their internalization, and subsequent degradation in the lysosome/vacuole, and, in yeast, this modification is mediated by the HECT ubiquitin ligase (E3) Rsp5p, usually in lysine residues of the target protein. Despite the prevailing role of Rsp5 in ubiquitylation of yeast plasma membrane proteins, there is no evidence for a direct interaction between the ligase and these cargoes. The majority of Rsp5 substrates do not carry proline rich sequences (PY elements) that are recognized directly by the tryptophan rich domains (WW) of Rsp5 and may depend on PY containing adaptor proteins for their ubiquitylation by Rsp5. Several yeast transporters have been shown to rely on arrestin-like adaptor proteins to undergo ubiquitylation. These PY motif-containing arrestin-like proteins are found in many species and were suggested to regulate protein trafficking. In this work we show that the arrestin like protein Art4 is crucial for the HECT-ubiquitin ligase Rsp5-dependent Jen1 ubiquitylation, required for its glucose-induced endocytosis. Moreover we have demonstrated that phosphorylation of Jen1-Ser_{606}, is also required for Jen1 endocytosis.

Introduction

In yeast, the downregulation of transporters requires their ubiquitylation, leading to their endocytosis and sorting to the multivesicular bodies (MVBs) pathway for posterior degradation in the vacuole (Katzmann *et al.*, 2002; Hicke and Dunn, 2003). The ubiquitylation of these cargoes is performed by the Rsp5 ubiquitin-ligase (E3) (Horak, 2003). Rsp5 possesses a C2 domain, involved with membrane association (Dunn *et al.*, 2004), three WW domains and a catalytic C-terminal HECT domain. The three WW domains can be related to the recognition of PPXY motifs (or related proline-containing sequences), the PY elements. Some cargoes present those endogenous PY motifs and bind directly to Rsp5, like Sna3 (Stawiecka-Mirota *et al.*, 2007). However, most Rsp5 plasma membrane endocytic cargoes lack these sequences and seem to require the presence of adaptor proteins (Nikko *et al.*, 2008; Leon and Haguenauer-Tsapis, 2009). Phosphorylation also plays a role in this process, and it is required for the internalization of several transporters, either by changing the transporter conformation,

facilitating the direct access to the Lys residues, or by stimulating the direct interaction of Rsp5 with the endocytic cargo and the adaptor protein (Belgareh-Touze *et al.*, 2008; Paiva *et al.*, 2009).

Several adaptor proteins involved in endocytic internalization and in the sorting at MVBs of a broad range of substrates have, recently, been characterized. There are indications that not only the adaptors themselves but also the specific combination between them dictate the substrate specific ubiquitylation by Rsp5. For instance, downregulation of the metal transporter Smf1, directly from the Golgi to the vacuole (VPS pathway), requires the action of two sets of adaptor proteins, that facilitate the access of Rsp5 to Smf1: Bsd2, a PY-motif containing protein, and two other adaptors without PY motifs, Tre1/Tre2 (Hettema *et al.*, 2004). Nevertheless, these adaptors are not required for the endocytosis of Smf1 from the plasma membrane (Nikko *et al.*, 2008). Moreover, Bsd2 has also been shown to facilitate ubiquitylation of other targets, without the help of the Tre1/Tre2 adaptors (Hettema *et al.*, 2004). Ear1/Ssh4 are also PY-motif containing adaptors that contribute to the sorting of MVB cargoes, like the amino acid permease Gap1, the uracil transporter Fur4, the iron/siderophore transporter Sit1 and the vacuolar polyphosphatase Phm5, but not Smf1(Leon *et al.*, 2008). However, a mutant deleted in these pair of adaptors was able to efficiently internalize Fur4, from the plasma membrane, but presented an impaired ability to correctly sort the transporter to the MVB pathway. This observation reinforces the requirement for consecutive Rsp5-dependent ubiquitylation steps, at the plasma membrane and at the MVBs, and suggests that these processes might involve different adaptor proteins (Leon and Haguenauer-Tsapis, 2009).

Recently, homology searches identified an arrestin like family, present in several organisms, including yeast and mammals (Alvarez, 2008). These ARTs (arrestin-related-trafficking-adaptors) have sequences quite distantly related to the β-arrestin family, which is composed of soluble proteins that mediate the downregulation of ligand-activated G-protein coupled receptors, in animal cells (Lefkowitz *et al.*, 2006; Marchese *et al.*, 2008). β-arrestins act by recognizing conformational features of the receptors and phosphorylated residues, and by recruiting clathrin and other components of the endocytic machinery, or even specific ubiquitin-ligases that will modify the arrestin and the receptor (Lefkowitz *et al.*, 2006; Marchese *et al.*, 2008; Shenoy *et al.*, 2008). The ARTs seem to conserve the characteristic arrestin folding and typically have

one or more PY-motif at the C-terminal, unlike the β-arrestins (Alvarez, 2008; Lin *et al.*, 2008; Nikko *et al.*, 2008), indicating the probable interaction with HECT domain ubiquitin ligases.

Nine ARTs are encoded within the *S. cerevisiae* genome: Art1 to Art9, although only eight have typical arrestin sequence signatures: Art2/Ecm21, Art3/Aly2, Art4/Rod1, Art5/Ygr068c, Art6/Aly1, Art7/Rog3, Art8/Csr2 and Art9/Rim8. The Art9/Ldb19 is significantly more distantly related, but it is still considered an arrestin family member. ARTs have been shown to mediate the ubiquitylation and endocytosis of several transporters, in response to stress or to the presence of their substrates, acting as E3 ligase adaptors that recruit Rsp5, facilitating the ubiquitylation of the specific cargo and of the ART protein itself (Lin *et al.*, 2008; Nikko *et al.*, 2008). The involvement of distinct ARTs in the downregulation of the metal transporter Smf1 (after prior phosphorylation), of the amino acid transporters Can1, Mup1, Lyp1, Tat2, of the inositol transporter Itr1, of the uracil transporter Fur4 and of the hexose transporter Hxt6 has been experimentally demonstrated (Lin *et al.*, 2008; Nikko *et al.*, 2008; Nikko and Pelham, 2009), and seems to depend on the type of stimuli that triggers the endocytosis of the specific cargo. Stress-induced endocytosis, upon cyclohexamide treatment, seems to require one or both of the related Art2/Art8 pair (Nikko and Pelham, 2009). The arrestin Art1 is likely to be related to amino acid transporters downregulation, and somehow recognize a specific conformational state, since all the tested amino acid transporters require this arrestin for substrate-induced endocytosis. Additionally, Fur4 and Tat2 use the arrestin Art2 for both stress and substrate-induced endocytosis, and require the action of the Bul proteins and of other adaptors (Nikko and Pelham, 2009). These results indicate that the recognition of a wide amount of endocytic substrates by Rsp5 is dependent on the individual or collective action of several adaptor proteins, and that each cargo requires a specific combination of these proteins to be correctly sorted for degradation in the vacuole, by endocytosis or by the VPS pathway.

Previously, we have shown that casein kinase 1-dependent phosphorylation and HECT-ubiquitin ligase Rsp5-dependent ubiquitylation of lysine 338 are required for glucose-induced endocytosis of Jen1 (Paiva *et al.*, 2009). Here we show that the arrestin like protein, Art4, is involved in the Rsp5-dependent ubiquitylation of Jen1.

Additionally, we show that S606 of Jen1 is required for its efficient downregulation, upon glucose treatment.

Material and Methods

Media and Culture conditions

Growth was carried out at 30ºC, with 200 rpm agitation, either on synthetic minimal medium YNB: 0.67% (w/v) yeast nitrogen base (Difco), supplemented with appropriate aminoacids to fulfill the auxotrophic requirements; or on YP rich medium: 1% (w/v) yeast extract and 1% (w/v) peptone, supplemented either with 2% (w/v) D-glucose or 0.5% (v/v) lactic acid pH 5.0. Cell cultures were maintained on solid YPD media with 2% (w/v) agar. Carbon sources included 2% (w/v) glucose, 2% (w/v) raffinose, 2% (w/v) galactose or 0.5% (v/v) lactic acid pH 5.0. Cells were always harvested at mid exponential phase. For growth under repression conditions, cells encoding *JEN1*, under the control of its own promoter were grown on 2% (w/v) glucose. For derepression conditions, glucose-grown cells were harvested, washed twice, with ice-cold deionised water, and resuspended in minimum YNB media, supplemented with 0.5% (v/v) lactic acid, pH 5.0. Medium containing raffinose was used to promote growth of cells encoding *JEN1* from the GAL promoter, under non-induction conditions. To induce *JEN1* expression, 2% (w/v) galactose was added to raffinose-grown cells.

Strains construction

S. cerevisiae strains used in this work are listed in table 1. A collection of *S. cerevisiae* BY strains disrupted individually in the nine arrestin-like coding genes was purchased from EUROSCARF. *art4* mutant strain, carrying an *art4::kanMX4* locus was transformed with the hygromycin resistance gene *HphMX4* resulting in a marker switch producing the *art4::HphMx4 locus* (strain SP8) (Goldstein and McCusker, 1999). The *S. cerevisiae* strain BLC 491-U2 (Table 1), was used to amplify the genetic chimaera, *JEN1::GFP::KANr,* using the primers W303-1A forward and W303.1A reverse (Table 2). The *art4::HphMx4* strain was subsequently transformed with the 2.8 kB *JEN1::GFP::KANr* PCR product, resulting in strain SP9. Additionally, the *S. cerevisiae* BY4741 *end3* strain was used to amplify a 1.8 Kb *end3::KanMX4* cassette, using the primers A END3 and D END3 (Table 2). This fragment was transformed in the *art4::HphMx4* strain, originating an *end3art4* strain (SP10). Transformed cells were

grown in YPD media for 4 h and spread on YPD plates, containing 200 mg/L of Geneticin (G418 from Invitrogen) and 300 mg/L of Hygromycin (Hygromycin B from Invitrogen). The obtained transformants were confirmed, by analytical PCR with primers: A1/K2 for the *art4* mutant strain harboring Jen1-GFP; and A END3/K2 or K3/D END3 for the double *end3art4* mutant strain (Table 3) (Kruckeberg *et al.*, 1999). Cloning and PCR amplification analyses were performed, as described previously (Sambrook, 1989). Subsequently, the strain SP10 was transformed with the plasmid *pJEN1-GFP-6His*, originating strain SP12. The pSP2 plasmid (*pJEN1-JEN1-GFP*), (Paiva *et al.*, 2009) expresses a C- terminal Jen1-GFP fusion protein, under the control of the native *JEN1* promoter. Mutations that replace serines by alanines were introduced in the plasmid pSP2 through site directed mutagenesis, as previously described (Ansaldi *et al.*, 1996), using the primers listed in table 3. The resulting plasmids pSP6 to pSP10 express the obtained mutant alleles of *JEN1* (Tab. 2). All the mutations were confirmed by sequencing. The strain W303-1A *jen1* was then transformed with the resultant plasmids pSP6-pSP10, originating strains SP13 to SP17.

Table 1 – *S. cerevisiae* strains used in this work.

Strains	Genotype	Reference
Art1	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art1:: KanMX4*	EUROSCARF
Art2	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art2:: KanMX4*	EUROSCARF
Art3	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art3:: KanMX4*	EUROSCARF
Art4	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art4:: KanMX4*	EUROSCARF
Art5	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art5:: KanMX4*	EUROSCARF
Art6	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art6:: KanMX4*	EUROSCARF
Art7	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art7:: KanMX4*	EUROSCARF
Art8	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art8:: KanMX4*	EUROSCARF
Art9	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art9:: KanMX4*	EUROSCARF
SP8	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 art4::HphMx4*	This work
BLC 491-U2	*MAT*a *ura3-52 JEN1::GFP::KAN*^{r}	(Paiva *et al.*, 2002)
SP9	SP8,*JEN1-GFP-KanMX4*	This work
BY4741*end3*	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 end3::KanMX4*	EUROSCARF
SP10	SP8, *end3::KanMX4*	This work
SP11	*end3::KanMX4, pGAL-JEN1-GFP*-6_*HIS*	(Paiva *et al.*, 2009)
SP12	SP10, *pGAL-JEN1-GFP*-6_*HIS*	This work
W303-1A *jen1*	*MAT*a *ura3-52 trp1Δ2 leu2-3-112 his3-11 ade2-1 can1-100 jen1::KanMX4*	(Paiva *et al.*, 2004)
SP13	W303-1A *jen1*, *pJEN1-S4A-GFP*	This work
SP14	W303-1A *jen1*, *pJEN1-S11A-GFP*	This work
SP15	W303-1A *jen1*, *pJEN1-S81A-GFP*	This work
SP16	W303-1A *jen1*, *pJEN1-S584-GFP*	This work
SP17	W303-1A *jen1*, *pJEN1-S606A-GFP*	This work

Table 2 – Plasmids used in this study.

Plasmids	Genotype	Source or Reference
pJEN-GFP-6HIS	*CEN, URA3, pGAL-JEN1-GFP-6_HIS*	(Paiva *et al.*, 2009)
pSP2	*CEN, URA3, pJEN1-JEN1-GFP*	(Paiva *et al.*, 2009)
pSP6	*CEN, URA3, pJEN1-JEN1-S4A-GFP*	This work
pSP7	*CEN, URA3, pJEN1-JEN1-S11A-GFP*	This work
pSP8	*CEN, URA3, pJEN1-JEN1-S81A-GFP*	This work
pSP9	*CEN, URA3, pJEN1-JEN1-S584A-GFP*	This work
pSP10	*CEN, URA3, pJEN1-JEN1-S606A-GFP*	This work

Table 3 – Primers used in this work.

Primers	Sequences
W303-1A forward	GATTTGTCCTCTCCTGTTATGAAG
W303-1A reverse	ATCTTGCTAGTGTTAACGGCTGTTA
A END3	TAACATCAAGTTCCTTGAACAAACA
D END3	CATCATCAAGATTTAACACAAGCAC
A1	GGCCTATCCAAGGATGCTGTC
GFP_rev	AACATCACCATCTAATTCAAC
K2	CGA TAG ATT GTC GCA CCT G
K3	CCA TCC TAT GGA ACT CCC TC
S4 forward	GAA AAT ATG TCG TCG GCA ATT ACA AAA ATA TC
S4 reverse	GAT ATT TTC TCA TCT GTA ATT GCC GAC ATA ATA TTT TC
S11 forward	CAA TTA CAG ATG AGA AAA TAG CTG AAC AGC AAC
S11 reverse	GTT GCT GTT CAC CAG CTA TTT TCT CAT CTG TAA TTG
S81 forward	CTG GAA AGA GTT TAT GCC CAG GAT CAA GGT GTA G
S81 reverse	CTA CAC CTT GAT CCT GGG CAT AAA CTC TTT CCA G
S584 forward	CAT TGT TGA ACA AAA GAC GGA ATC TGC TTC AGT GAA GAT
S584 reverse	TTC CGT CTT TTG TTC AAC AAT GTC ACT AAT CGC AAG ACC ATC GGC
S606 forward	AAC GGT CTC AAT ATG CTC CTC ATA TGT CTT TGC GAC GTT CGA ATC
S606 reverse	TGA GGA GCA TAT TGA GAC CGT TTA ATC ACT TTT CAT TGC

Transport assays

Transport assays, using labelled D,L-[U-^{14}C] Lactic acid (sodium salt; CFB97-Amersham Biosciences; 4000 dpm/nmol, at pH 5.0), were carried out as described previously (Paiva *et al.*, 2009).

Microscopy

S. cerevisiae cells were grown under derepression conditions and promptly examined, without fixation, with a LEICA Microsystem DM-5000B epifluorescence microscope with appropriated filter settings. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

Total membrane extracts and Immunoblotting

Strains were grown in the complex medium YP Lactic acid 0.5% (v/v), pH 5.0 till an OD_{640nm} of approximately 0.5. The cells were then subjected to a pulse of 2% (w/v) glucose, and samples were collected, over time. Total protein extracts were prepared using the previously described NaOH-TCA lysis technique (Volland *et al.*, 1994). Lysates were prepared as previously described (Paiva *et al.*, 2009). Proteins were resuspended in sample buffer, heated for 15 min, at 37ºC, and subsequently resolved by SDS-PAGE in 10% acrylamide gels, using Tricine buffer, and transferred to PVDF membranes. The membranes were then probed with monoclonal antibodies: anti-GFP antiserum (Roche diagnostics), anti-Ub (clone P4D1)-HRP conjugate (Santa Cruz Biotechnology), anti-PGK (Molecular Probes), for protein loading control. Horseradish peroxidase-conjugated anti-mouse immunoglobulin G was used as the secondary antibody (Sigma, St Louis, MO USA) and detected by enhanced chemiluminescence (ECL).

His6-tagged Jen1-GFP Purification—His6-tagged Jen1-GFP

Purification experiments were performed essentially as previously described (Morvan *et al.*, 2004), except that Ni^{2+}-NTA resin was used in batch rather than in column. 6-7x 10^8 cells were harvested, and lysate was prepared. The lysate was subjected to centrifugation at 13,000 *g* for 30 min to generate the supernatant and pellet fractions. The pellet was resuspended in 300 µl of buffer A (lysis buffer supplemented with 5 mM imidazole, 0.1% (w/v) SDS, and 1% (v/v) Triton X-100). The suspension was incubated on ice, for 30 min, and then diluted, by adding 300 µl of buffer B (lysis buffer supplemented with 5 mM imidazole and 1% (v/v) Triton X-100), and centrifuged for 10 min at 13,000 *g*, to remove the remaining insoluble material. The supernatant was added to 200 µl of Ni^{2+}-NTA Superflow resin (Qiagen Inc., Hilden, Germany) and incubated, with mixing, for 1 h at 4°C. The unbound fraction was collected, and the resin was washed three times with 200 µl of buffer B. Jen1-GFP-6His was eluted three times with 200 µl of elution buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 200 mM imidazole). Aliquots of different fractions were prepared for Western blot analysis.

Results

Endocytosis of Jen1 is mediated by Art4

To assess the role of the members of the arrestin-like protein family (Lin *et al.*, 2008; Nikko *et al.*, 2008) in glucose-induced Jen1 endocytosis, a collection of strains disrupted individually in the nine known yeast arrestin-like proteins was used. The ability to transport labelled lactic acid was then monitored after glucose addition to lactic acid-induced wild-type and mutant cells. The addition of glucose caused a sharp decrease in lactic acid uptake, in the wild-type cells (Fig. 1A). Nevertheless, the decrease in lactic acid uptake was far less pronounced in the *art4* mutant strain. This relative protection against the loss of transport activity indicated that the disruption of *ART4* stabilized the Jen1 transporter at the plasma membrane (Fig. 1A). Extracts from cells collected at different time points, upon glucose addition, were then analyzed by immunobloting against GFP (Fig. 1B). The transporter immunoreactivity declined in parallel with the drop in the transport activity, in the wild-type cells, whereas a significant protection against degradation was observed in the *art4* mutant cells (Fig. 1B). Moreover, the subcellular localization of Jen1-GFP was followed by fluorescence microscopy before and after the addition of glucose to lactic acid-induced wild-type and *art4* mutant cells. Jen1-GFP was correctly internalized and sorted to the vacuolar lumen, over time, in the wild-type cells. In contrast, Jen1-GFP stabilized at the plasma membrane in the *art4* mutant cells (Fig. 1C). Therefore, glucose-induced downregulation of *S. cerevisiae* Jen1 is dependent on the arrestin like Art4 protein.

Art4 dependent Jen1 ubiquitylation

Adaptor proteins are believed to facilitate the ubiquitylation of target proteins by binding simultaneously to the endocytic cargoes and to the E3 ubiquitin ligases, and thus, enable the access of ubiquitin ligases to the endocytic cargo (Shearwin-Whyatt *et al.*, 2006). In this manner, it is possible to predict that Art4 binds to Jen1 upon glucose addition, and that through its PY motifs interacts with Rsp5, stimulating Jen1 ubiquitylation. We set up to determine whether Rsp5-dependent Jen1 ubiquitylation, triggered by glucose, requires Art4. A mutant *end3,* impaired in the internalization step of endocytosis, was used to construct a double *art4end3* mutant strain harboring the p*JEN1-GFP-6HIS* plasmid (Paiva *et al.*, 2009). *end3* and *art4end3* cells were induced in galactose for the production of Jen1-GFP-6His, and then subjected to a 10 min glucose

pulse, to trigger endocytosis of Jen1. Immunoblots of cell extracts revealed with anti-GFP antibody are represented in figure 2. Two to three more slowly migrating bands of immunoreactive material are visible above the main Jen1-GFP signal in *end3* cell extracts (Fig. 2), as previously reported (Paiva *et al.*, 2009). These bands are hardly visible at time zero, before glucose addition, and far less clear in the *art4end3* mutant cell extracts (Fig. 2). These results suggest a putative impairment or delay of glucose-induced Jen1 ubiquitylation in the double *art4end3* mutant strain.

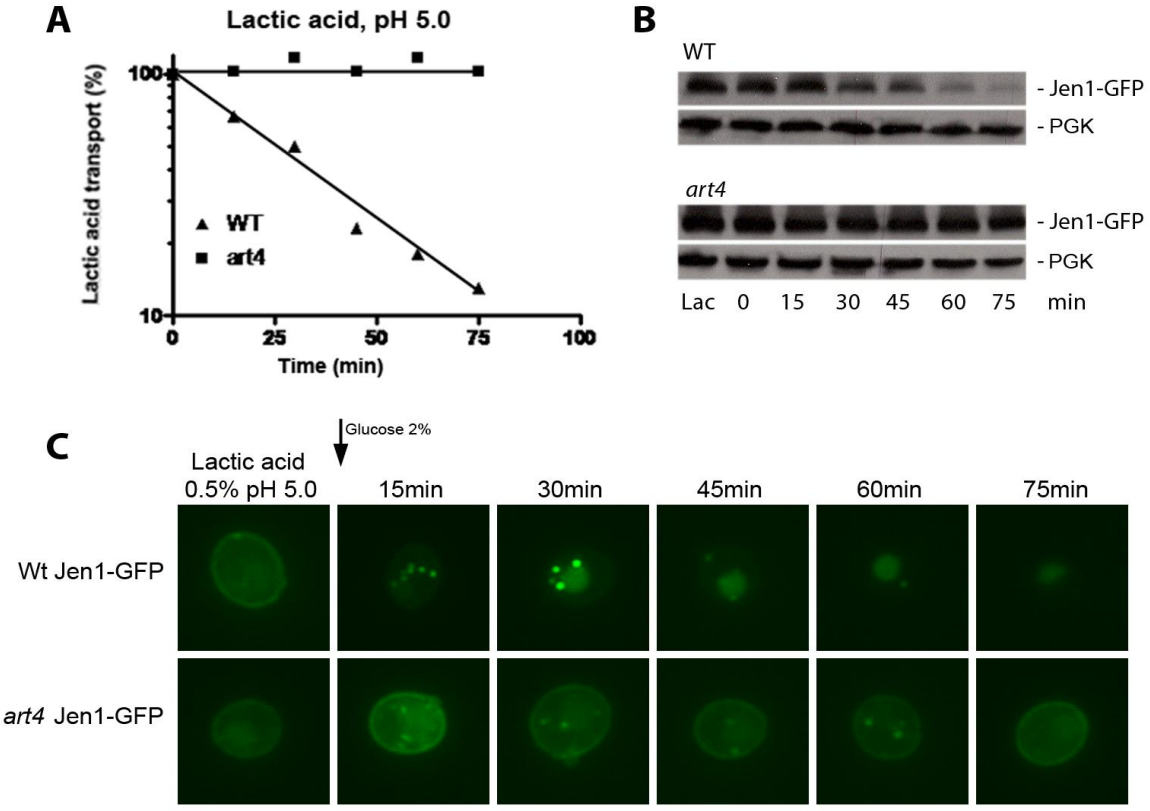

Figure 1 – Jen1 degradation requires Art4. *S. cerevisiae* wt and *art4* mutant strains harboring Jen1-GFP chimaera were induced for the production of Jen1-GFP in lactic acid, for 4 h at 30ºC. A – Lactic acid uptake was measured at the times indicated after the addition of 2% of glucose. The results are expressed as percentages of initial activities.▲, Jen1-GFP; ■, *art4* Jen1-GFP. B – Protein extracts collected at the same time points were analysed by Western immunoblotting with an anti-GFP antibody. The blots were reprobed with an anti-phosphoglycerol kinase (PGK) antibody for loading controls. C – Subcellular localization of Jen1-GFP in living cells at the time points indicated after the addition of 2% glucose. Wt, wild-type; *art4*, mutant strain.

A biochemical characterization of Jen1-GFP-6His more slowly migrating bands was further attempted, in an effort to confirm our previous findings. Again, aliquots of the *end3* and *art4end3* cells were collected after glucose addition to galactose-induced cells, and the respective cell extracts subjected to cell fractionation. Membrane enriched fractions were solubilized and incubated with Ni^{2+}-NTA beads. Aliquots of the bound

material were resolved by electrophoresis and immunobloted against anti-ubiquitin and anti-GFP antibodies. Immunobloting with anti-GFP antibody proved that Jen1-GFP-6His was specifically retained on nickel beads (Fig. 3). The slower migrating bands were detected with anti-ubiquitin antibodies, in the *end3* cells, indicating that they correspond to ubiquitin conjugated to Jen1-GFP-6His. However, the slowly migrating bands detected with anti-ubiquitin antibodies were far less abundant in the Ni^{2+}-NTA retained fractions of *art4end3* cells, showing that Art4 is involved in the ubiquitylation of the transporter. Overall, these results suggest that the arrestin-like adaptor, Art4 mediates Rsp5-dependent Jen1 ubiquitylation, upon glucose addition.

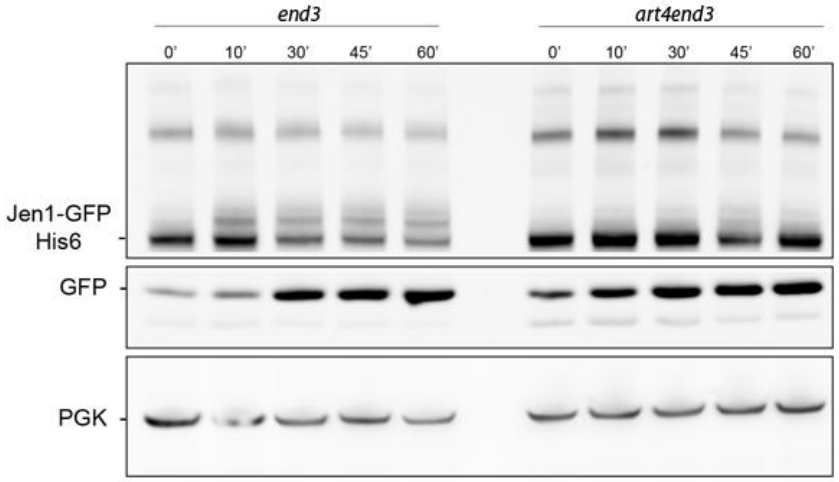

Figure 2 –Jen1-GFP-6His immunodetection in *end3* and *art4end3* cells. *end3* and *art4end3* cells previously transformed with *pJEN1-GFP-HIS6* plasmid were induced in galactose for 2h at 30ºC. Protein extracts were prepared from induced cells before and after glucose addition, over time, and analysed by Western immunoblotting with an anti-GFP antibody.

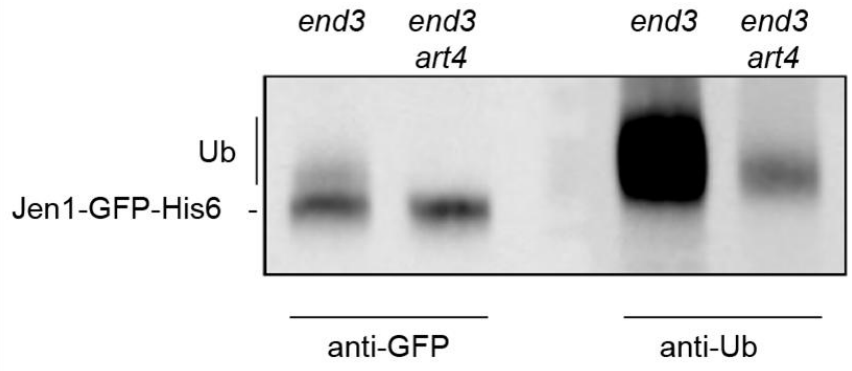

Figure 3 – Ubiquitylation of Jen1-GFP-6His in *end3* and *art4end3* cells. Lysates of galactose-induced *end3* and *art4end3* cells incubated, for 10 min, with glucose were fractioned, as previously described. Solubilized membranes were incubated with Ni^{2+}-NTA beads. The bound fraction corresponding to His6-tagged ubiquitylated proteins were eluted by 200 mM Imidazole containing buffer. Aliquots of bound fractions were resolved by electrophoresis and analyzed by Western immunoblotting both with anti-GFP and anti-Ub antibodies.

Jen1-GFP phosphorylation upon glucose addition

Phosphorylation of Jen1, Ste2 and Fur4 transporters, at several Ser residues, directly or indirectly by the casein kinase 1 isoforms Yck1/Yck2, was demonstrated to be essential for the ubiquitylation and endocytosis of these transporters (Hicke *et al.*, 1998; Marchal *et al.*, 1998; Marchal *et al.*, 2000; Paiva *et al.*, 2009). A phosphoproteome analysis by mass spectrometry revealed that Jen1 harbors several cytoplasmic serines that could constitute putative phosphorylation targets for Casein Kinase 1: S4, 11, 81, 584, 606 (Reinders *et al.*, 2007). In an effort to determine if phosphorylation of these serines was involved in Jen1 endocytosis, mutations that substitute *JEN1* serine codons by conservative but non-phosphorylated alanine residues were introduced in the plasmid pSP2 (*pJEN1-JEN1-GFP*), using site-directed mutagenesis (as described in (Paiva *et al.*, 2009)). The pSP2 plasmid expresses a C-terminal Jen1-GFP fusion protein, under the control of the native *JEN1* promoter. The plasmids expressing the resulting mutant alleles of *JEN1* were sequenced and transformed into a *S. cerevisiae jen1* mutant strain. The cellular trafficking and protein stability of the correspondent variant proteins were determined (Fig. 4). All the variant proteins were expressed in lactic acid-induced cells, and endocytosis was triggered by glucose addition. After derepression in lactic acid, at time zero, fluorescence was mainly detected at the plasma membrane, in the wild-type and S606A cells (Fig. 4C), as well as in all the other cell types (data not shown). The results obtained showed that all the variant proteins were correctly expressed and delivered to the cell surface. Upon a glucose pulse to wild-type lactic acid-induced cells, Jen1-GFP fluorescence signal progressively disappeared from the plasma membrane and intracellular spots were visualized, probably corresponding to early and late endosomes. Additionally, some staining of the vacuole was observed (Fig. 4C). These results were similar to the ones observed for cells harboring S4A, S11A, S81A and S584A Jen1-GFP variant proteins, (data not shown). In contrast, the internalization of Jen1-GFP in the strain harboring the S606A variant transporter seemed slightly delayed (Fig. 4C). To follow the downregulation of the transporter variants more accurately, their stability was assessed at the plasma membrane by measuring Jen1 transport activity, after the glucose addition to lactic acid-induced cells. Glucose treatment triggered a drop in lactic acid uptake in the wild-type cells (Fig. 4A). However, the decrease in the transport activity was less significant in cells producing the S606A variant transporter. Additionally, cell extracts

from cells withdrawn at various times after glucose addition were analysed by immunoblotting (Fig. 4B). The immunoreactivity of the wild-type species declined in parallel to the drop in transport activity. Nevertheless, S606A variant transporters were more slowly degraded than the wild-type strain. The relative protection against loss of transport activity (Fig. 4A) and the relative stabilization of S606A variant proteins (Fig. 4B) suggest that serine 606, is probably, a target for Casein kinase 1 Jen1 phosphorylation, at the plasma membrane.

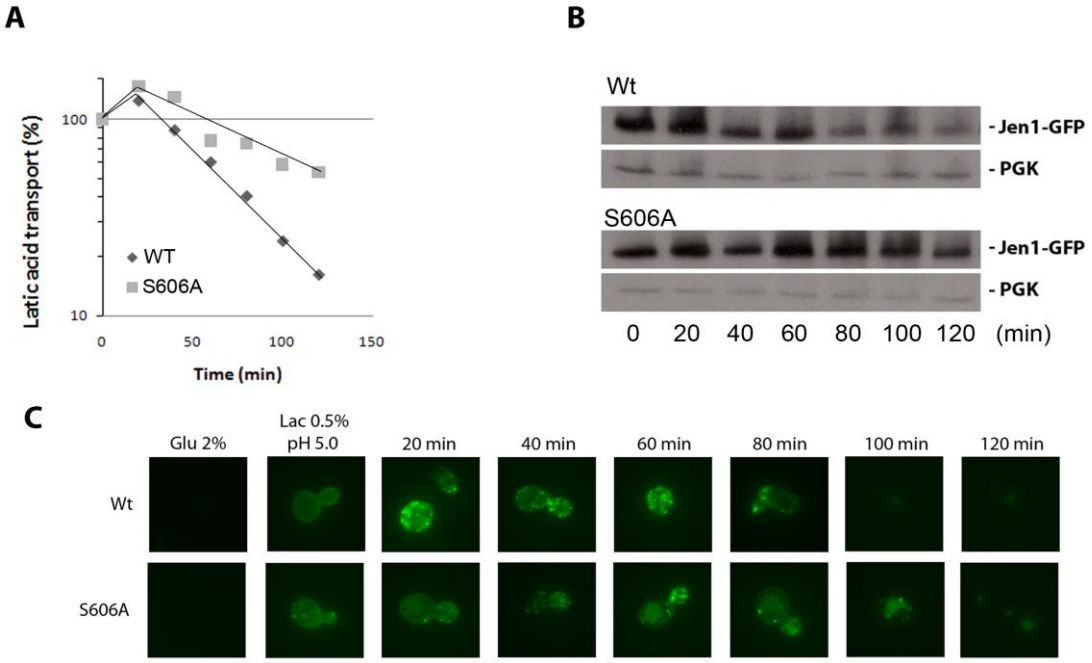

Figure 4 – Impact of Ser to Ala mutations on Jen1-GFP stability. W303-1A *jen1* cells transformed with pSP2 (*JEN1-GFP*) and pSP10 (*JEN1-S606A-GFP*) were induced for the production of Jen1-GFP in lactic acid, during 4h at 30ºC. A- Lactic acid uptake was followed over time, upon glucose addition. The results correspond to percentages of initial uptake activities. B – Protein extracts, collected at the same time points, were analysed by Western immunoblotting with anti-GFP antibody. The blots were reprobed with anti-PGK antibody for loading controls. C – Microscopy images of Jen1-GFP in living cells over time upon glucose treatment of lactic acid-induced cells.

Discussion

The focus of this work was to determine the adaptors involved in the endocytosis of Jen1 from the plasma membrane, after glucose addition to lactic acid induced cells.

Recent data evidenced the significance of the arrestin-like protein family in promoting the specificity underlying Rsp5 dependent ubiquitylation of several transporters.

In the present work we demonstrated that the arrestin-like protein, Art4, previously associated with the high affinity glucose transporter (Hxt6) downregulation,

is involved in the endocytosis of Jen1 from the plasma membrane, also triggered by glucose addition. These results suggest that possibly, Art4 activity and/or expression increases upon glucose addition, to lactic acid induced cells, and that this adaptor protein is recruited to the plasma membrane, in these conditions. Subcellular localization studies were performed in a Art4-GFP strain (where Jen1-mediated lactic acid transport is not affected; data not shown), in order to test this hypothesis. However, the level of Art4-GFP induction was extremely poor in lactic acid induced cells, and thus, the results were inconclusive (data not shown). It is possible that glucose-triggered phosphorylation of Jen1 can result in conformational modifications that will stimulate the interaction between Rsp5 and Art4, or that the glucose signal also triggers Art4 phosphorylation, allowing its recruitment to the plasma membrane. This still remains to be determined. Nevertheless, the disruption of Art4 impaired the ubiquitylation of Jen1, at the cell surface, upon a pulse of glucose to induced cells, supporting the role of Art4 in the downregulation of a membrane transporter. Further experiments will be performed to determine whether Art4 recruitment involves not only its phosphorylation but also its ubiquitylation.

Our group has recently demonstrated that phosphorylation of Jen1, directly or indirectly by the yeast Casein kinase 1, is required prior to its internalization, in the glucose induced endocytosis process {Paiva, 2009 #143}. Jen1 harbors several cytoplasmic serines that could constitute putative phosphorylation targets for Casein Kinase 1 {Reinders, 2007 #669}. However, serine 4, 11 and serine 81 are not real concensus sites for Yck1: MSSS4; DEKIS11; ERVYS81. Usually, sites for Yck1 are acidic prior the serine to be phosphorylated. Typically, the minus 2 and/or minus 3 aminoacid prior the serine must be acidic (ex. EXXS). That renders S584 and S606 (ADGLS584; DSNVS606) more likely to be the sites for phosphorylation by YcK1/Yck2 rather than the other potential serines. In the present report we demonstrated that at least Jen1-Ser^{606} is a target for phosphorylation, upon glucose addition, possibly by Casein kinase 1. Nevertheless, other serines can constitute additional Casein kinase 1 targets for phosphorylation, namely Jen1-Ser^{81} (data not shown). Moreover, Kinasephos, (KinasePhos, http://kinasephos.mbc.nctu.edu.tw/) envisages that different potential catalytical kinases, besides Yck1/Yck2 phosphorylate Jen1 at specific sites, and thus, other kinases can be responsible for Jen1 phosphorylation upon glucose treatment.

Furthermore, non-arrestin like adaptor proteins involved in the endocytic internalization and in the sorting at MVBs of a broad range of substrates, have recently been characterized. Transporters seem to require different adaptors proteins to undergo endocytosis from the cell surface, Rsp5-dependent ubiquitylation in the Golgi (shortly after synthesis) and sorting into the MVBs {Nikko, 2008 #165}. Hence, Jen1-GFP endocytosis should be followed in parallel in wild type cells, and in cells deleted for the various PY-containing adaptors identified, so far, regarding their role in MVB sorting (Bsd2, Tre1/2, Ear1/Ssh4) {Hettema, 2004 #226}{Leon, 2008 #227}. This might unravel other putative adaptors involved in glucose-induced Jen1 sorting at MVBs. Moreover, whether ubiquitylation occurring at plasma membrane is sufficient for subsequent MVB sorting of Jen1, or whether the internalized transporter undergoes deubiquitylation, and new ubiquitylation for MVB sorting, is not yet determined. Art4-Rsp5-dependent Jen1 ubiquitylation may or not be sufficient for Jen1 MVB sorting. If not, additional rounds of ubiquitylation can occur in internal compartments, after potential deubiquitylation occurring in the route from plasma membrane to endosomes. Therefore, the role of the deubiquitylating enzyme Ubp2, which has been shown to be associated with Rsp5, and to be involved in potential deubiquitylation events, should be assessed, since it might affect Jen1 trafficking {Kee, 2005 #690}{Kee, 2006 #691}.

References

Alvarez, C. E. 2008. On the origins of arrestin and rhodopsin. *BMC Evol Biol.* **8**: 222.

André, B., and Haguenauer-Tsapis, R. 2004. Membrane trafficking of yeast transporters: mechanisms and physiological control of downregulation. *Top. Curr. Genet.* **9**: 273–323.

Ansaldi, M., Lepelletier, M. and Mejean, V. 1996. Site-specific mutagenesis by using an accurate recombinant polymerase chain reaction method. *Anal Biochem.* **234**: 110-111.

Belgareh-Touze, N., Leon, S., Erpapazoglou, Z., Stawiecka-Mirota, M., Urban-Grimal, D. and Haguenauer-Tsapis, R. 2008. Versatile role of the yeast ubiquitin ligase Rsp5p in intracellular trafficking. *Biochem Soc Trans.* **36**: 791-796.

Dunn, R., Klos, D. A., Adler, A. S. and Hicke, L. 2004. The C2 domain of the Rsp5 ubiquitin ligase binds membrane phosphoinositides and directs ubiquitination of endosomal cargo. *J Cell Biol.* **165**: 135-144.

Goldstein, A. L. and McCusker, J. H. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast.* **15**: 1541-1553.

Hettema, E. H., Valdez-Taubas, J. and Pelham, H. R. 2004. Bsd2 binds the ubiquitin ligase Rsp5 and mediates the ubiquitination of transmembrane proteins. *EMBO J.* **23**: 1279-1288.

Hicke, L. and Dunn, R. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol.* **19**: 141-172.

Hicke, L., Zanolari, B. and Riezman, H. 1998. Cytoplasmic tail phosphorylation of the alpha-factor receptor is required for its ubiquitination and internalization. *J Cell Biol.* **141**: 349-358.

Horak, J. 2003. The role of ubiquitin in down-regulation and intracellular sorting of membrane proteins: insights from yeast. *Biochim Biophys Acta.* **1614**: 139-155.

Katzmann, D. J., Odorizzi, G. and Emr, S. D. 2002. Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol.* **3**: 893-905.

Kee, Y., Lyon, N. and Huibregtse, J. M. 2005. The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. *EMBO J.* **24**: 2414-2424.

Kee, Y., Munoz, W., Lyon, N. and Huibregtse, J. M. 2006. The deubiquitinating enzyme Ubp2 modulates Rsp5-dependent Lys63-linked polyubiquitin conjugates in *Saccharomyces cerevisiae*. *J Biol Chem.* **281**: 36724-36731.

Kruckeberg, A. L., Ye, L., Berden, J. A. and van Dam, K. 1999. Functional expression, quantification and cellular localization of the Hxt2 hexose transporter of *Saccharomyces cerevisiae* tagged with the green fluorescent protein. *Biochem J.* **339**: 299-307.

Lefkowitz, R. J., Rajagopal, K. and Whalen, E. J. 2006. New roles for beta-arrestins in cell signaling: not just for seven-transmembrane receptors. *Mol Cell.* **24**: 643-652.

Leon, S., Erpapazoglou, Z. and Haguenauer-Tsapis, R. 2008. Ear1p and Ssh4p are new adaptors of the ubiquitin ligase Rsp5p for cargo ubiquitylation and sorting at multivesicular bodies. *Mol Biol Cell.* **19**: 2379-2388.

Leon, S. and Haguenauer-Tsapis, R. 2009. Ubiquitin ligase adaptors: regulators of ubiquitylation and endocytosis of plasma membrane proteins. *Exp Cell Res.* **315**: 1574-1583.

Lin, C. H., MacGurn, J. A., Chu, T., Stefan, C. J. and Emr, S. D. 2008. Arrestin-related ubiquitin-ligase adaptors regulate endocytosis and protein turnover at the cell surface. *Cell.* **135**: 714-725.

Liu, J., Sitaram, A. and Burd, C. G. 2007. Regulation of copper-dependent endocytosis and vacuolar degradation of the yeast copper transporter, Ctr1p, by the Rsp5 ubiquitin ligase. *Traffic.* **8**: 1375-1384.

Marchal, C., Haguenauer-Tsapis, R. and Urban-Grimal, D. 1998. A PEST-like sequence mediates phosphorylation and efficient ubiquitination of yeast uracil permease. *Mol Cell Biol.* **18**: 314-321.

Marchal, C., Haguenauer-Tsapis, R. and Urban-Grimal, D. 2000. Casein kinase I-dependent phosphorylation within a PEST sequence and ubiquitination at nearby lysines signal endocytosis of yeast uracil permease. *J Biol Chem.* **275**: 23608-23614.

Marchese, A., Paing, M. M., Temple, B. R. and Trejo, J. 2008. G protein-coupled receptor sorting to endosomes and lysosomes. *Annu Rev Pharmacol Toxicol.* **48**: 601-629.

Morvan, J., Froissard, M., Haguenauer-Tsapis, R. and Urban-Grimal, D. 2004. The ubiquitin ligase Rsp5p is required for modification and sorting of membrane proteins into multivesicular bodies. *Traffic.* **5**: 383-392.

Nikko, E. and Pelham, H. R. 2009. Arrestin-mediated endocytosis of yeast plasma membrane transporters. *Traffic.* **10**: 1856-1867.

Nikko, E., Sullivan, J. A. and Pelham, H. R. 2008. Arrestin-like proteins mediate ubiquitination and endocytosis of the yeast metal transporter Smf1. *EMBO Rep.* **9**: 1216-1221.

Paiva, S., Devaux, F., Barbosa, S., Jacq, C. and Casal, M. 2004. Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. *Yeast.* **21**: 201-210.

Paiva, S., Kruckeberg, A. L. and Casal, M. 2002. Utilization of green fluorescent protein as a marker for studying the expression and turnover of the monocarboxylate permease Jen1p of *Saccharomyces cerevisiae*. *Biochem J.* **363**: 737-744.

Paiva, S., Vieira, N., Nondier, I., Haguenauer-Tsapis, R., Casal, M. and Urban-Grimal, D. 2009. Glucose-induced ubiquitylation and endocytosis of the yeast Jen1 transporter: role of lysine 63-linked ubiquitin chains. *J Biol Chem.* **284**: 19228-19236.

Reinders, J., Wagner, K., Zahedi, R. P., Stojanovski, D., Eyrich, B., van der Laan, M., Rehling, P., Sickmann, A., Pfanner, N. and Meisinger, C. 2007. Profiling phosphoproteins of yeast mitochondria reveals a role of phosphorylation in assembly of the ATP synthase. *Mol Cell Proteomics.* **6**: 1896-1906.

Sambrook, J., Fritsch, E.F., and Maniatis, T., (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY

Shearwin-Whyatt, L., Dalton, H. E., Foot, N. and Kumar, S. 2006. Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. *Bioessays.* **28**: 617-628.

Shenoy, S. K., Xiao, K., Venkataramanan, V., Snyder, P. M., Freedman, N. J. and Weissman, A. M. 2008. Nedd4 mediates agonist-dependent ubiquitination, lysosomal targeting, and degradation of the beta2-adrenergic receptor. *J Biol Chem.* **283**: 22166-22176.

Stawiecka-Mirota, M., Pokrzywa, W., Morvan, J., Zoladek, T., Haguenauer-Tsapis, R., Urban-Grimal, D. and Morsomme, P. 2007. Targeting of Sna3p to the endosomal pathway depends on its interaction with Rsp5p and multivesicular body sorting on its ubiquitylation. *Traffic.* **8**: 1280-1296.

Volland, C., Urban-Grimal, D., Geraud, G. and Haguenauer-Tsapis, R. 1994. Endocytosis and degradation of the yeast uracil permease under adverse conditions. *J Biol Chem.* **269**: 9833-9841.

Chapter IX

Final Considerations

Final Considerations

Cellular homeostasis relies on the maintenance of an equilibrated intracellular environment, which is strongly dependent on the functionality of multiple transport systems located mainly at the plasma membrane level. The understanding of these systems involved in the uptake, compartmentalization and recruitment of specific compounds has evolved significantly, in the past years. With the development of gene sequencing technologies, complete genome sequences of several organisms, ranging from bacteria to human were made available, enabling the study of several proteins, in the three domains of life. This has boosted the field of comparative genomics leading to a more precise and complete view of the overall cell proteome. The work developed in the scope of this thesis contributed to the clarification of the regulatory mechanisms involved in the uptake of carboxylic acids in the yeast *S. cerevisiae*. Moreover, the functional characterization of predicted carboxylate transporters homologs, present in different microorganisms, was attempted in an effort to improve the knowledge on the phylogeny of carboxylate transporters.

In *S. cerevisiae* activities for at least two monocarboxylate proton symporters have been described. Jen1 was the first monocarboxylate transporter to be identified in fungi (Casal *et al.*, 1999), and belongs to the lactate/pyruvate:H^+ symporter family, (TC 2.A.1.12.2), with homologs in different Hemiascomycetes and Euascomycetes fungi. Ady2 identification resulted from DNA microarray analysis of the transcriptome of yeast cells shifted from glucose to acetic acid (Paiva *et al.*, 2004). According to the transport classification database TCDB, Ady2 belongs to the YaaH family (TC 2.A.96.), which has been recently considered. This family was previously classified as the Gpr1/Fun34/YaaH membrane protein family (TC 9.B.33), currently abolished.

In this thesis, we have focused on the characterization of *S. cerevisiae* monocarboxylate permeases homologs, in different microorganisms (Section I). With the rising clinical interest in pathogenic yeasts and the emergence of data connecting carbon metabolism and the pathogenicity of these microorganisms, we decided to functionally characterize the homologs identified in the two most prevalent fungal pathogens in humans: *C. albicans* and *C. glabrata* (Chapter II, III and IV). Moreover, after the sequencing of the acetotrophic methanogen *M. acetivorans* two Ady2 homologs were identified, allowing us to extend our studies to the Archaea domain of

life, in an effort to clarify Ady2 true function (Chapter V). Another aim of this work was to study, in more detail, the transcriptional and post-transcriptional regulation of Jen1 (Section II, Chapter VI), as well as the trafficking and turnover of this permease, in response to glucose addition to the culture medium (Section III, Chapter VII and VIII). Finally, in this chapter, we'll briefly address the most important results and conclusions derived from the data obtained in the past years, and delineate some suggestions for the further development of our current research.

The complete sequence of *C. albicans* diploid genome was released by the Stanford Genome Technology Center, in 2002. In a previous work, carried out by our group, a mediated transport system involved in the uptake of lactate was characterized, in *C. albicans* lactic acid-grown cells, with the following kinetic parameters: K_m 0.33 mM and V_{max} 0.85 nmol s^{-1} mg dry wt^{-1}, obtained for labelled lactic acid uptake, at pH 5.0. In an effort to identify the *C. albicans* lactate permease encoding gene, a sequence homology search was performed against ScJen1. CaJen1 was identified with 41% identity to the ScJen1, and subsequently characterized as a lactate-pyruvate-propionate proton symporter in this pathogen. Nevertheless, *C. albicans* had another ScJen1 homolog of unknown function, CaJen2, annotated in the Candida Genome Database as a putative lactate-pyruvate transporter, and with 36% of identity to the ScJen1. In Chapter II we have demonstrated that *CaJEN2* encodes a succinate-malate transporter induced after growth in succinic and malic acids, with the following kinetic parameters: K_m 0.49 mM and V_{max} 0.25 nmol s^{-1} mg dry wt^{-1}; and K_m 0.12 mM and V_{max} 0.18 nmol s^{-1} mg dry wt^{-1}, for labelled succinic and malic acid, at pH 5.0, respectively. The deletion of both *CaJEN2* alleles resulted in a complete loss of measurable succinate and malate permease activity, although having no effect in the mediated uptake of lactate or acetate. Expression of *CaJEN1* and *CaJEN2*, in different carbon and energy sources, was assessed at the transcriptional level, by qRT-PCR, as well as at the protein level, by Western blot analysis against tagged CaJen1-GFP and Jen2-GFP and by epifluorescence. The results showed that CaJen1 and CaJen2 are expressed after growth in the presence of mono- and dicarboxylic acids and repressed in the presence of glucose. However, monocarboxylic acids induce *CaJEN1* expression to a greater degree, than dicarboxylic acids, whereas the expression of *CaJEN2* was stronger in the presence of dicarboxylic acids. This is in accordance to their function, within the yeast cell. The heterologous expression of *CaJEN2* was carried out in a *S. cerevisiae jen1*

strain that acquired the ability to actively transport dicarboxylates, such as succinic acid. Furthermore, based on the knowledge that genes related to non-fermentable carbon sources metabolism are significant for the pathogen survival within the host, especially while thriving in glucose poor niches, the virulence of the double mutant, *jen1jen2,* was assessed in a murine model of systemic candidiasis. Despite the induction of CaJen1/2-GFP in *in vivo* and *ex vivo* models of infection, no attenuated virulence was observed for the *jen1jen2* double mutant strain, in comparison to the wild-type strain. These results indicate that, most probably, other means of acquiring carbon sources are active in this fungus, and hence, the impairment of the mediated transport of lactate, succinate and malate has no severe effects on the virulence of this pathogen.

The functional characterization of *C. albicans* carboxylate permeases involved the genetic cloning of *CaJEN2* in the CIp20 *E. coli*/*C. albicans* shuttle vector. The ligation of *CaJEN2* to CIp20 and its reintegration in the *RPS1* genomic locus of the *jen2* mutant strain were fundamental, in order to abolish *URA3* positional effects, that could influence the virulence of the reintegrant strain (Brand *et al.*, 2004). Due to the inability to propagate this gene in *E. coli*, several alternative experimental attempts were performed, aiming at the cloning of *CaJEN2* in the CIp20 plasmid. These results are summarized in Chapter III. This work resulted in the construction of a collection of plasmids suitable for the *in vivo* cloning of genes in *S. cerevisiae,* by gap repair: CIp10-2µ, CIp20-2µ and CIp30-2µ. This is particularly useful for genes which are difficult to clone in *E. coli* (like the ones encoding several membrane proteins).

We attempted to functionally characterize some of the predicted Ady2 homologs in the second most prevalent yeast pathogen in humans, *C. glabrata*. The complete genome sequence of this yeast became available in 2004, as a result of the Génolevures Consortium. *C. glabrata* shares with *S. cerevisiae* the common Whole Genome Duplication in its ancestry, but shows a significantly greater degree of genetic loss, which resulted in a regressive evolution, with loss of specific functions. Although this pathogen has no homologs of Jen1 encoded in its genome, it presents two predicted homologs of *S. cerevisiae* Ady2 transporter and one of Ato3. Physiological studies performed in the wild-type *C. glabrata* strain, ATCC2001, evidenced the presence of a low affinity mediated transport system, for the uptake of acetic acid with the following kinetic parameters: V_{max} of 8,08±1,21 nmol s-1 mg dry wt.-1; and a K_m of 8,16±2,13 mM (in a range of concentrations between 0.5 mM and 10 mM). Deletion of the

phylogenetically closest ScAdy2 homolog, *CAGLOMO3465g (CgADY2a),* affected the mediated uptake of acetate in acetic acid-grown cells. Nevertheless, it proved to be insignificant in what respects to the ability of the mutant strain to use distinct carbon sources, when compared to the wild-type cells. Most probably the second Ady2 homolog, *CAGL0L07766g (CgADY2b),* can influence the transport of this acid, across the plasma membrane and in this manner compensate for the disruption of *CAGLOMO3465g.* Expression studies of *CgADY2a/b* and *CgATO3* were followed by RT-PCR (reverse transcriptase), which enables the detection of very low amounts of RNA. All homologs were expressed in the different tested media, including glucose. These results were unexpected since both monocarboxylate transporters are strongly repressed by glucose in *S. cerevisiae*. Nevertheless, previous gene expression studies performed by Northern blot analyses in the yeast *K. lactis* evidenced the expression of *KlJEN1* and *KlJEN2* in glucose-grown cells (Queiros *et al.*, 2007). Primary carbon metabolism in this Crabtree-negative yeast is markedly diverse from that in *S. cerevisiae,* displaying very different mechanisms of catabolite regulation, under aerobic and anaerobic conditions (Snoek and Steensma, 2006). In *K. lactis,* glucose repression is less pronounced and it is strain-dependent (Ferrero *et al.*, 1978; Breunig *et al.*, 2000). Little is known about glucose repression in *C. glabrata*. In fact in the literature, contradictory information is found (Petter and Kwon-Chung, 1996; Bialkova and Subik, 2006). Moreover, since RT-PCR represents a very low quantitative method one cannot infer the level of induction of these genes. The results presented in this chapter are still preliminary and should be further developed.

In section II we have also designed an experimental strategy aiming at the functional characterization of predicted Ady2 homologs, in the Archaea domain of life. The availability of genetic tools to dissect genomic sequences together with the genetic, physiological and environmental diversity of the archaeon *M. acetivorans* made possible the study of this methanogen. In 2002 the complete genome sequence of M. *acetivorans* became available, revealing an unexpected genetic diversity. Two homologs of ScAdy2, Ma0103 and Ma4008 were found in this acetotrophic methanogen. The heterologous expression of both homologs was attempted in *S. cerevisisae jen1ady2,* after correct cloning in an expression plasmid under the control of the *PMA1* promoter, ptYEplac181. Although Ma0103 protein was expressed and detected at the predicted size, by Western blot analysis in *S. cerevisiae jen1ady2* strain,

no mediated transport activity was found for labelled acetic acid, in acetic acid derepressed cells. Most probably, the significant differences between these organisms might have impaired the correct expression of the proteins in a heterologous eukaryotic system. Moreover, the fact that the UAG stop codon can be translated into an unusual amino acid, the pyrrolysine, but which can also be used as a stop codon in this methanogen, could have terminated prematurely the translation of some UAG containing mRNA. However, we believe this was not the case, since both predicted Ma0103 and Ma4008 reading frames present no TAG codons.

Section III of this thesis focused on the post-transcriptional regulation of *JEN1* mRNA in *S. cerevisiae*. Different transcripts can be regulated, within the cell, at the post-transcriptional level. This regulation is achieved by means of RNA binding proteins involved in different processes, such as, alternative splicing, alternative polyadenylation, RNA editing, RNA degradation (nuclear and cytoplasmic), nuclear export and storage in P-bodies, among others. We have shown that the cytoplasmic deccaping activator Dhh1, co-localized to P-bodies, is involved in the regulation of *JEN1* and *ADY2* expression, directly influencing their mRNA decay rates, upon a glucose pulse to the culture media. Northern blot analysis showed that, in contrast to what was observed for the wild-type strain, there was expression of *JEN1* in a *dhh1* mutant, when formic or propionic acids were used, as sole carbon and energy sources. Furthermore, global transcription analyses performed in the wild-type and mutant *dhh1* strains, shifted from glucose to formic acid containing media, indicated that the deletion of *DHH1* had a relatively large effect, since 1000 genes were detected with a 2-fold RNA increase, in at least one of the tested conditions. Moreover, some genes subjected to glucose catabolite repression in the wild-type strain, have an increased expression in the absence of *DHH1*, suggesting that their glucose-driven negative regulation mainly occurs through post-transcriptional processes. On the other hand, other genes, like *JEN1*, were only derepressed in formic acid containing media, and hence, are not sensitive to the deletion of *DHH1* in the presence of glucose, suggesting that they are mainly repressed at the transcriptional level. The cases of *ADR1/CAT8* and *ACS1/ACH1* are particularly demonstrative since they are supposed to be similarly regulated but behaved differently in our experiments.

In the last section we have characterized the mechanisms underlying the downregulation of Jen1 induced by glucose, in *S. cerevisiae*. Previous studies,

developed by our group, demonstrated that the newly synthesized Jen1-GFP fusion protein co-localizes to the plasma membrane, in lactic acid-grown cells, in an active and stable form, and that a pulse of glucose triggers, within minutes, the *JEN1* gene repression and mRNA degradation (Andrade and Casal, 2001). Moreover, the loss of Jen1 activity is a result of its internalization, in an *END3* dependent manner, with subsequent degradation in the vacuole (Paiva *et al.*, 2002). Using the GFP-tagged version of this protein we have also demonstrated that like many plasma membrane proteins, inactivation of Jen1 requires its prior ubiquitylation. The downregulation of this monocarboxylate transporter was shown to be dependent on the HECT E3 ubiquitin ligase, Rsp5, which promotes Jen1 ubiquitylation at the cell surface. The protein turnover was shown to be dependent on the casein kinase 1 phosphorylation, indicating that most probably glucose-triggered phosphorylation plays a role in Jen1 endocytosis, whether by inducing a conformational change that renders certain Lys residues for direct ubiquitylation or by instigating the interaction of Rsp5 with the transporter, via an arrestin-like adaptor. We have identified the arrestin-like protein involved in the downregulation of Jen1, Art4, reinforcing the need for the presence of an adaptor to promote interactions between the E3 ubiquitin ligase Rsp5, and the non-PY motif containing Rsp5 plasma membrane endocytic cargoes. Data from large scale analyses based on mass spectrometry approaches led to the detection of two sites for Jen1 ubiquitylation and several sites of phosphorylation (Peng *et al.*, 2003; Reinders *et al.*, 2007). We have demonstrated that Jen1-Lys_{338} is one of the target residues modified by ubiquitin at the cell surface and that Jen1-Ser_{606} is one of the target residues possibly modified by phosphorylation, which are essential for the downregulation of this monocarboxylate permease. Using a collection of SUB stains, deleted in the four natural ubiquitin genes and expressing different ubiquitin Lys to Arg variants, as their only source of ubiquitin, we have also shown that Jen1 is modified by oligo-ubiquitylation, with ubiquitin-Lys63 linked chain(s). Jen1-GFP was correctly internalized and sorted to the vacuolar lumen, over time, after glucose addition, in all the SUB cells, expressing wild-type ubiquitin and the K6R, K11R, K27R, K29R and K33R variants. Nevertheless, in the SUB413 cells producing the Ub-K63R variant, Jen1-GFP fluorescence remained in the plasma membrane. Additionally, the ubiquitin-Lys63 linked chain(s) were shown to be required either directly or indirectly to sort Jen1 into Multivesicular Bodies, since Jen1-GFP stained the vacuolar rim even at time zero. This indicates that MVB sorting

was affected in the SUB413 cells, as a result of either impaired ubiquitylation of Jen1 itself or from a deficiency in MVB sorting machinery. Strikingly, we have demonstrated that ubiquitin-K63 linked chain(s) are required for correct trafficking of Jen1 at two stages of endocytosis: endocytic internalization and sorting at Multivesicular Bodies.

Future Prospects

The studies performed, in the scope of this thesis were mostly focused on carboxylate transporters in distinct microorganisms. In spite of the advances obtained in this work, there are still many questions unanswered. However, *S. cerevisiae* Jen1 and Ady2 transporters are certainly good model systems to fill in many missing gaps, underlying the capacity of cells to adapt and to respond to distinct environmental cues.

The physiological and genetical characterization of *C. albicans* Jen1 and Jen2 proteins and their induction in models of infection, suggests their involvement in the acquisition of non-fermentable carbon sources, in glucose poor conditions. Nevertheless, *CaJEN1/2* deletion mutant failed to directly affect the survival of this pathogen, within a murine model of systemic candidiasis. This indicated that most probably, additional non-fermentable carbon sources are available in those niches, and that other transport systems might be responsible for the uptake of those non-fermentable carbon and energy sources. In this manner, the characterization of the eight CaAdy2 homologs, as well as the construction of serial mutants, disrupted in all these genes, could contribute to unravel the significance of alternative carbon sources utilization in virulence, within the human host. It would also clarify the nutrient availability within a phagosome, which is still poorly characterized.

Presently, the YaaH family with homologs in the three domains of life: bacteria, archaea and eukaryota, still includes members with unclear functions, namely the *E. coli* Yaah, which gives name to this family, and the yeast *S. cerevisiae ADY2* (*YCR010c*) gene. *ADY2* has been shown to be crucial for the acetate uptake (Paiva *et al.*, 2004) and its C-termini demonstrated to be necessary for the triggering of acetic acid sensitivity (Gentsch *et al.*, 2007). Nevertheless, these assumptions are still a theme of debate since there are other authors hypothesizing the role of *ScATO1/ADY2*, as well as *ScATO2* (*YDR384c*) and *ScATO3* (*YNR002c*), as ammonium/H+ antiporters, important for ammonia production. In this manner, the functional elucidation of Ady2 homologs, both in the pathogenic yeast *C. glabrata* and in the methanogen *M. acetivorans*, should be

concluded. In the first case, the double mutant construction should be further attempted, since the disruption of both homologs seems crucial to the characterization of their role within the cell. Moreover, if in fact both genes are involved in the uptake of acetic acid, the study of their expression in models of systemic candidiasis should be accomplished, in order to unravel the metabolic behaviour of this pathogen, while thriving within the host. Additionally, physiological studies concerning the mediated uptake of distinct carboxylates should be developed to further characterize this system. Secondly, the *de novo* synthesis of *M. acetivorans* Ma0103 and Ma4008, from an optimized sequence, should be performed to overcome putative codon bias issues. Concomitantly, heterologous expression of both putative permeases, fused with soluble fusion tags, could be attempted in an *E. coli* codon plus strain (*E. coli* BL21-Codon Plus) that can supply additional copies of specific tRNA genes, which are rare in this bacteria, such as isoleucine, leucine and arginines, but present in high quantities in many archaea (Kim and Lee, 2006).

Regarding the second section of this thesis work, the role of Dhh1 in the regulation of carbon related genes still poses intriguing questions. The known pleiotropic behaviour of this DEAD-box RNA helicase, within the yeast cell, has been extensively documented. However, the accumulation of some glucose repressed mRNAs in the mutant *dhh1* gave rise to a new putative role of Dhh1 in the transcriptional repression of several genes, induced by glucose. Functional analysis of the promoter region of the gene *JEN1* and *ADY2* should be accomplished, aiming at the identification of the cis-elements and trans-factors regulating their transcription. Moreover, Dhh1 and the Ccr4-Not complex, a regulator of transcription involved in the control of the initiation, elongation and degradation of mRNA, are conserved in the pathogen *C. albicans*. It would be interesting to study the role of Dhh1 in the regulation of alternative carbon sources utilization, namely on the *C. albicans* carboxylate transporters. Moreover, the deletion of the Not4 protein, a subunit of the Ccr4-Not complex that has been suggested to act as an E3 ubiquitin ligase, based on functional analysis of the human Not4 (Albert *et al.*, 2002; Panasenko *et al.*, 2006), has been shown to affect *C. albicans* pathogenicity, probably by regulating the expression of a broad range of genes that directly or indirectly control cellular morphogenesis and virulence (Krueger *et al.*, 2004). In this manner, the Ccr4-Not complex that physically interacts with Dhh1 in *S. cerevisiae* must be capable of affecting *C. albicans* virulence

by regulating the expression of transcription factors and of virulence related genes. The role of Dhh1 in transcriptional repression should also be studied in this pathogen, as should its role in fungal virulence.

Finally, the monocarboxylate transporter Jen1 of *S. cerevisiae* has proven to be an excellent system for genetically dissecting mechanisms that regulate trafficking of an eukaryotic plasma membrane protein, according to physiological constraints. In this manner, the study of protein trafficking and turnover should be continued using Jen1 as a model, but also extended it to ScAdy2 and possibly to *C. albicans* Jen1 homologs. Focusing on the recent data concerning Jen1 trafficking, we intend to investigate the post-translational modifications of Rsp5 and of the arrestin-like adaptor protein Art4, upon glucose addition to lactic acid-induced cells. It would be particularly interesting to assess their level of phosphorylation and ubiquitylation, before and after glucose addition and how it affects Jen1 downregulation. Additionally, the clarification of whether Art4 is also involved in the sorting at Multivesicular Bodies, or if other adaptor(s) is/are involved should be accomplished. Finally, the identification of new functional domains of the Jen1 carrier, involved either in the trafficking or turnover of the protein or in its overall activity should be significant. Therefore, N- and C-terminal regions of Jen1 could be swapped with other transporters that have a different pattern of downregulation. This strategy is ideal to identify whether the Jen1 terminals include molecular determinants necessary and sufficient for glucose-elicited endocytosis and sorting to endosomes. We may also be able to find out the functional motifs mediating the recognition of Jen1 substrates by Art4.

References

Albert, T. K., Hanzawa, H., Legtenberg, Y. I., de Ruwe, M. J., van den Heuvel, F. A., Collart, M. A., Boelens, R. and Timmers, H. T. 2002. Identification of a ubiquitin-protein ligase subunit within the *CCR4-NOT* transcription repressor complex. *EMBO J.* **21**: 355-364.

Andrade, R. P. and Casal, M. 2001. Expression of the lactate permease gene *JEN1* from the yeast *Saccharomyces cerevisiae*. *Fungal Genet Biol.* **32**: 105-111.

Bialkova, A. and Subik, J. 2006. Biology of the pathogenic yeast *Candida glabrata*. *Folia Microbiol (Praha).* **51**: 3-20.

Brand, A., MacCallum, D. M., Brown, A. J., Gow, N. A. and Odds, F. C. 2004. Ectopic expression of *URA3* can influence the virulence phenotypes and proteome of *Candida albicans* but can be overcome by targeted reintegration of *URA3* at the *RPS10* locus. *Eukaryot Cell.* **3**: 900-909.

Breunig, K. D., Bolotin-Fukuhara, M., Bianchi, M. M., Bourgarel, D., Falcone, C., Ferrero, I. I., Frontali, L., Goffrini, P., Krijger, J. J., Mazzoni, C., Milkowski, C., Steensma, H. Y., Wesolowski-Louvel, M. and Zeeman, A. M. 2000. Regulation of primary carbon metabolism in *Kluyveromyces lactis*. *Enzyme Microb Technol.* **26**: 771-780.

Casal, M., Paiva, S., Andrade, R. P., Gancedo, C. and Leao, C. 1999. The lactate-proton symport of *Saccharomyces cerevisiae* is encoded by *JEN1*. *J Bacteriol.* **181**: 2620-2623.

Ferrero, I., Rossi, C., Landini, M. P. and Puglisi, P. P. 1978. Role of the mitochondrial protein synthesis is the catabolite repression of the petite-negative yeast *K.lactis*. *Biochem Biophys Res Commun.* **80**: 340-348.

Gentsch, M., Kuschel, M., Schlegel, S. and Barth, G. 2007. Mutations at different sites in members of the *Gpr1/Fun34/YaaH* protein family cause hypersensitivity to acetic acid in *Saccharomyces cerevisiae* as well as in *Yarrowia lipolytica*. *FEMS Yeast Res.* **7**: 380-390.

Kim, S. and Lee, S. B. 2006. Rare codon clusters at 5'-end influence heterologous expression of archaeal gene in *Escherichia coli*. *Protein Expr Purif.* **50**: 49-57.

Krueger, K. E., Ghosh, A. K., Krom, B. P. and Cihlar, R. L. 2004. Deletion of the *NOT4* gene impairs hyphal development and pathogenicity in *Candida albicans*. *Microbiology.* **150**: 229-240.

Paiva, S., Devaux, F., Barbosa, S., Jacq, C. and Casal, M. 2004. Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. *Yeast.* **21**: 201-210.

Paiva, S., Kruckeberg, A. L. and Casal, M. 2002. Utilization of green fluorescent protein as a marker for studying the expression and turnover of the monocarboxylate permease Jen1p of *Saccharomyces cerevisiae*. *Biochem J.* **363**: 737-744.

Panasenko, O., Landrieux, E., Feuermann, M., Finka, A., Paquet, N. and Collart, M. A. 2006. The yeast Ccr4-Not complex controls ubiquitination of the nascent-associated polypeptide (NAC-EGD) complex. *J Biol Chem.* **281**: 31389-31398.

Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D. and Gygi, S. P. 2003. A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol.* **21**: 921-926.

Petter, R. and Kwon-Chung, K. J. 1996. Disruption of the *SNF1* gene abolishes trehalose utilization in the pathogenic yeast *Candida glabrata*. *Infect Immun.* **64**: 5269-5273.

Queiros, O., Pereira, L., Paiva, S., Moradas-Ferreira, P. and Casal, M. 2007. Functional analysis of *Kluyveromyces lactis* carboxylic acids permeases: heterologous expression of *KlJEN1* and *KlJEN2* genes. *Curr Genet.* **51**: 161-169.

Reinders, J., Wagner, K., Zahedi, R. P., Stojanovski, D., Eyrich, B., van der Laan, M., Rehling, P., Sickmann, A., Pfanner, N. and Meisinger, C. 2007. Profiling phosphoproteins of yeast mitochondria reveals a role of phosphorylation in assembly of the ATP synthase. *Mol Cell Proteomics.* **6**: 1896-1906.

Snoek, I. S. and Steensma, H. Y. 2006. Why does *Kluyveromyces lactis* not grow under anaerobic conditions? Comparison of essential anaerobic genes of *Saccharomyces cerevisiae* with the *Kluyveromyces lactis* genome. *FEMS Yeast Res.* **6**: 393-403.