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Characterization of novel mechanisms involved in phosphate signalling in *Saccharomyces cerevisiae*

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't is dan een kwestie van door te zetten en positief te blijven...

"...I have not failed, I've just found 10000 ways that won't work"
(Thomas Edison)

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List of abbreviations

AM	arbuscular mycorrhizal
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AP-1	Activator Protein – 1
cAMP	3',5'-cyclic adenosine monophosphate
bp	base pair(s)
CDC	cell division cycle
CDK	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor
Da	Dalton
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
FGF-23	Fibroblast Growth Factor – 23
FGM	fermentable growth medium induced
GAAC	general amino acid control
GAP	GTPase-activating protein
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
GTP	guanine triphosphate
Hsp	heat shock protein
IP_3	inositol triphosphate
IP_4	inositol tetraphosphate
IP_5	inositol pentaphosphate
IP_6	inositol hexaphosphate
kDa	kilo Dalton
K_m	Michaelis-Menten constant
M	molar

mM	millimolar
μM	micromolar
MAPK	mitogen-activated protein kinase
Mat**a**	mating type a
Matα	mating type α
mRNA	messenger RNA
NES	nuclear export sequence
NLS	nuclear localization sequence
nt	nucleotide
OD	optical density
ORF	open reading frame
P	phosphorus
PAS	Per-Arnt-Sim
PCR	polymerase chain reaction
PDS	post-diauxic shift element
PHM	PHosphate Metabolism
Pi	inorganic phosphate
PIP_2	phosphatidylinositol bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
polyP	inorganic polyphosphate
PP1	protein phosphatase type 1
PP2A	protein phosphatase type 2A
PP-IP_4	diphosphoinositol tetraphosphate
PP-IP_5	diphosphoinositol pentaphosphate
$[PP]_2$-IP_3	bisdiphosphoinositol triphosphate
$[PP]_2$-IP_4	bisdiphosphoinositol tetraphosphate
PTH	parathyroid hormone
RGS	regulator of G-protein signalling
RIM	regulator of *IME2*
RNA	ribonucleic acid
SD	standard deviation

STRE	stress responsive element
TOR	target of rapamycin
TORC1	TOR complex one
TORC2	TOR complex two

List of genes names and their products

ADO1	adenosine kinase
ADK1	adenylate kinase
ARG82	dual specificity inositol 1,4,5-trisphosphate 6-kinase/inositol 1,4,5,6-tetrakisphosphate 3-kinase (IP3 6-/IP4 3-kinase)
BCY1	regulatory subunit of PKA (Bypass of CYr1 requirement)
BMH1,2	14-3-3 proteins involved in signal transduction by anchoring proteins in the cytoplasm
CDC24	Cdc42 exchange factor, involved in cell morphology and polarised growth
CDC25	Ras guanine nucleotide exchange factor
CDC28	CDK, controls cell cycle progression
CDC35/CYR1	adenylate cyclase
CLG1	Pho85 cyclin
CRZ1	transcription factor involved in calcium signalling
ELM1	Snf1 activating kinase, partially redundant with Pak1 and Tos3
GAL83	regulatory β-subunit of Snf1 kinase complex
GAP1	general amino acid permease, involved in amino acid signalling
GCN4	basic leucine zipper (bZIP) transcription factor required for general amino acid control (GAAC)
GIS1	C_2H_2 zinc finger transcription factor required for transcription of genes controlled by the PDS (Post Diauxic Shift)-element
GLC7	protein phosphatase type I
GLC8	activator of protein phosphatase Glc7
GLK1	glucokinase
GPA2	heterotrimeric G-protein α-subunit, involved in glucose and sucrose signalling together with its receptor Gpr1
GPR1	G-protein coupled receptor, involved in glucose and sucrose signalling
GRE1	Hydrophilin of unknown function, induced by various stress signals
GSY1	glycogen synthase, isoforms 1
GSY2	glycogen synthase, isoforms 2
HSP12	12 kDa heat shock protein

HSP26	26 kDa heat shock protein
HXK1	hexokinase I
HXK2	hexokinase II
IME2	serine/threonine protein kinase, positive regulator of early, middle and late meiotic genes
IPK1	inositol 1,3,4,5,6-pentakisphosphate 2-kinase
IRA1,2	Ras GTPase-activating protein
KCS1	inositol polyphosphate kinase
LCB4	sphingoid long-chain base kinase
MEP2	ammonium permease, involved in ammonium signalling
MIG1	C_2H_2 zinc finger transcriptional repressor involved in glucose-repression
MSN2-MSN4	C_2H_2 zinc finger transcriptional activator for genes controlled by the STRE (STress REsponsive)-element
MSN5	importin-β family member required for nuclear export of Pho4, Rim15 and Crz1
NTH1	neutral trehalase
PAK1	Snf1 activating kinase, partially redundant with Elm1 and Tos3
PCL	Pho85 CycLin (e.g. *PCL1*,*2*,*5*,*6*,*7*,*8*, *9* and *10*)
PDE1	low-affinity cAMP phosphodiesterase
PDE2	high-affinity cAMP phosphodiesterase
PHO2	homeodomain protein, activates PHO gene transcription in cooperation with Pho4
PHO4	basic helix-loop-helix (bHLH) transcriptional activator of PHO genes, negatively regulated by Pho85-Pho80 dependent phosphorylation
PHO5	secreted acid phosphatase
PHO80	Pho85 cyclin, involved in phosphate signalling
PHO81	CKI, Pi-dependent inhibitor of Pho85-Pho80 and Pho85-Pcl7 kinases
PHO84	high-affinity H^+/Pi symporter, induced by Pi starvation
PHO85	cyclin-dependent kinase, can interact with 10 different cyclins (Pcl)
PHO86	ER-resident protein required for proper ER-exit of Pho84
PHO87	low-affinity H^+/Pi symporter, expression is independent of Pi
PHO89	high-affinity Na^+/Pi symporter, induced by Pi starvation

PHO90	low-affinity H^+/Pi symporter, expression is independent of Pi
PHO91	low-affinity H^+/Pi symporter, expression is independent of Pi
PLC1	phospholipase C
PPH21,22	protein serine/threonine phosphatase type 2A (PP2A), catalytic subunit
PSE1	importin-β family member required for nuclear import of Pho4
RAS1,2	small G-proteins, positive regulators of adenylate cyclase
REG1	regulatory subunit of protein phosphatase Glc7, involved in glucose repression
RGS2	Gpa2 GTPase activating protein
RIM15	serine/threonine protein kinase, contains N-terminal PAS domain
RPL25	ribosomal protein L25
RPS33	ribosomal protein S33
RVS167	protein involved in regulation of actin dynamics, endocytosis and response to starvation
SCH9	serine/threonine protein kinase, homologous to mammalian PKB
SDC25	Ras guanine nucleotide exchange factor
SIC1	inhibitor of Cdc28-Clb kinases
SIP1	regulatory β-subunit of Snf1 kinase complex
SIP2	regulatory β-subunit of Snf1 kinase complex
SNF1	serine/threonine protein kinase required for glucose repression, catalytic α-subunit of Snf1 kinase complex
SNF4	regulatory γ-subunit of Snf1 kinase complex
SPL2	Pho81 homologue, induced by Pi-starvation
SSA3	70 kDa heat shock protein (Stress Seventy family)
TOR1,2	Target Of Rapamycin proteins
TOS3	Snf1 activating kinase, partially redundant with Elm1 and Pak1
TPK1,2,3	cAMP-dependent protein kinase A, catalytic subunits
TPS1	trehalose-6-phosphate synthase
TPS2	trehalose-6-phosphatase
TPS3	regulatory subunit of the trehalose synthase complex
TSL1	regulatory subunit of the trehalose synthase complex
VTC4/PHM3	vacuolar membrane protein involved in polyP accumulation

Summary

Inorganic phosphate (Pi) constitutes an essential nutrient required by all living cells. As Pi cannot be synthesized by cells, it must be acquired from the external environment, a process that requires specialized plasma membrane proteins, the Pi transporters. Perception of Pi availability in the environment must be integrated with information regarding the cell's internal phosphate status in order to regulate cellular metabolism and growth.

Under Pi-rich conditions, the budding yeast *Saccharomyces cerevisiae* obtains Pi via a low-affinity transport system, composed of the partially redundant Pho87, Pho90 and Pho91 proteins. However, free Pi is rather limited in many terrestrial ecosystems (typically in the μM range) and may therefore often constitute a limiting factor for yeast growth. Low Pi conditions are sensed by yeast via the PHO signalling pathway, composed of the central Pho85-Pho80 CDK-cyclin kinase, its upstream regulator Pho81 and the downstream Pho4 transcription factor. Limiting Pi results in a Pho4-dependent transcriptional activation of genes involved in phosphate acquisition, allowing for maximal proliferation under conditions of low external Pi. When all available Pi-sources become exhausted the yeast cells enter the quiescent or stationary phase (G_0) due to Pi starvation. Entry into the G_0 phase is a general response to starvation for an essential nutrient and encompasses many transcriptional and metabolic rearrangements in the yeast cell. Some of these (such as the induction of stress-responsive genes and the accumulation of the reserve carbohydrates trehalose and glycogen) serve to acquire stress resistance and ensure optimal survival during the starvation period. Other changes such as the repression of genes involved in protein synthesis result in the controlled downregulation of growth. All these traits are known to be tightly regulated by the activity of the yeast protein kinase PKA. Therefore, these properties are often annotated as "PKA targets".

In this study we investigated the regulation of G_0 properties specifically in response to Pi, thereby keeping all other nutrients in sufficient amounts and using glucose as a carbon source. We demonstrated that in addition to augmenting Pi-acquisition the PHO pathway is also involved in the control of some typical G_0 properties in response to Pi availability. More specifically, in the absence of Pi, Pho81-mediated inhibition of Pho85 activity is required for proper induction of PDS-controlled stress responsive genes and accumulation of trehalose. While the transcriptional response solely requires the Pho80 cyclin, additional Pho85 cyclins are involved in correct regulation of trehalose metabolism. Interestingly, the Pho4 transcription does not directly mediate transcriptional induction of stress responsive genes, as its effects were found to be solely dependent on its positive action on Pho81 function. Rather, the Rim15 protein kinase was found to constitute a physiological target of the Pho85-Pho80 kinase responsible for the transcriptional regulation of PDS-driven genes, thereby mediating entry into G_0 in response to Pi starvation. (this study and (Wanke et al., 2005)). Consequently, this study expanded the role of Rim15 in the regulation of G_0-entry in response to nutrient starvation as this kinase was previously shown to integrate signals derived from the nutrient-regulated protein kinases PKA, Sch9 and TORC1.

Re-addition of Pi to yeast cells previously starved for this nutrient in the presence of glucose results in a rapid activation of PKA targets, which requires an activation of the Pho85-Pho80 kinase. In addition, rapid activation of PKA targets is highly dependent on the Pi transport capacity of the yeast cells. Interestingly, whereas rapid phosphate consumption and signalling in wild-type yeast cells depends on the Pho84 high-affinity Pi transporter, Pho84-independent phosphate signalling was observed in a *pho4Δ* strain which specifically requires the Pho87 low-affinity Pi transporter. As *PHO87* transcription is not affected by Pi and/or Pho4 activity, Pho87 function is regulated at the posttranscriptional level via an as yet unknown Pho4-dependent component. These results imply that Pho87 may be involved in Pi-signalling under conditions in which Pho4 is inactive. In this respect we found that Pho4 is inactivated upon glucose removal from Pi-starved cells through nuclear exclusion depending on a functional Pho85-Pho80 kinase. As Pho87 mediates

phosphate transport/signalling in a *pho4Δ* strain, this phosphate transporter may be specifically activated when both Pi and carbon source are growth-limiting.

It thus seems that yeast cells produce differential responses to limiting Pi depending on the composition of the nutrient environment. When Pi is the sole limiting nutrient, yeast cells activate, through Pho4, a system allowing for high-affinity Pi signalling through the Pho84 transporter. Upon additional glucose exhaustion the yeast cell inactivates this high-affinity Pi-transport/signalling system in order to avoid futile energy expenditure when Pi is no longer the sole limiting nutrient. Under these conditions, the yeast cell monitors the Pi-status of the environment using a low-affinity system, i.e. Pho87. Interestingly, we found that the Rim15 protein kinase is involved in nuclear exclusion of Pho4 upon glucose removal. Thus, while Rim15 function is modulated in response to phosphate by the PHO pathway, Rim15 in turn is able to adjust the activity of the PHO pathway in response to glucose. Hence, a model emerges wherein the Rim15 protein kinase does not solely control G_0-entry by functioning downstream of nutrient-regulated signalling pathways but also plays a crucial role in the dynamical cross-talk between its own upstream signalling pathways. This will allow the yeast to produce an appropriate response to a wide variety of nutrient conditions.

Samenvatting

Anorganisch fosfaat (Pi) is een essentieel nutrient voor alle levende cellen. Aangezien Pi niet kan worden aangemaakt door de cel, moet het uit de omgeving worden opgenomen, een proces dat afhankelijk is van gespecialiseerde membraanproteïnen; de Pi-transporters. Regulatie van het cellulair metabolism en de groei vereist een integratie van informatie van enerzijds extracellulaire Pi beschikbaarheid en anderzijds de interne fosfaatstatus.

Onder condities van overvloedig Pi zal de gist *Saccharomyces cerevisiae* Pi opnemen via een lage-affiniteits transportsysteem, bestaande uit de Pho87, Pho90 en Pho91 proteïnen. Pi is echter maar beperkt aanwezig in vele landelijke ecosystemen (typisch in de grootte-orde van μM) waardoor het vaak een limiterende faktor kan vormen voor de groei van de gist. Lage Pi condities worden door de gist waargenomen via de PHO signaalweg, welke is opgebouwd uit het centrale Pho85-Pho80 CDK-cycline kinase samen met zijn doelwit proteïne, de transcriptiefaktor Pho4, en zijn stroomopwaartse regulator Pho81. Lage Pi-concentraties leiden tot een Pho4-afhankelijke transcriptionele inductie van genen betrokken bij fosfaatverwerving, zodoende dat een maximale proliferatie wordt bereikt onder deze condities. Wanneer uiteindelijk alle beschikbare fosfaatbronnen zijn uitgeput zal de gistcel overgaan naar de stationaire fase (G_0). Deze overgang is een algemene respons op nutrientdeprivatie en omvat vele transcriptionele en metabolische aanpassingen in de gistcel. Sommige van deze aanpassingen, zoals de inductie van stress-gevoelige genen en de accumulatie van de reservekoolhydraten trehalose en glycogeen, leiden tot het verkrijgen van stress-resistentie en verzekeren een optimale overleving gedurende de deprivatieperiode. Andere veranderingen, zoals de repressie van genen betrokken in proteïnesynthese, resulteren in een gecontroleerde groeistop. Al deze kenmerken worden gereguleerd door de activiteit van het proteïne kinase PKA en worden dan ook aangeduid als “PKA doelwitten”.

In deze studie hebben we de regulatie van G_0-eigenschappen onderzocht specifiek in respons op Pi, waarbij alle andere essentiële nutriënten in voldoende hoeveelheden aanwezig zijn en glucose als koolstofbron werd gebruikt. We toonden aan dat onder deze conditie de PHO signaalweg sommige typische G_0-kenmerken controleert. Meer specifiek, in de afwezigheid van Pi is een Pho81-gemedieerde inhibitie van Pho85-activiteit vereist voor een correcte inductie van PDS-gecontroleerde stress-gevoelige genen en de accumulatie van trehalose. Terwijl de transcriptionele respons enkel afhankelijk is van het Pho80 cycline, zijn bijkomende Pho85-cyclines betrokken bij de Pi-afhankelijke regulatie van het trehalosemetabolisme. Hoewel de Pho4 transcriptiefaktor een doelwitproteïne is van het Pho85-Pho80 kinase, is Pho4 niet direct betrokken bij de transcriptie van PDS-afhankelijke genen maar oefent zijn invloed uit via stimulatie van het Pho81 proteïne. Het proteïne kinase Rim15 werd geïdentificeerd als het doelwitproteïne van Pho85-Pho80 kinase dat verantwoordelijk is voor de transcriptionele regulatie van PDS-afhankelijke genen en dat zodoende de overgang regelt naar de G_0-fase in respons op fosfaatbeschikbaarheid (deze studie en {Wanke, 2005 #1360}). Vermits eerder was aangetoond dat Rim15 signalen integreert afkomstig van de nutriëntafhankelijke proteïne kinase PKA, Sch9 en TORC1, heeft deze studie geleid tot een uitbreiding van de rol van Rim15 in de regulatie van G_0-eigenschappen in respons op nutriëntbeschikbaarheid.

Het toevoegen van Pi aan gistcellen gedepriveerd voor dit nutriënt induceert op een glucose-bevattend medium een snelle repons van typische PKA doelwitten. Deze respons vereist een activatie van het Pho85-Pho80 kinase en is bovendien sterk afhankelijk van de Pi-transportcapaciteit van de gistcel. Pi-geïnduceerde signalering alsmede Pi-consumptie in wild-type gistcellen is afhankelijk van de Pho84 hoge-affiniteits fosfaattransporter. Deletie van het *PHO4* gen zorgt echter voor een Pho84-onafhankelijke fosfaatsignalering, specifiek gemedieerd door de Pho87 lage-affiniteits fosfaattransporter. Transcriptie van *PHO87* is onafhankelijk van zowel Pi als Pho4-activiteit, wat erop duidt dat de functie van Pho87 wordt gereguleerd op posttranscriptioneel niveau via een nog onbekende, Pho4-afhankelijke component. Deze resultaten wijzen erop dat Pho87 bijdraagt tot fosfaatsignalering onder condities waarin Pho4 inactief is. Hieromtrent tonen we aan dat Pho4 activiteit niet enkel door fosfaat maar ook door glucose wordt geregeld. Wanneer glucose wordt verwijderd

van Pi-gedepriveerde cellen transloceert Pho4 van de celkern naar het cytoplasma en deze translocatie vereist een actief Pho85-Pho80 kinase.

De respons van de gistcel op Pi-deprivatie is dus afhankelijk van de nutriëntensamenstelling van het medium. Wanneer Pi het enige limiterende nutriënt is, activeert de gistcel, via Pho4, een systeem dat hoge-affiniteits fosfaatsignalering toelaat via de Pho84 transporter. Bijkomende glucose-uitputting leidt echter tot inactivatie van Pho4 zodat energie wordt gespaard vermits Pi niet langer het enige limiterende nutriënt is. Onder deze conditie zal fosfaatsignalering gebeuren de Pho87 lage-affiniteits fosfaattransporter. Interessant is dat ook Rim15 betrokken is bij de glucose-afhankelijke regulatie van Pho4 localisatie. Dit wijst op een duale rol van Rim15 in fosfaatsignalering, waarbij enerzijds de functie van Rim15 op een Pi-afhankelijke manier wordt gemoduleerd door de PHO signaalweg en anderzijds Rim15 de activiteit van diezelfde PHO signaalweg afstemt op de beschikbaarheid van glucose. Deze en andere resultaten leiden tot een model waarin het proteïne kinase Rim15 niet enkel stroomafwaarts staat van verschillende nutriënt-geïnduceerde signaalwegen en nodig is voor een correcte overgang naar G_0-fase in respons op nutriëntdeprivatie, maar waarbij Rim15 ook een cruciale role speelt in de uitwisseling van informatie tussen diezelfde signaalwegen. Dit dynamisch netwerk van signaleringsmodules stelt de gist in staat om een gepaste respons te formuleren op een grote variëteit aan nutriëntcondities.

Chapter I

A literature overview

Introduction

All living organisms require nutrients to provide the essential building blocks and energy supply to make the necessary cellular components and drive metabolism. Therefore, the presence of nutrients is essential to survive, proliferate, and be productive. As a result, cells have developed sophisticated mechanisms (signalling pathways) to sense nutrient availability and produce appropriate responses. In general, nutrient sensing can occur through detection of the nutrient as such via receptor proteins, either at the plasma membrane or inside the cell. Alternatively, the presence of a certain nutrient can be detected indirectly via its metabolism.
Nutrient-induced signalling pathways allow for optimal nutrient consumption in a dynamic manner and particularly in unicellular organisms it enables coordinated induction of a resting phase where the cells cease proliferation upon nutrient limitation, but rapidly resume the process once conditions improve (Roosen et al., 2004). In higher multicellular organisms, most cells experience relative environmental homeostasis, and endocrine (and nervous) systems are often regarded as having the dominant role in regulating the responses of tissues to altered nutrient availability. Still, in these organisms specialised cell types continually monitor the level of key nutrients and produce appropriate metabolic and/or behavioural responses, typically via altered secretion of certain hormones and/or neurotransmitters (Best and McLaughlin, 2004; Proud et al., 2004). A key example is the regulation of human blood glucose via the insulin and glucagon hormones produced in specialised pancreatic islet cells. In addition, evidence is mounting that particular nutrients can initiate cell-signalling events independently of hormonal influences.

The unicellular eukaryote *Saccharomyces cerevisiae* (or budding yeast) is often used as a model system to study nutrient-induced signalling in eukaryotic cells, as many signal transduction mechanisms are highly conserved between lower and higher eukaryotes (Manning et al., 2002). In yeast, information about the nutritional situation of the environment must be transduced across the plasma membrane and integrated with information regarding intracellular nutrient levels in order for the yeast to regulate its growth, proliferation and metabolism (Winderickx et al., 2003). Starving yeast cells for essential nutrients will induce entry into a quiescent state, commonly

termed the stationary (G_0) phase. Within the G_0 phase, many transcriptional and metabolic rearrangements take place, all directed to ensure optimal survival of the yeast under these harsh conditions. Interestingly, many of these adjustments, such as transcriptional profiles, are also found in yeast cells subjected to other environmental stresses, e.g. heat stress, indicating that nutritional deprivation *per se* may be considered as a stress condition.

In this work, we investigated the mechanisms involved in the signalling of inorganic phosphate availability in the yeast *Saccharomyces cerevisiae*. As phosphate acts in concert with glucose as a nutrient signal for yeast, we will start this literature overview with an introduction of glucose signalling in yeast, after which the concepts of signal integration of glucose and other nutrients are explored ("FGM signalling"). We will then go over the phosphate metabolism and signalling process in yeast focussing on the phosphate transporters and the PHO signalling pathway. Finally, the broad role of the Pho85 cyclin-dependent kinase in yeast will be discussed.

1. Glucose-induced signalling

Carbon sources are of central importance to living organisms. Like many other organisms, the yeast *Saccharomyces cerevisiae* can utilise a variety of compounds as carbon and energy sources, however glucose is preferred. When glucose is present in sufficient amounts, yeast will favour fermentation of this sugar into ethanol and CO_2 instead of respiration to produce CO_2 and H_2O. Although the energy (ATP) yield of fermentation is considerably less than that obtained by respiration, the high amounts of ethanol produced by fermentation inhibits growth of most other microorganisms, allowing *S. cerevisiae* to dominate in spontaneous fermentation. In addition, the ethanol - and acetate - produced during fermentation will be metabolised in the post-diauxic growth phase following glucose depletion, so nearly al of the available carbon source will eventually be used. The metabolic preference for fermentable sugar is mediated through a variety of regulatory pathways, all directed to the optimal and exclusive use of this preferred carbon source. Two of these glucose-induced signal transduction pathways, the glucose repression pathway (or catabolite repression pathway) and the Ras-cAMP pathway, will be discussed below. Activation of these pathways is dependent on both transport and intracellular phosphorylation of the fermentable sugar but, interestingly, no further metabolism beyond the step of phosphorylation seems to be necessary.

1.1 The main glucose repression pathway

The catabolite repression or main glucose repression pathway is a rapid response system that, when activated by glucose, represses transcription of genes encoding proteins involved in the uptake and utilisation of alternative carbon sources (Gancedo, 1998; Ronne, 1995; Schuller, 2003). The serine-threonine protein kinase Snf1 plays an essential role in this signalling pathway (Fig. I.1). As its mammalian orthologue, the AMP-activated protein kinase (AMPK), the Snf1 protein kinase functions as a heterotrimer composed of a catalytic α–subunit (Snf1), a regulatory β–subunit (Gal83, Sip1 or Sip2) and a γ–subunit (Snf4) (Celenza and Carlson, 1989; Hardie and Carling, 1997; Jiang and Carlson, 1997; Yang et al., 1994). The Snf1 protein kinase itself consists of an N-terminal catalytic domain and a C-terminal regulatory domain. In

high glucose conditions, an auto-inhibitory interaction occurs between the regulatory domain and the catalytic domain, keeping the Snf1 kinase in an inactive state. Activation of Snf1 under low glucose conditions basically requires two steps (McCartney and Schmidt, 2001). First of all, activation requires the phosphorylation by an upstream kinase of a conserved threonine residue (Thr210) in the activation loop of the catalytic domain. Secondly, an additional conformational change is mediated by the Snf4 γ–subunit, through interaction with the regulatory domain, allowing for maximal activity of the Snf1 kinase (Jiang and Carlson, 1996). These two steps will disrupt the interaction between the catalytic domain with the regulatory domain, thereby relieving auto-inhibition and activating the Snf1 kinase. The β–subunits (Gal83, Sip1 or Sip2) play a structural role in the Snf1-Snf4 complex and also define substrate specificity by regulating the subcellular localization of their accompanying Snf1 kinase subunit and mediating the interaction of Snf1 with specific targets (Ashrafi et al., 2000; Chu et al., 1998; Hubbard et al., 1992; Jiang and Carlson, 1997; Schmidt and McCartney, 2000; Vincent and Carlson, 1999; Vincent et al., 2001; Yang et al., 1992).

Three upstream kinases, Pak1, Tos3 and Elm1, can activate the Snf1 kinase *in vivo* via phosphorylation of its Thr210 residue (Hong et al., 2003; Nath et al., 2003; Sutherland et al., 2003). These three kinases appear to be partially redundant in their activation of Snf1 as only a *pak1Δ tos3Δ elm1Δ* can give a *snf1*$^{-}$ mutant phenotype and completely abolishes the Snf1 catalytic activity (Hong et al., 2003; Sutherland et al., 2003). However, the different Snf1 kinase kinases make different contributions to cellular regulation under different growth conditions (Hedbacker et al., 2004; Hong et al., 2005; Kim et al., 2005). Furthermore, the different isoforms of the Snf1 kinase (i.e. Snf1-Gal83, Snf1-Sip1 and Snf1-Sip2) display stress-dependent preferences for the Pak1, Tos3 and Elm1 kinases (McCartney et al., 2005).

Inactivation of Snf1 activity occurs via dephosphorylation mediated by the yeast protein phosphatase type 1 (PP1), which plays an essential role in several cellular processes such as carbon catabolite repression, regulation of glycogen accumulation, sporulation, cell cycle progression, and translation (Cannon et al., 1994; Feng et al., 1991; Hisamoto et al., 1994; Wek et al., 1992). Specificity of PP1, of which the

catalytic subunit is encoded by the *GLC7* gene, towards its various substrates is governed through interaction with specific regulatory subunits (Frederick and Tatchell, 1996; Hisamoto et al., 1995; Lenssen et al., 2005; MacKelvie et al., 1995; Tu and Carlson, 1995; Tu et al., 1996; Zhang et al., 1995). In the glucose repression pathway Glc7 interacts with the regulatory subunit Reg1 (Gancedo, 1998; Huang et al., 1996a). Reg1 interacts with the catalytic domain of Snf1 and recruits the catalytic Glc7 to dephosphorylate Snf1 and presumably other components of the kinase complex (Ludin et al., 1998; Sanz et al., 2000). In this way, the auto-inhibited state of the Snf1 kinase complex is reintroduced.

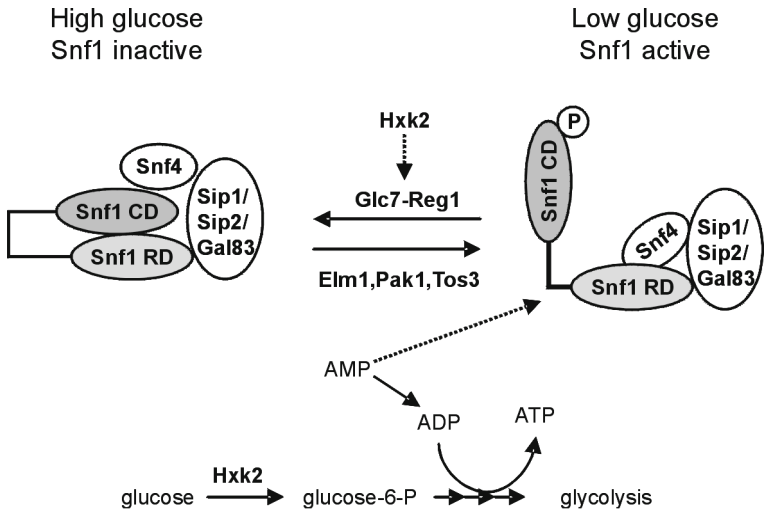


Figure I.1: The Main Glucose Repression Pathway in *S. cerevisiae*. In glucose-grown cells, the Snf1 kinase complex exists predominantly in an inactive state through auto-inhibition of the catalytic domain (CD) by the regulatory domain (RD). Activation upon glucose exhaustion depends on phosphorylation of Snf1 at its Thr210 residue in the catalytic domain by one of the upstream kinases, Elm1, Pak1 or Tos3. In addition the γ–subunit Snf4 mediates a conformational change leading to full Snf11 activity. Substrate specificity of the Snf1 kinase is established via the associated β–subunits; Sip1, Sip2 or Gal83. Deactivation of the Snf1 kinase occurs via a Glc7-Reg1 mediated dephosphorylation. The roles of the Hxk2 protein and/or the metabolite AMP in mediating the glucose signal are still unclear. See text for details.

Many of the transcriptional effects exerted by the Snf1 kinase complex operate via the Mig1 protein. Mig1 constitutes a glucose-dependent transcriptional repressor which binds to specific sites in the promoter of numerous glucose-repressed genes. In the presence of glucose, Mig1 is localised in the nucleus and prevents transcription by recruiting the general co-repressor proteins Ssn6 and Tup1 (De Vit et al., 1997;

Keleher et al., 1992; Treitel and Carlson, 1995; Vallier and Carlson, 1994). Depletion of glucose triggers Snf1-dependent phosphorylation of Mig1, leading to a loss of interaction with the Ssn6 and Tup1 co-repressors and subsequent Mig1 translocation to the cytoplasm, thereby relieving glucose repression of Mig1-dependent genes (DeVit and Johnston, 1999; Papamichos-Chronakis et al., 2004; Smith et al., 1999; Treitel et al., 1998). In addition, Snf1 stimulates the activity of the transcriptional activators Cat8 and Sip4, both being specific activators of gluconeogenic genes (Lesage et al., 1996; Randez-Gil et al., 1997).

Although no further metabolism beyond glucose phosphorylation is required for signalling glucose repression, the nature and target of the initial glucose signal that affects Snf1 function remains a matter of discussion. Based on the similarity between the Snf1, Snf4, and Sip proteins and the subunits of the mammalian AMP-activated protein kinase, it was proposed that AMP levels or the AMP/ATP ratio can act as a signal for the sensing of glucose phosphorylation and metabolic activity. Although the Snf1 activity correlates well with the AMP/ATP ratio upon glucose withdrawal (Wilson et al., 1996) or when cells approach stationary phase (Rudoni et al., 2001), AMP and ATP levels were shown to be very similar during growth on glycerol and glucose (Banuelos et al., 1977) and are therefore unlikely to be the main signal for glucose repression. Also, several studies indicate that the Snf1 protein kinase is not directly activated by AMP *in vitro* (Mitchelhill et al., 1994; Woods et al., 1994), due to substitutions in essential amino acids required for AMP binding when compared to the mammalian AMPK (Adams et al., 2004; Bateman, 1997; Carling, 2004; Daniel and Carling, 2002; Hardie et al., 2003).

In another model, a specific regulatory role is attributed to the hexokinase 2 (Hxk2) (Hohmann et al., 1999; Kraakman et al., 1999b; Mayordomo and Sanz, 2001) as Hxk2, but not hexokinase 1 (Hxk1) or glucokinase (Glk1), is required to maintain glucose repression during growth on glucose (De Winde et al., 1996). Hxk2 might exert its regulatory role via stimulation of the Glc7-Reg1 protein phosphatase (Herrero et al., 1989; Sanz et al., 2000). In addition, a fraction of the Hxk2 pool is located in the nucleus (Randez-Gil et al., 1998) where it may be directly involved in the glucose repression process through associating with DNA-protein complexes (Herrero et al., 1998).

Other possible targets to receive the glucose signal are the Glc7-Reg1 protein phosphatase (Hong et al., 2005) and the Snf1 upstream kinases. Alternatively, the glucose signal may result from a combination of the abovementioned possibilities. For instance, when glucose becomes exhausted, the AMP signal may activate Snf1. During the following period, when growth is adjusted to the new, alternative carbon source, ATP levels will recover and another signalling component may take over the function of keeping Snf1 active. Further studies are required to clarify the nature of the glucose signal affecting Snf1 function.

1.2 The Ras-cAMP pathway

The Ras-cAMP pathway plays a major role in the control of stress resistance, metabolism and proliferation. Cyclic AMP has been implicated as an important second messenger in the nutrient response, accomplishing the metabolic adaptation from respiratory to fermentative growth conditions through modulation of the activity of the cAMP-dependent protein kinase A (PKA). Synthesis of cAMP from ATP is catalysed by adenylate cyclase (*CYR1*/*CDC35*) (Kataoka et al., 1985; Matsumoto et al., 1984) of which the activity is controlled by the Ras-cAMP pathway (Fig. I.2) (Thevelein et al., 2000; Thevelein and de Winde, 1999). The activity of adenylate cyclase is stimulated by two types of G proteins: the small G proteins Ras1 and Ras2 and the G_α protein Gpa2. These G proteins act as molecular switches, cycling between a GTP-bound, active conformation and a GDP-bound inactive state.

The two small G proteins Ras1 and Ras2 are essential for adenylate cyclase activity and cell viability (Field et al., 1988; Toda et al., 1985). Activation of the Ras proteins (by exchanging the bound GDP for GTP) is catalysed by the ‘guanine nucleotide exchange factor (GEF)’ Cdc25 and to a lesser extent Sdc25 (Broek et al., 1987; Camonis and Jacquet, 1988; Camus et al., 1994; Jones et al., 1991). Inactivation (via hydrolysis of the bound GTP to GDP) on the other hand is stimulated by the ‘GTPase activating proteins (GAP)’ Ira1 and Ira2 (Tanaka et al., 1991; Tanaka et al., 1989; Tanaka et al., 1990a; Tanaka et al., 1990b).

The second G protein involved in adenylate cyclase activation is encoded by the *GPA2* gene which was originally cloned as a yeast homologue of mammalian heterotrimeric G_α proteins (Nakafuku et al., 1988). Together with the upstream

receptor-like protein, Gpr1, Gpa2 constitutes a G protein coupled receptor system (GPCR) (Kraakman et al., 1999a; Xue et al., 1998; Yun et al., 1997). Gpa2 is negatively regulated by Rgs2, a member of the family of regulators of G protein signalling (RGS) which acts as a GTPase for Gpa2 (Versele et al., 1999).

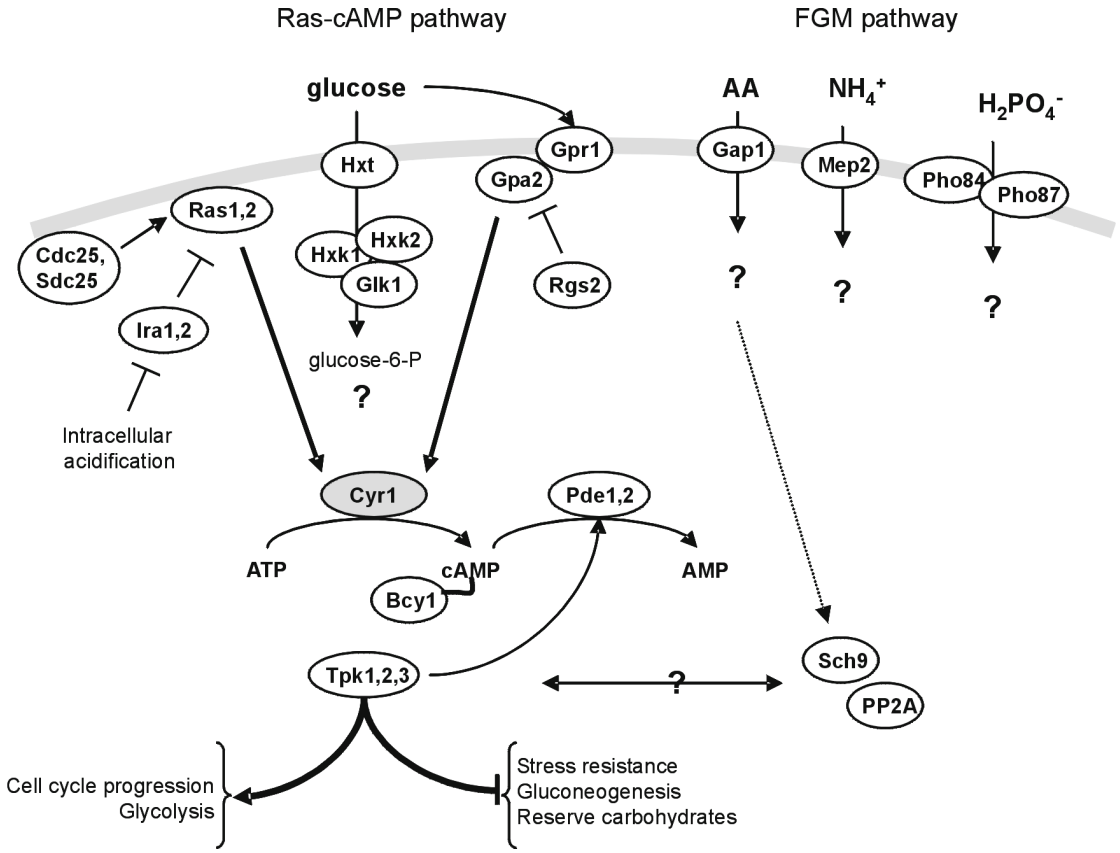


Figure I.2: The Ras-cAMP and FGM pathway in *S. cerevisiae*. Addition of glucose to derepressed cells triggers the rapid synthesis of cAMP leading to activation of PKA, which mediates the adaptation to glucose presence. Two glucose-sensing systems are involved in cAMP signalling: (i) detection of extracellular glucose by the Gpr1-Gpa2 system and (ii) detection of intracellular glucose via the glucose phosphorylation event. The second system may operate by regulating the GTP-loading of Ras proteins. The FGM (Fermentable Growth Medium induced) pathway regulates the same targets as PKA, though acts in a cAMP-independent manner. FGM signalling starts with the detection of nutrients by specific transport proteins. The signalling of amino acids (AA) requires the Sch9 protein kinase, which may direct signals of the protein phosphatase PP2A. . The grey bar represents the plasma membrane. See text for details.

Only two stimuli are known to activate cAMP synthesis in yeast: intracellular acidification and addition of glucose (or sucrose) to derepressed cells. Intracellular

acidification increases the GTP/GDP ratio on Ras, possibly via inhibition of the GTPases Ira1 and Ira2 and independent of Cdc25 and Sdc25 (Colombo et al., 1998). However, at least one other target of intracellular acidification exists for stimulation of cAMP synthesis, possibly adenylate cyclase itself. Gpa2 appeared not to be involved in the cAMP signal provoked by intracellular acidification. A potential role for this intracellular acidification control on cAMP synthesis may lie in the maintenance of a proper intracellular pH and ATP level during carbon starvation (Thevelein and de Winde, 1999).

Glucose-induced cAMP synthesis requires two sensing systems. The first system is an intracellular glucose-sensing mechanism that requires glucose uptake and phosphorylation and appears to be an absolute prerequisite for activation of the Ras-cAMP pathway by glucose (Rolland et al., 2000). Further metabolism of the glucose, however, is not required (Beullens et al., 1988). In contrast to the glucose repression pathway, any of the three sugar phosphorylating enzymes (Hxk1, Hxk2, and Glk1) can activate the Ras-cAMP pathway. Yet, neither glucose-6-phosphate (Glc-6-P) nor ATP, the substrate of adenylate cyclase, appears to act as metabolic trigger for cAMP signalling (Rolland et al., 2001). The glucose phosphorylation requirement may act through Ras to sustain cAMP signalling (Mbonyi et al., 1988). Indeed, glucose phosphorylation has been shown to be necessary for glucose-induced Ras-GTP loading, likely by inhibition of the Ira proteins (Colombo et al., 2004). The second sensing system comprises extracellular glucose detection mediated by the GPCR system, consisting of Gpr1 and Gpa2. Addition of glucose to derepressed cells activates the low affinity Gpr1 receptor, which in turn stimulates the exchange of GDP for GTP on Gpa2 (Kraakman et al., 1999a; Lemaire et al., 2004; Rolland et al., 2000). Gpr1 displays a high specificity for D-glucose and sucrose, with an apparent Km for activation of 75 mM and 1 mM, respectively. An intriguing item that remains to be resolved is how both glucose-sensing systems are integrated to control adenylate cyclase activity. Another important aspect of cAMP signalling involves its glucose-repressible nature (Thevelein, 1991), suggesting a connection with the main glucose repression pathway. Indeed, no glucose-induced cAMP increase can be observed in *snf1* mutants, which are unable to derepress (Arguelles et al., 1990).

The sole function of cAMP in yeast appears to be activation of PKA (Matsumoto et al., 1985). PKA is a hetero-tetramer consisting of two catalytic subunits encoded

redundantly by *TPK1*, *TPK2* ad *TPK3* (Toda et al., 1987b), and two regulatory subunits encoded by *BCY1* (Toda et al., 1987a). Inactivation of any of the PKA catalytic subunits is viable, whereas deletion of all three genes is lethal, suggesting largely overlapping functions of Tpk1-3 for viability, which is also reflected by a high degree of homology (Toda et al., 1987b). Nonetheless, there are examples demonstrating that the catalytic subunits also exhibit separate, specific functions (Chevtzoff et al., 2005; Pan and Heitman, 1999; Ptacek et al., 2005; Robertson et al., 2000; Robertson and Fink, 1998; Singh et al., 2004). Cyclic AMP activates PKA by binding to its regulatory subunit, thereby releasing and activating the catalytic subunits (Kuret et al., 1988). Cyclic AMP in turn is hydrolysed by the low- and high-affinity phosphodiesterases, encoded by *PDE1* and *PDE2*, respectively (Nikawa et al., 1987; Sass et al., 1986; Wilson and Tatchell, 1988). The nature of the cAMP signal and the subsequent boost in PKA activity are transient due to a strong and fast feedback inhibition on cAMP levels. The low-affinity phosphodiesterase Pde1 has been shown to be specifically involved in this feedback regulation (Ma et al., 1999), whereas the high-affinity phosphodiesterase Pde2 is shown to be the major determinant of cAMP levels and thus stress tolerance in stationary phase (Park et al., 2005). Other components involved in feedback regulation of cAMP signalling may include the adenylate cyclase itself as well as the Ras proteins or one of its regulators (Colombo et al., 2004; Thevelein et al., 2000). In addition to cAMP-mediated control, PKA activity is also regulated by modulating the abundance, phosphorylation state and intracellular localisation of its subunits (Griffioen et al., 2000; Griffioen et al., 2001; Portela and Moreno, 2006; Schmelzle et al., 2004; Werner-Washburne et al., 1991).

The main function of the Ras-cAMP pathway is to signal the presence of glucose in the medium and, in turn, to switch cells from respiration to fermentation (Boy-Marcotte et al., 1996; Jiang et al., 1998). It is important to note that this signal transduction cascade can only be activated in glucose-derepressed cells. Once the switch from respiration to fermentation has been achieved and cells are glucose-repressed, they exhibit a ‘high PKA phenotype’ and the Ras-cAMP pathway is no longer responsive. In general, a ‘high PKA phenotype’ includes an upregulation of glycolytic enzymes and a downregulation of gluconeogenic enzymes, a mobilisation of the storage carbohydrates trehalose and glycogen (Thevelein, 1992), repression of

stress-responsive genes (Ruis and Schuller, 1995) and an induction of ribosomal protein genes (Griffioen et al., 1996; Kraakman et al., 1993). As a consequence of these changes, cells lose their stress resistance (Thevelein, 1994). In the absence of a fermentable carbon source, cells exhibit a 'low PKA phenotype' with opposite characteristics.

1.2.1 Transcriptional regulation of stress responsive genes by PKA through the Rim15-Msn2/4-Gis1 module

As a response to nutrient starvation, yeast will induce the expression of a multitude of stress responsive genes, contributing to the higher stress resistance of the yeast in this state (Winderickx et al., 2003). The multiple stress responses have been correlated with the activity of the transcriptional activators Msn2 and Msn4 (Schmitt and McEntee, 1996). Activity of these transcriptions factors is required for the expression of STRE (Stress Response Element)-controlled genes and is inhibited by PKA (Boy-Marcotte et al., 1998; Moskvina et al., 1998; Smith et al., 1998). During growth on glucose, Msn2 and Msn4 are phosphorylated and retained in the cytosol. When glucose becomes exhausted, cells undergo a diauxic shift, which precedes respiration. Upon this diauxic transition, the proteins become hyperphosphorylated, translocated to the nucleus and thus activated. This hyperphosphorylation is prevented by high PKA activity (Garreau et al., 2000; Gorner et al., 1998; Gorner et al., 2002). PKA directly phosphorylates Msn2 on its nuclear localisation domain, thereby stimulating nuclear exclusion of Msn2 and thus inhibiting its function. Another effector of PKA in mediating transcriptional regulation of the STRE-controlled genes is the Ccr4-Not complex. It has been suggested that this complex contributes to repress transcription by Msn2/4, possibly through direct interaction with Tpk2 and modulation of the phosphorylation status of Msn2 via the Glc7-Bud14 protein phosphatase (Lenssen et al., 2005; Lenssen et al., 2002). Proper entry into the diauxic shift also requires the transcriptional activator Gis1. This factor binds to the so-called post-diauxic shift (PDS)-elements. The activity of Gis1 as well as that of Msn2 and Msn4 depends on the protein kinase Rim15, whose kinase activity is negatively regulated by PKA, presumed through direct phosphorylation (Cameroni et al., 2004; Pedruzzi et al., 2000; Reinders et al., 1998). Interestingly, deletion of either *RIM15* or both *MSN2* and

MSN4 can rescue the lethality of an otherwise lethal *tpk null* strain (Roosen et al., 2004).

Rim15 as an integration point for nutrient signalling

Besides the negative regulation of its kinase activity through PKA-mediated phosphorylation, Rim15 function is additionally regulated at the level of its subcellular localisation by the nutrient-regulated kinases TORC1, Sch9 and Pho85-Pho80. The Tor pathway is highly conserved from yeast to mammalian cells and controls cellular growth and proliferation in response to several nutrients, especially carbon sources and nitrogen (Schmelzle and Hall, 2000). The Sch9 protein kinase has been implicated as a glucose- and nitrogen-responsive regulator of the fermentable growth medium-induced pathway (Crauwels et al., 1997), while the Pho85-Pho80 kinase is the central player in the phosphate-responsive PHO pathway in yeast (Lenburg and O'Shea, 1996). In cells growing exponentially on a complete glucose-containing medium, Rim15 is sequestered in the cytoplasm via its interaction with 14-3-3 proteins (Wanke et al., 2005), allowing inactivation of Rim15 by PKA (Reinders et al., 1998). When starved for essential nutrients, Rim15 translocates to the nucleus to prepare the yeast cell for entry into the quiescent G_0 phase (Pedruzzi et al., 2003; Swinnen et al., 2005; Wanke et al., 2005). Active Rim15 also leads to autophosphorylation, which accelerates its nuclear export by Msn5 (Wanke et al., 2005). The TORC1 kinase, Sch9 as well as Pho85-Pho80 all function to promote cytoplasmic retention of Rim15 under conditions of abundant nutrient supply. Indeed, inhibition of the Tor kinases by treatment with rapamycin or deletion of *SCH9*, *PHO85* or *PHO80* results in a nuclear localisation of a kinase-dead Rim15 on glucose medium (Pedruzzi et al., 2003; Wanke et al., 2005). Rim15 is shown to be a direct *in vivo* target of the Pho85-Pho80 kinase (Wanke et al., 2005). Interestingly, the Pho85-Pho80 and TORC1 kinases converge on a single amino acid in Rim15, Thr1075, phosphorylation of which induces interaction with the cytoplasmic 14-3-3 anchor proteins. As TORC1 and Pho85-Pho80 localise to the cytoplasm and nucleus, respectively (Harris and Lawrence, 2003; Kaffman et al., 1998a), these kinases may act on different pools of Rim15. The mechanism by which Sch9 regulates Rim15 localisation is not known yet. Rim15 thus constitutes an integration point of at least

four major nutrient signalling pathways in yeast involving the protein kinases PKA, TORC1, Sch9 and Pho85-Pho80. Rim15 therefore plays an essential role in the integration of several nutrient signals allowing the yeast cell to properly adapt to the availability of a large battery of nutrients, ranging from carbon and nitrogen sources to inorganic phosphate (Fig. I.3).

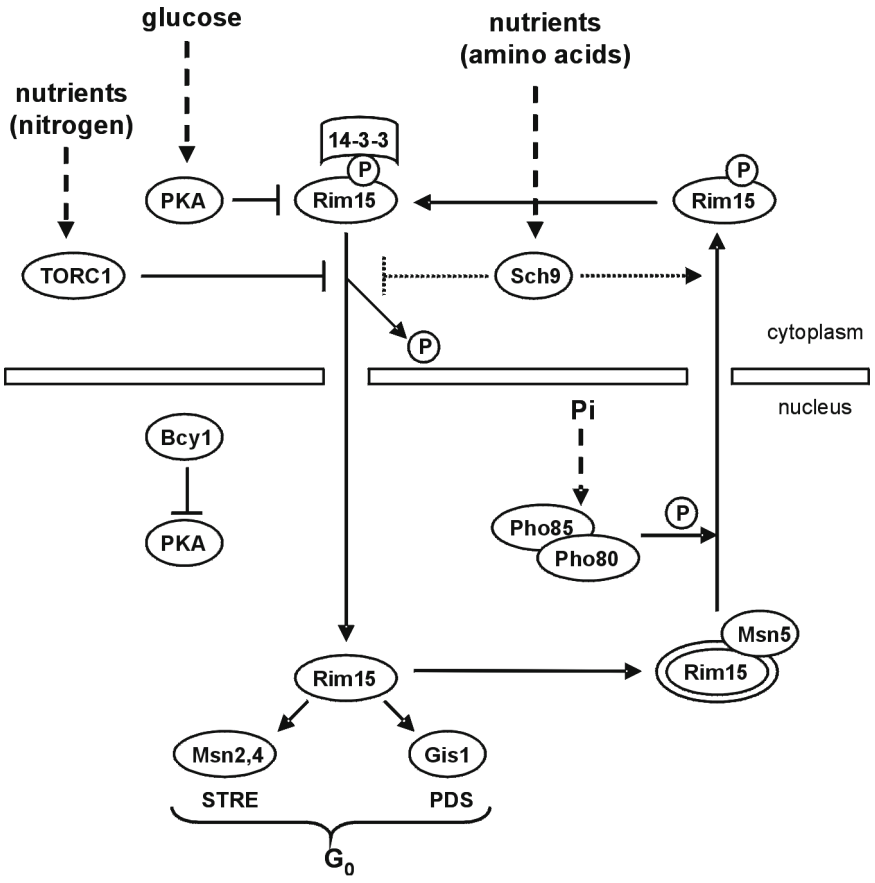


Figure I.3: Rim15 is a nutritional integrator in *S. cerevisiae*. Rim15 activity is regulated by at least four nutrient-regulated protein kinases. Cytoplasmic Rim15, anchored through its binding 14-3-3 proteins, is kept inactive through PKA-mediated phosphorylation. TORC1 inactivation results in dephosphorylation of phospho-Thr1075 (indicated with P) in Rim15 with concomitant abrogation of cytoplasmic retention of Rim15 by 14-3-3 proteins. Following its nuclear import, Rim15 presumably escapes further PKA-mediated inhibition (as Bcy1 inhibits PKA activity in the nucleus). Active Rim15 regulates entry into the stationary (G_0) phase via regulation of the Msn2,4 and Gis1 transcription factors. Msn2,4 regulates expression of stress responsive (STRE) genes while Gis1 induces transcription of post-diauxic shift (PDS) genes. Active Rim15 also initiates an autophosphorylation process (illustrated by a circle around Rim15) which stimulates its nuclear export via Msn5. The nuclear Pho85-Pho80 kinase stimulates phosphorylation of Thr1075 on Rim15, thereby promoting cytoplasmic retention of nuclear exported Rim15. The mechanism by which Sch9 regulates Rim15 localisation is not known. See text for details.

Additionally, the N-terminus of Rim15 contains a PAS (Per-Arnt-Sim) domain (Cameroni et al., 2004), a small regulatory module represented in all kingdoms of life which functions as a *cis*-acting regulatory domain in proteins, responding to a wide variety of stimuli, such as changes in light, redox potential, oxygen, small ligands and overall energy level of the cell (Amezcua et al., 2002; Gilles-Gonzalez and Gonzalez, 2004; Rutter et al., 2001; Rutter et al., 2002; Taylor and Zhulin, 1999). Thus, in addition to the nutrient-induced signalling pathways described above, Rim15 activity might be regulated by additional factors via its PAS domain.

1.2.2 Regulation of reserve carbohydrates metabolism by PKA

Another common response of yeast cells to nutrient starvation is the accumulation of trehalose and glycogen (Lillie and Pringle, 1980). Trehalose is a widely distributed reserve carbohydrate, found in bacteria, fungi, plants and insects but is apparently absent in vertebrates (Chen and Haddad, 2004; Gancedo and Flores, 2004). Yeast cells accumulate trehalose when deprived for e.g. glucose, nitrogen or phosphate, which suggests a function as a reserve carbohydrate. In *Saccharomyces cerevisiae*, trehalose synthesis is catalysed by the trehalose synthase complex encoded by *TPS1*, *TPS2*, *TPS3* and *TSL1* (Francois and Parrou, 2001). Tps1 catalyses the first step of trehalose synthesis; the formation of trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate. Tps2 dephosphorylates trehalose-6-phosphate to yield trehalose. Tps3 and Tsl1 probably function to stabilise the trehalose synthase complex and are required for optimal enzyme activity (Bell et al., 1998). Trehalose degradation is accomplished through hydrolysis catalysed by the trehalase enzyme. Three different trehalase enzymes have been identified in yeast, encoded by the *ATH1*, *NTH1* and *NTH2* genes. *ATH1* encodes a vacuolar acid trehalase and is essential for growth on trehalose as sole carbon source (Nwaka et al., 1996; Plourde-Owobi et al., 1999). Nth1 and Nth2 are two neutral cytosolic trehalases. Nth1 is primarily responsible for trehalose hydrolysis in response to nutrient presence, whereas Nth2 may be involved in the degradation of trehalose during recovery from heat stress (Nwaka and Holzer, 1998; Nwaka et al., 1995).

All genes of the trehalose synthase complex, *TPS1*, *TPS2*, *TPS3* and *TSL1*, contain STRE-elements in their promoter, allowing for coordinated expression of these genes

under stress conditions. High PKA activity inhibits their expression, through its negative action on the Msn2 and Msn4 transcription factors and possibly also via the Sok2 repressor (Enjalbert et al., 2004). However, of these genes, only *TSL1* shows an expression pattern like that of typical STRE-controlled genes (Winderickx et al., 1996). The other genes are less sensitive to downregulation of transcription during growth on glucose, which may relate to the involvement of trehalose biosynthesis in regulating growth on glucose (Teusink et al., 1998). In addition, the Tps1 protein is inactivated *in vitro* by PKA and is also subject to catabolite inactivation (Francois et al., 1991; Panek et al., 1990; Panek et al., 1987), providing additional mechanisms for regulating trehalose levels in response to nutrient levels.

Like the *TPS* genes, the *NTH1* gene also possesses STRE promoter elements (Zahringer et al., 1997). However, despite induction of *NTH1* expression during the diauxic shift, the Nth1 protein is maintained in an inactive state suggesting a tight but opposite control of the enzyme at the transcriptional and post-translational levels (Coutinho et al., 1992; Zahringer et al., 2000). Apparently, the trehalase enzyme has to be activated post-translationally, most likely via phosphorylation (App and Holzer, 1989). Several studies indicate that Nth1 activity correlates well with PKA activity *in vivo* (Durnez et al., 1994; Mbonyi et al., 1990; Zahringer et al., 1998) and trehalase can be activated *in vitro* through phosphorylation by PKA (App and Holzer, 1989; Ortiz et al., 1983; Uno et al., 1983). However, all attempts to demonstrate phosphorylation of neutral trehalase *in vivo*, e.g. during nutrient-induced activation, have been unsuccessful and therefore it is uncertain if nutrient-induced activation of trehalase is mediated through direct phosphorylation by PKA *in vivo*. Furthermore, activation of trehalase may be mediated by phosphorylation of more than one site (Wera et al., 1999) and require the involvement of additional protein kinases (Roosen et al., 2005; Souza et al., 2002; Swinnen et al., 2005). Also, while overexpression of the yeast protein phosphatase 2A inactivates the trehalase enzyme *in vitro*, the same overexpression leads to a 'high PKA phenotype' with elevated basal trehalase activity *in vivo* (Sugajska et al., 2001) illustrating that *in vitro* results have to be interpreted with caution.

Besides functioning as a storage carbohydrate, trehalose has also been shown to act as a stress protectant (Singer and Lindquist, 1998). In addition, trehalose metabolism, especially the Tps1 protein, is involved in the regulation of glycolytic fluxes in response to the availability of rapid fermentable sugars (Teusink et al., 1998;

Thevelein and Hohmann, 1995), a function which may depend on the metabolite trehalose-6-phosphate (Blazquez et al., 1993) and/or a free Tps1 fraction which is not part of the trehalose synthase complex (Bell et al., 1998). This regulation of glycolytic flux has also been suggested to play an important role in the adaptation to several stress conditions, by introducing ATP futile cycles leading to a stabilisation of glycolytic rates under slow or non-proliferating stress conditions (Blomberg, 2000; Godon et al., 1998; Hottiger et al., 1987; Parrou et al., 1997). Finally, besides serving as storage factors under carbon starvation, trehalose and also glycogen reserves may function to provide auxiliary carbon and ATP sources when conditions improve, thereby enabling the yeast cell to enter a new cell cycle more rapidly (Sillje et al., 1999; Sillje et al., 1997).

Glycogen accumulates during exponential growth, when ample glucose is still available, whereas trehalose is not synthesized until the glucose level is nearly exhausted (Lillie and Pringle, 1980; Parrou et al., 1999). At the end of stationary phase, glycogen accumulation stops and even turns into mobilisation, in contrast to trehalose production which continues further in stationary phase. Thus, in contrast to trehalose, glycogen behaves like a typical reserve carbohydrate. The glycogen synthesis involves initiation, elongation and ramification steps. Glycogen synthesis is initiated by self-glucosylating proteins, encoded by *GLG1* and *GLG2*, and continued by glycogen synthase, encoded by *GSY1* and *GSY2*. The chains are ramified by the branching enzyme Glc3. Enzymes involved in degradation are encoded by *GPH1* and *GDB1*, encoding respectively a glycogen phosphorylase and debranching enzyme (reviewed by (Francois and Parrou, 2001). Similar to trehalose accumulation, high PKA activity inhibits glycogen accumulation, by repressing transcription of genes encoding the key enzymes involved in glycogen biosynthesis and inactivating their protein products (Enjalbert et al., 2004; Francois and Parrou, 2001; Hardy et al., 1994). However, obtained data on *in vitro* activities of Gsy2 and Gph1 (Hardy and Roach, 1993; Wingender-Drissen and Becker, 1983) has not yet led to convincing evidence that PKA directly phosphorylates these two enzymes *in vivo* and the effect of PKA on the activity of Gsy2 and Gph1 is more likely indirect through modulation of other signalling pathways.

2. The Fermentable Growth Medium-induced (FGM) pathway

The Ras-cAMP pathway triggers multiple responses upon the addition of glucose to glucose-derepressed cells. However, the activation of PKA by cAMP is only transient due to feedback mechanisms (see above) and the active state of the Ras-cAMP pathway seems to be restricted to the transition from respiratory to fermentative growth (Jiang et al., 1998). Nevertheless, fermenting cells growing on a complete growth medium exhibit a typical 'high PKA phenotype'.

Readdition of any essential nutrient, such as nitrogen sources, phosphate or sulphate to cells starved for this nutrient on glucose-containing medium, triggers a rapid activation of trehalase with concomitant trehalose mobilisation (Hirimburegama et al., 1992). These rapid changes in trehalase activity are independent on protein synthesis and hence occur at the posttranslational level, presumably by protein phosphorylation (Durnez et al., 1994; Giots et al., 2003; Hirimburegama et al., 1992). Other typical PKA targets are also affected in a way similar to the situation with activation of the Ras-cAMP pathway, i.e. STRE-controlled genes are repressed and ribosomal protein genes are induced (Giots et al., 2003; Pernambuco et al., 1996). Notably however, the effects triggered by readdition of an essential nutrient in a fermentable medium do not appear to be triggered by a cAMP signal. Under fermentative conditions, no transient increase in the cAMP level is generated, and the effects mentioned are also observed in mutants lacking the regulatory subunit of PKA, Bcy1, further ruling out a cAMP-mediated effect (Durnez et al., 1994; Giots et al., 2003; Hirimburegama et al., 1992; Thevelein, 1991). Despite of the independence of cAMP, the effects do seem to require the presence of PKA (Durnez et al., 1994; Giots et al., 2003). Hence, it appears that during fermentative growth PKA is activated in a novel, non-cAMP mediated way. Since this activation is dependent on the presence of glucose or another rapidly-fermentable sugar, and concomitantly on all other nutrients required for growth, the pathway involved was called the 'Fermentable Growth Medium-induced (FGM) pathway' (Fig. I.2) (Thevelein, 1994). This model hypothesises that during fermentative growth the FGM pathway may control PKA activity by acting directly on free catalytic subunits. The cAMP requirement is restricted to a basal level necessary to provide a certain level of these free catalytic subunits. Obviously, the FGM pathway must be complex with distinct nutrient-sensing systems signalling to a

common information-integrating system, such as the Rim15 protein kinase (see above). Although the presence of a rapidly fermentable sugar is an absolute requirement for FGM signalling (Durnez et al., 1994), many of the FGM signalling effects are independent of sugar phosphorylation (Giots et al., 2003; Pernambuco et al., 1996). Intriguingly, the glucose sensing step in the FGM pathway can be fulfilled either through the glucose-phosphorylation step (mediated by Hxk1, Hxk2 or Glk1) or via detection by the Gpr1-Gpa2 GPCR (Giots et al., 2003; Winderickx et al., 2003), indicating a fundamental difference with the requirements for glucose sensing in the Ras-cAMP pathway. According to the concept of FGM-signalling, the role of the Ras-cAMP pathway would be confined to the transition from non-fermentable to fermentable growth conditions. The transient cAMP increase triggers a rapid dissociation of the regulatory subunits of PKA resulting in a temporary boost in PKA activity. This PKA boost facilitates resetting of different regulatory mechanisms (Jiang et al., 1998). Due to feedback inhibition, the cAMP level falls back to about its basal level but at the same time the FGM pathway takes over the function as main determinant of PKA activity. At the end of fermentation, before the diauxic shift, the FGM activity also drops and an increased expression and/or modification of the regulatory subunit Bcy1 reset PKA activity level (Werner-Washburne et al., 1991). Consequently, the cells reprogram to respiratory growth and subsequently to stationary phase (Boy-Marcotte et al., 1996; Werner-Washburne et al., 1996).

Several data implicate the Sch9 protein kinase as an essential component for nitrogen-induced FGM signalling. Sch9 shares sequence similarity with the catalytic subunits of PKA (Jin et al., 1995; Toda et al., 1988) and it is a functional homologue of mammalian protein kinase B (PKB/Akt) (Geyskens et al., 2000). *SCH9* was originally isolated as a multicopy suppressor of strains deficient in PKA activity (Toda et al., 1988). An *sch9Δ* strain exhibits a slow growth and small colony phenotype which can be suppressed by activation of the Ras-cAMP pathway (Hartley et al., 1994; Toda et al., 1988). Also, the growth defect of the *sch9Δ* strain is seriously exacerbated when combined with deletions of *GPR1*, *GPA2* and *RAS2*, which are all implicated in glucose-induction of the cAMP signal (Kraakman et al., 1999a; Lorenz et al., 2000). These data suggest that Sch9 might act as an activator of the free catalytic subunits. More recent data, however, revealed that Sch9 acts in parallel to PKA to control the

activity of Rim15, another kinase important for FGM signalling (Fig. I.3 (Roosen et al., 2005)).

The role of Sch9 for FGM signalling was confirmed by the observation that glucose-repressed *sch9Δ* cells are defective for nitrogen-induced trehalase activation, repression of STRE-controlled genes and induction of ribosomal protein genes while derepressed *sch9Δ* cells are still glucose-responsive for all these parameter (Crauwels et al., 1997). It was also observed that the positive effect of Sch9 on PKA activity is restricted to fermentative growth conditions. In derepressed cells growing on nonfermentable carbon sources, Sch9 appears to act as an inhibitor of PKA. This carbon source dependent switch in function may relate to differences in phosphorylation of Sch9. The protein phosphatase PP2A was identified as a second component of the FGM pathway. Although PP2A could dephosphorylate and thereby inactivate trehalase *in vitro*, overexpression of one of the subunits of the phosphatase, i.e. Pph22, resulted in a 'high PKA phenotype' *in vivo* when glucose- and nitrogen-induced responses were examined, including a defect in trehalose accumulation, high trehalase activity and constitutive repression of STRE-controlled genes (Sugajska et al., 2001). Overexpression of *PPH22* in a *sch9Δ* strain had no effect on the glucose-induced responses indicating that Sch9 is necessary to mediate the effects of PP2A. Furthermore, since overexpression of *PPH22* had no effect on the glucose-induced cAMP signal, PP2A seems to act on a component downstream of cAMP synthesis. In glucose-repressed cells, deletion of *SCH9* by itself causes the same defect in nitrogen-induced signalling as that mediated by overexpression of *PPH22* (Crauwels et al., 1997; Sugajska et al., 2001). Thus, Pph22 might regulate the function of Sch9, possibly through direct dephosphorylation. Finally, phosphoinositides appear to act as second messengers in nitrogen signalling (Bergsma et al., 2001; Schomerus and Kuntzel, 1992; Wera et al., 2001). However, a connection between phosphoinositide signalling and the activation of Sch9 still remains to be established.

Inorganic phosphate is proven to be also able to activate the FGM pathway (Giots et al., 2003). Phosphate-induced trehalase activation occurs without a cAMP signal and is independent from protein synthesis. Furthermore, ATP does not act as a second messenger in this pathway. Strikingly, phosphate-induced FGM signalling is not affected by a deletion of *SCH9*, similar to the situation in derepressed *sch9Δ* cells (Crauwels et al., 1997; Giots et al., 2003). In a *sch9Δ* strain, basal levels of trehalase

activity were somewhat higher after phosphate starvation and basal expression of the stress responsive genes *SSA3* and *HSP12* were lower, consistent with higher basal PKA activity under these conditions (Giots et al., 2003). Apparently, phosphate and nitrogen signalling use different pathways for activation of the PKA targets. Intriguingly, the function of Sch9 in phosphate-induced FGM-signalling resembles the role it plays in glucose-induced signalling, although the cells were starved for phosphate in the presence of excess glucose and no cAMP signal is generated after phosphate re-addition. The cyclin-dependent protein kinase (CDK) Pho85, a central component of the phosphate responsive PHO signalling pathway (Lenburg and O'Shea, 1996), is recently shown to be involved in phosphate-induced signalling controlling typical PKA targets such as trehalase activity, trehalose accumulation and expression of stress responsive genes and ribosomal protein genes (Swinnen et al., 2005). Regulation of many of these properties occurs via a Pho85-dependent regulation of Rim15 function (Fig. I.3). Interestingly, a *pho85Δ sch9Δ* strain displays a synthetic lethal phenotype, indicating that they operate in parallel, but converging pathways.

The FGM pathway must integrate information about the presence of various nutrients. The sensing of these nutrients appears to start at the level of the plasma membrane (Fig. I.2) (Roosen et al., 2004; Winderickx et al., 2003). More specifically, particular nutrient transporters activate FGM signalling, having a sensing function in addition to their transport function. Activation of the FGM pathway by amino acids is triggered by the general amino acid permease Gap1 (Donaton et al., 2003), ammonium-induced signalling requires the Mep2 ammonium permease (Van Nuland et al., 2006), while specifically the Pho84 and Pho87, but not the Pho89, Pho90 or Pho91 phosphate permeases can sustain rapid phosphate-induced FGM signalling (Giots et al., 2003).

The initial, rapid activation of the FGM pathway is independent of the yeast's metabolism. Indeed, nitrogen- or phosphate-induced activation of trehalase is independent of glucose phosphorylation and several non-metabolisable nutrient analogues, such as D-amino acids or the phosphate analogue arsenate, are capable of sustaining trehalase activation (Donaton et al., 2003; Giots et al., 2003; Pernambuco et al., 1996). This indicates that the yeast cell can directly detect the presence of a nutrient, independent of its further metabolism. In contrast, long-term maintenance of

the 'high PKA phenotype' appears to be very closely connected to the growth and consequently metabolism of the cells.
Nutrient-activation of the FGM pathway thus clearly affects typical PKA targets without the involvement of a cAMP signal although PKA activity is required. The central question remains whether the free catalytic subunits of PKA are directly modulated by nutrient signals generated by the FGM pathway or if the PKA requirement is indirect, for instance that PKA might be necessary for phosphorylation and basal activity of some of the essential FGM components. Data obtained so far favour the latter and indicate Rim15 as central signal integrator (Fig. I.3 (Roosen et al., 2005; Swinnen et al., 2005; Wanke et al., 2005)). Nevertheless, in addition to generating a cAMP signal, glucose addition to derepressed cells has been shown to cause phosphorylation of Tpk1, resulting in activation of this kinase (Portela and Moreno, 2006).

3. Phosphate metabolism and transport in *Saccharomyces cerevisiae*

Inorganic phosphate (Pi) is an essential nutrient for all organisms, being required for structural and metabolic needs, such as the biosynthesis of nucleic acids, phospholipids and cellular metabolites. Maintaining proper phosphate homeostasis is therefore critical to ensure survival of cells and organisms. Besides its nutrient function, inorganic phosphate also exerts an important signalling function, regulating not only its own transport and metabolism but also a pleiotropic set of properties related to the growth rate and general metabolic state as well as developmental capacities of the cells.

Saccharomyces cerevisiae, like most organisms, uses inorganic phosphate as the preferred phosphorus source (Johnston and Carlson, 1992). When extracellular Pi becomes limiting, extracellular acid phosphatases can hydrolyse phosphate units from a broad spectrum of phospho-ester substrates, thereby increasing the available Pi in the medium (Bostian et al., 1980; Bostian et al., 1983). In addition, yeast can utilise alternative phosphate sources, such as glycerophosphoinositol and glycerophosphocholine (Almaguer et al., 2004; Fisher et al., 2005).

Once transported into or generated in the yeast cell, Pi is incorporated into numerous compounds and metabolites. A major fate of free cellular Pi consists of the incorporation into inorganic polyphosphate (polyP), a linear polymer consisting of a few to several hundreds of Pi residues linked together by high-energy phosphoanhydride bonds. PolyPs are widespread in living organisms, being found in the cells of microorganisms, plants and animals (Kulaev and Kulakovskaya, 2000). Its exact role depends on the cell type, age, subcellular compartment and environmental conditions (Kornberg et al., 1999). The majority of yeast polyP is stored in the vacuole (Saito et al., 2005; Urech et al., 1978) and this vacuolar polyP plays a predominant role in the regulation of phosphate homeostasis and survival of the yeast cell under conditions of limiting Pi (Shirahama et al., 1996). Synthesis of polyP in the vacuole is thought to be mediated by a vacuolar membrane-bound enzyme system consisting of the Phm1-4 proteins (Cohen et al., 1999; Ogawa et al., 2000). It is postulated that the Phm1-4 system may use the energy stored in the proton motive force across the vacuolar membrane to directly synthesize the high-energy phosphoanhydride bonds in polyP from Pi, without an ATP intermediate (Ogawa et al., 2000). A similar situation of coupling of polyP synthesis to Pi transport may operate in lysosomes of animal cells (Pisoni and Lindley, 1992).

In addition to the vacuole, polyPs are present in the cytosol, mitochondria, nucleus and cell periphery. The presence of polyP in all these compartments suggests either that enzymes involved in the synthesis/degradation are also present in these organelles or, alternatively, that specific mechanisms mediate uptake/extrusion of polyP (Kulaev and Kulakovskaya, 2000; Lichko et al., 1998). To date, the *PPX1* gene is the only known gene encoding an exopolyphosphatase (Wurst and Kornberg, 1994; Wurst et al., 1995). Exopolyphosphatases degrade polyP by a stepwise removal of the terminal Pi residue ($polyP_n \rightarrow polyP_{n-1} + Pi$). More general phosphatases are located in multiple organelles and may also play a role in polyP turnover, e.g. the acid phosphatases Pho5, Pho11 and Pho12 are located in the extracellular periplasmic space (Haguenauer-Tsapis et al., 1986), while the alkaline phosphatase, Pho8, is localized in the vacuole (Plankert et al., 1991) and glycerol phosphatases such as Hor2 are located in the cytosol (Norbeck et al., 1996). These enzymes are capable of hydrolyzing a variety of substrates, including nucleic acids, phosphosugars, phospholipids and phosphoproteins (Oshima, 1997). More exopolyphosphatases have

been characterized although the corresponding genes have not yet been identified (Lichko et al., 2003). In addition, the *PHM5*/*PPN1* gene encodes an vacuolar endopolyphosphatase, responsible for internal cleavage of long chain polyP of several hundred residues to generate progressively shorter chains (Ogawa et al., 2000; Shi and Kornberg, 2005). Ppn1 activity is inhibited by Pi and pyrophosphate (PPi), creating a negative feedback regulation thereby preventing wasteful degradation of polyP during times when the end products, i.e. Pi and PPi, are abundant in the cell (Kumble and Kornberg, 1996). The mechanisms for polyP accumulation at many subcellular sites still remain elusive. Only for the yeast vacuole and cell envelope, mechanisms for polyP synthesis have been proposed (Kulaev and Kulakovskaya, 2000; Ogawa et al., 2000).

Regulation of polyP levels is controlled not only by the availability of exogenous Pi, but also by the presence of carbon source, the cell cycle and the specific growth phase of the yeast (Bostian et al., 1983; Gillies et al., 1981; Schuddemat et al., 1989; Vagabov et al., 2000; Vagabov et al., 1998; Werner et al., 2005). Aside from its obvious role as a phosphate reserve, polyP also functions as an energy source (Beauvoit et al., 1989; Bostian et al., 1983; Thomas and O'Shea E, 2005), it is involved in the detoxification and osmoregulation of the cytosol by trapping cations in vacuoles (Beeler et al., 1997; Dunn et al., 1994; Klionsky et al., 1990), buffering pH variations of the cytosol (Castro et al., 1995; Castrol et al., 1999; Pick et al., 1990), in nuclei their function is probably connected with the regulation of gene expression (Kornberg et al., 1999) and polyPs in the yeast cell envelope, take part in the formation of a negative charge on the cell surface (Kulaev and Kulakovskaya, 2000). Intriguingly, a *phm5Δ* strain not only shows a defect in vacuolar endopolyphosphatase activity (Ogawa et al., 2000), but also reduced growth and loss of viability in stationary phase in synthetic medium (Sethuraman et al., 2001), failure to grow on the non-fermentable carbon sources ethanol and lactate, and their mitochondria are defective in respiration functions (Pestov et al., 2005). How the absence of a vacuolar endopolyphosphatase causes these dramatic properties is not known. Perhaps several short-chain polyPs may play a role in these processes as signalling molecules. As described here, proper regulation of (poly)phosphate metabolism is crucial to the yeast cell for adaptation not only to different levels of external Pi but also a large number of other environmental conditions.

3.1 Phosphate transport across the plasma membrane

A crucial step in maintaining phosphate homeostasis encompasses the transport of Pi across the plasma membrane into the cell. Since cellular membranes are impermeable to Pi, specialized integral membrane transport proteins are required to transport this essential compound across these barriers. Metabolic reactions typically require millimolar concentrations of Pi whereas most environmental concentrations are substantially lower. In yeast cells, phosphate can be transported into cells against a large concentration gradient (Goodman and Rothstein, 1957). Pi-uptake depends on active metabolism of the cells and is greatly influenced by the available carbon source, with a great preference of fermentable sugars.

In *Saccharomyces cerevisiae*, two symport systems of phosphate transport have been kinetically characterized (Borst-Pauwels, 1981; Persson et al., 1998; Persson et al., 1999; Tamai et al., 1985). First, a low-affinity system exists with a K_m for external Pi of approximately 1 mM. This system is proposed to be expressed constitutively and satisfies the cellular need for Pi at normal or high concentrations. Secondly, a derepressible high-affinity transport system with a K_m of about 10 µM is activated when extracellular levels of Pi are scarce. Both these acquisition systems use the free energy stored in the electrochemical ion gradient across the plasma membrane for active sequestration of phosphate across this membrane.

3.1.1 High-affinity phosphate uptake

The high-affinity phosphate transport system consists of the Pho84 and Pho89 phosphate transporters. Expression of their correlating genes is induced under conditions of limiting Pi concentrations through the activation of the PHO pathway (Bun-Ya et al., 1991; Martinez and Persson, 1998).

The *PHO84* gene was cloned in 1991 by Bun-ya *et al.* based on the ability to complement the constitutive synthesis of repressible acid phosphatase of a *pho84* mutant. The Pho84 transporter is highly homologous to identified or proposed phosphate transporters of yeast, fungi and plants belonging to the phosphate:H^+ symporter (PHS) family, a member of the major facilitator superfamily (MFS) of

transporters (Pao et al., 1998; Paulsen et al., 1998). Interestingly, the Pho84 protein has also been shown to be highly homologous to several HXT sugar transporters of mammalian, yeast, and bacterial origin (Andre, 1995; Bun-Ya et al., 1991; Henderson, 1993; Nelissen et al., 1997; Nelissen et al., 1995). Therefore, Pho84 is also classified within the family of sugar permease homologues (Bun-Ya et al., 1991; Nelissen et al., 1997). Within *Saccharomyces cerevisiae*, Pho84 does not share significant amino acid similarity with other Pi transporters (Martinez and Persson, 1998; Nelissen et al., 1997). It does, however, share homology with the Git1 protein, a broad specificity glycerophosphoinositol permease (Patton-Vogt and Henry, 1998), which is also classified in the PHS family along with Pho84 (Paulsen et al., 1998).

Based on hydropathic analysis of the primary amino acid sequence and its homology with other members of the MFS family, a secondary structure model has been proposed (Bun-Ya et al., 1991; Fristedt et al., 1996; Lagerstedt et al., 2004; Persson et al., 1998; Persson et al., 1999). Pho84 consists of 12 transmembrane (TM) domains with a central, large hydrophilic loop of 74 amino acids separating TM6 and TM7. This central loop as well as the N-and C-termini are proposed to reside on the cytoplasmic side of the plasma membrane.

According to a mobile-carrier model, phosphate transport across the plasma membrane is energized by the proton motive force, Δp (Borst-Pauwels, 1981). The direction of phosphate transport catalysed by the Pho84 protein is determined by the direction of the driving force rather than by the orientation of the protein in the membrane, as for many other secondary transport systems which operate bidirectionally (Fristedt et al., 1996; Fristedt et al., 1999a; Fristedt et al., 1999b). Furthermore, both components of the proton motive force, i.e. the membrane potential ($\Delta\psi$) and the proton gradient (ΔpH) are competent and interchangeable in driving phosphate uptake (Fristedt et al., 1999a). In a proteoliposome system, Pho84 catalyzes an uncoupler-sensitive phosphate accumulation with an estimated K_m for phosphate of 24μM, which is close to the value found in intact yeast cells (Berhe et al., 1995). Finally, it was observed that bivalent metal ions such as Mn^{2+} and Co^{2+} stimulate the phosphate uptake process while addition of a chelator such as EDTA eliminates phosphate transport without affecting the $\Delta\psi$ across the membrane (Fristedt et al., 1999a). This suggests that the Pho84 transport activity is dependent on the presence of bivalent ions and that a metal-phosphate complex might be the translocated substrate.

Interestingly, the Pho84 has been proposed to act as a low-affinity metal transporter being involved in manganese homeostasis (Jensen et al., 2003).

The second derepressible high-affinity phosphate transporter is encoded by the *PHO89* gene (Martinez and Persson, 1998), which was identified based on its high sequence homology with the *PHO4* gene of the fungus *Neurospora crassa*, encoding a Na^+-Pi symporter (Versaw and Metzenberg, 1995). The Pho89 transporter belongs to the inorganic phosphate transporter family (PiT family), in which it is the sole representative in *Saccharomyces cerevisiae* (Nelissen et al., 1997). Within the PiT family, Pho89 is an orthologue of the mammalian type III Na^+-Pi transporters, which function as a housekeeping membrane transport system (Werner and Kinne, 2001).
Hydropathy analysis suggests that Pho89 is an integral membrane protein containing 12 TM, similar to the Pho84 transporter, although its large hydrophilic loop is not symmetrically positioned, but connects TM segments 7 and 8 (Martinez and Persson, 1998; Persson et al., 1999). Based on its homology with the mammalian Pit-2 transporter, the large hydrophilic loop is proposed to be located intracellular, while the N- and C-termini will be located outside the cell (Bottger and Pedersen, 2005; Salaun et al., 2001).
Genetic analysis demonstrated that the Pho89 is responsible for Na^+-dependent Pi-uptake in yeast, a phenomenon that was already kinetically characterized (Martinez and Persson, 1998; Roomans et al., 1977). Pho89 has an apparent K_m for Pi of 0.5μM and a pH optimum of 9.5 (Martinez and Persson, 1998). The observation that phosphate accumulation catalyzed by Pho89 is stimulated by low Na^+ concentrations has led to the suggestion that the transport is of an electrogenic nature, where transport of a positively charged complex resulting from the combination of one monovalent negative phosphate ion with two Na^+ ions may be driven by the prevailing electrical gradient, $\Delta\psi$, across the membrane (Roomans et al., 1977). However, at alkaline pH conditions, the membrane H^+ gradient is disrupted and ion-pumping of the plasma membrane Na^+-ATPases (Ena1 and/or Ena2) is required for growth (Garciadeblas et al., 1993; Lamb et al., 2001). Ena1 hydrolyzes ATP to pump Na^+ out of the cell and may thus generate a Na^+ motive force across the membrane, which would also be able to drive Na^+-dependent phosphate uptake by Pho89.

3.1.2 The low-affinity phosphate transport system

The fact that the *pho84Δ pho89Δ* strain, deleted in its high-affinity Pi-transport system, is viable and even still grows under low-Pi conditions (albeit very slow) indicates the presence of a low-affinity Pi-transport system encoded by genes other than *PHO84* and *PHO89* (Martinez and Persson, 1998). This low-affinity Pi transport process had been observed previously in kinetic studies of *S. cerevisiae* cells and was proposed to be due to a constitutively expressed system with a K_m value for external phosphate of approximately 1 mM (Borst-Pauwels, 1981; Tamai et al., 1985). Moreover, the K_m of this system is unaffected by Na^+, suggesting that the low-affinity transporter comprises a H^+-coupled Pi transport system (Persson et al., 1999; Roomans et al., 1977). The low-affinity phosphate transport system is now believed to be composed of the partially redundant Pho87, Pho90 and Pho91 phosphate transporters (Wykoff and O'Shea, 2001). Analysis of the Pi-uptake capacity of strains expressing either the native *PHO87*, *PHO90* or *PHO91* gene as the sole Pi-transporter demonstrated a K_m of 216μM for Pho87, 205μM for Pho90 and 181μM for Pho91, which are much higher than those found for the high-affinity Pi-transporters Pho84 and Pho89 (Berhe et al., 1995; Martinez and Persson, 1998; Wykoff and O'Shea, 2001).

The Pho87, Pho90 and Pho91 Pi-transporters belong to the family of divalent anion:Na^+ symporters (DASS) and none of the proteins displays a significant degree of homology to the Pho84 protein (Paulsen et al., 1998; Wykoff and O'Shea, 2001). The low-affinity Pi-transporters share a common topology of 12 TM domains preceded by a large N-terminal cytoplasmic domain, which contains sequences highly conserved in proteins involved in phosphate signalling (Pho81p, Pho85p) and polyphosphate homeostasis (Phm1/Vtc2, Phm2/Vtc3 and Phm3/Vtc4) (Bun-ya et al., 1996; Cohen et al., 1999; Ogawa et al., 2000; Ogawa et al., 1995). Interestingly, this amino-terminal extension is also conserved in the Syg1 protein, the fourth member of the DASS family in yeast (Paulsen et al., 1998), and this domain of Syg1 has been shown to interact with the γ-subunit (Ste4p) of the heterotrimeric G-protein complex involved in mating (Spain et al., 1995). Therefore, these low-affinity Pi-transporters are also classified as a family of putative sensors (Van Belle and Andre, 2001) and it has been proposed that these low-affinity Pi-transporters could be important for

signalling the presence of extracellular phosphate under different circumstances (Giots et al., 2003; Pinson et al., 2004).

3.2 Regulation of phosphate transport in yeast

Since inorganic phosphate is an essential nutrient for living cells, which is required in substantial amounts, it is important for organisms to be able to obtain sufficient amounts of this nutrient. Especially for microorganisms, such as *S. cerevisiae*, the amount of Pi in the environment may fluctuate substantially, being scarce in many occasions. Besides having Pi-transporters with different uptake properties, the yeast also employs several regulatory mechanisms to ensure optimal uptake of Pi under these fluctuating conditions. Transport activity can be regulated at the level of transcription (induction/repression) or at a post-transcriptional level such as phosphorylation, ubiquitination, etc.

In yeast, one major mechanism for regulating Pi-transport activity is performed by the PHO regulatory pathway. Under conditions of limiting Pi availability, a plethora of genes is induced in order to optimally scavenge Pi from the environment (Ogawa et al., 2000; Persson et al., 2003). Among the genes induced are the *PHO84* and *PHO89* genes encoding the high-affinity Pi-transporters (Bun-Ya et al., 1991; Martinez and Persson, 1998). This transcriptional induction is responsible for the higher capacity for Pi-uptake seen in yeast cells grown under low-Pi conditions.

Besides this transcriptional regulation of the high-affinity Pi-transport system executed by the PHO pathway, several studies have shed light on yet other mechanisms by which the high-affinity phosphate transporters are regulated.

3.2.1 Regulation of the Pho84 transporter

Under conditions of limiting phosphate, Pho84 is responsible for the majority of Pi-transport. Indeed, Pi-transport assays in a *pho84* mutant grown in low-Pi medium indicates an almost complete lack of Pi-transport, while transport of glucose and sulphate were unaffected (Bun-Ya et al., 1992; Bun-Ya et al., 1991). Cells deleted for *PHO84* therefore grow slower than a wild type strain under low-Pi conditions. Intriguingly, in cells grown in a low-Pi environment at acidic pH the Pi-transport

capacity changes with cell growth, where Pho84 transport activity is maximally active in mid-log phase (Martinez et al., 1998). Upon depletion of extracellular Pi and carbon source, Pho84 activity and abundance decrease again. This decrease in Pho84 abundance is due to a rerouting of plasma membrane localised Pho84 to the vacuole were it is subsequently degraded (Martinez et al., 1998; Petersson et al., 1999). In addition, a shut-down of transcription is observed when the low-Pi grown cells reached stationary phase (Martinez et al., 1998). This repression of *PHO84* transcription is also seen when cells grown in rich medium are approaching the diauxic shift (DeRisi et al., 1997). So, under conditions of non-limited carbon supply, the extracellular Pi concentration serves as a major factor controlling the expression and activity of the Pho84 transporter (Swinnen et al., 2005). In contrast, limitations in the carbon source results in rapid degradation of the transporter in the presence of derepressible levels of Pi (Martinez et al., 1998). Hence, the functionality of the Pho84 transporter depends on the availability of both derepressible Pi concentrations and abundant carbon source, not necessarily glucose (Persson et al., 2003). How the presence of a carbon source influences the transcription of *PHO84* gene and activity of the Pho84 protein is presently unknown.

Similarly to a limitation in carbon source, the addition of a repressible amount of Pi to cells grown in limiting amounts of Pi also triggers internalization, via the process of endocytosis, and degradation of plasma membrane localized Pho84 (Lau et al., 2000). Interestingly, the endocytosis of Pho84 is independent of the PHO pathway (Lau et al., 2000) and is mediated, at least in part, by the PKA signalling pathway (Mouillon and Persson, 2005). Furthermore, the effects on Pho84 localization and degradation can be mimicked by methylphosphonate, a non-hydrolysable and non-utilisable phosphate analogue (Pratt et al., 2004). Thus, the Pho84 transporter is regulated not only at the transcriptional level but also by a direct molecule-sensing mechanism at the protein level. As for other proteins, covalent modification with ubiquitin and/or phosphorylation may be required for the initiation of the endocytosis and degradation processes (Hicke, 1999; Rotin et al., 2000). The region of Pho84 required for its endocytosis and degradation has not been identified yet (Lagerstedt et al., 2002). However, a genome-wide screening identified several *in vivo* phosphorylation sites in the Pho84 protein as well as two ubiquitinated lysine residues (Peng et al., 2003), which may lead the way to the elucidation of the regulated endocytosis of Pho84. Interestingly, in a *pho87Δ pho89Δ pho90Δ pho91Δ* quadruple Pi-transporter deletion,

a strong stabilization of Pho84 at the plasma membrane is seen which cannot be explained solely by the mild increase in transcription of *PHO84* in this strain (Wykoff and O'Shea, 2001). Thus, a compensatory effect is seen for the lack of a non-Pho84 mediated Pi-uptake pathway through enhanced Pho84 activity, partly due to its accumulation in the plasma membrane.

Integral membrane proteins such as Pho84 depend on the activities of many proteins and pathways for correct localisation. In this regulation, Pho86 has been shown to be an endoplasmic reticulum (ER) resident protein required for the correct packaging of Pho84 into COPII vesicles (Lau et al., 2000; Yompakdee et al., 1996a). In the absence of Pho86, Pho84 is localized to the ER and fails to be targeted for its plasma membrane destination. Pho86 lacks any recognizable retrieval signal and is not itself packaged into the COPII vesicles, but remains associated with the ER, therefore belonging to a class of outfitters: resident ER proteins that facilitate the loading of cargo into transport vesicles (Herrmann et al., 1999; Lau et al., 2000). Pho86 may play a critical role in assisting Pho84 to fold and correctly attain tertiary structures required for ER exit. In cells lacking Pho86, Pho84 appears to aggregate and consequently fail to exit the ER membrane (Kota and Ljungdahl, 2005). The localization of Pho86 does not depend on the Pi-status of the cell, however, Pi-limitation results in an induced expression of *PHO86* (Lau et al., 2000; Ogawa et al., 2000; Yompakdee et al., 1996a). This induction of Pho86 function when phosphate in the environment becomes limiting will enable the cell to ensure proper packaging and plasma membrane targeting of the increased amounts of Pho84 under this condition.

Finally, the Pho88 and Gtr1 proteins have been implicated in the regulation of Pho84 function (Bun-Ya et al., 1992; Lagerstedt et al., 2005; Yompakdee et al., 1996b), but it is still unclear how these proteins regulate the phosphate transport process.

3.2.2 Regulation of the Pho89 transporter

Similar to the *PHO84* gene, transcription of *PHO89* is induced when phosphate becomes limiting (Martinez and Persson, 1998). Although both genes are co-

ordinately induced, the activity of both high-affinity transporters is regulated independently.

The activation of Na^+-coupled Pi-uptake via Pho89 in cells grown in low-Pi media at acidic pH was shown to precede and partially overlap the activity of Pho84, which is mobilized later during cell growth (Pattison-Granberg and Persson, 2000). This difference in regulation may be controlled by the intracellular levels of free Pi and/or polyPi. As the maximal activity of Pho89 is at least 100-fold lower than that of the Pho84 transporter (assayed at their respective activity optima) when grown in low-Pi conditions, it is clear that Pho84 represents the predominant Pi-transport activity of yeast cells under these conditions. The differential regulation of the activity of the Pho84 and Pho89 Pi transporters, along with their different pH optima, may enable the yeast cell to optimally scavenge Pi across a broad range of pH conditions and growth phases, creating a fine tuning of the Pi-uptake process relative to the availability of external Pi. The mechanisms underlying the regulation of activity of Pho89 are currently still unknown. Processes similar to those seen with Pho84, including specific ER chaperones, interalization, etc., are plausible considerations.

Interestingly, although expression of *PHO84* is downregulated at the diauxic shift, expression of *PHO89* was induced (DeRisi et al., 1997), indicating that expression of both genes is differentially regulated by available carbon source. This also makes it unlikely that the transcriptional regulation by carbon source availability is performed solely through Pho4, since in that case we would expect that all PHO genes would be similarly regulated. More likely, other promoter elements and transcription factors will contribute to the differential transcriptional regulation of several PHO genes under a variety of conditions. For example, transcription of *PHO89* but not of *PHO84* is regulated by the calcium signalling pathway (Serrano et al., 2002). Although the PHO genes are classified as one group, regulation of their expression in response to environmental conditions (other than Pi-starvation) may thus be very different, as can be the regulation of the activity of their gene products.

3.2.3 Regulation of the low-affinity Pi-transport system

In contrast to the genes for the high-affinity Pi-transporters, the expression of *PHO87*, *PHO90* and *PHO91* is independent of Pi-availability and the activity of Pho4, the major transcription factor involved in the PHO pathway (Auesukaree et al., 2003).

Although the low-affinity Pi-uptake system is believed to be expressed constitutively, the activity of this low-affinity system changes when cells are grown in low-Pi and in high-Pi medium, being higher in the latter (Martinez and Persson, 1998). This indicates that the low-affinity Pi-transport system is also subject to some regulatory control. Although it was proposed that Pho87, Pho90 and Pho91 have redundant functions in Pi-transport (Auesukaree et al., 2003; Wykoff and O'Shea, 2001), the activities of these three Pi-transporters are not strictly equivalent and each of these transporters may play its role at specific stages of growth (Giots et al., 2003; Pinson et al., 2004). This differential regulation of activity may be executed at the level of transcription by conditions other than Pi-availability (DeRisi et al., 1997) or at the post-transcriptional level via phosphorylation and/or ubiquitination (Peng et al., 2003).

4. Signalling phosphate starvation in *Saccharomyces cerevisiae* – the PHO pathway

Most micro-organisms live under conditions of nutrient-limited growth. Therefore it is very important that these organisms can use these scarce nutrients efficiently. To achieve this, microorganisms have evolved complex signal transduction networks that enable them to integrate information about the availability of a large number of nutrients and use this information to regulate their growth, proliferation and metabolism.

When inorganic phosphate becomes limiting, *Saccharomyces cerevisiae* activates a regulatory mechanism known as the PHO pathway (Lenburg and O'Shea, 1996; Ogawa et al., 2000; Persson et al., 2003). This signalling cascade results in the increased expression of a multitude of genes involved in the acquisition, uptake and storage of Pi. Transcriptional induction of these genes in response to phosphate starvation depends on the Pho4 and Pho2 transcription factors. Pho4 is negatively regulated, through phosphorylation, by the Pho85 CDK in association with the Pho80 cyclin, and this kinase complex is under negative control of the Pho81 CDK inhibitor (CKI) (Fig. I.4).

The Pho4 transcription factor contains a C-terminal basic helix-loop-helix motif as DNA-binding domain (Berben et al., 1990; Ogawa and Oshima, 1990). This motif creates a DNA-interaction surface upon dimerisation and it is shown that Pho4 binds

to DNA as a homodimer, stabilised by an intermolecular disulfide bond between two Pho4 molecules (Robinson and Lopes, 2000; Shao et al., 1998). An acidic transcription-activation domain is located in the N-terminal part of the Pho4 protein (Ogawa and Oshima, 1990). The *PHO2* gene encodes a homeodomain protein with 2 DNA-binding domains and a putative nuclear localisation signal in its N-terminal part (Berben et al., 1988; Burglin, 1988). Pho4 binds to the consensus sequence 5'-CACGTG/T-3', while the Pho2 protein appears to bind A/T-rich segments flanking the Pho4 binding site (Magbanua et al., 1997a; Robinson and Lopes, 2000). The mechanism of transcriptional activation by Pho4 and Pho2 is most extensively studied for the *PHO5* promoter, and involves DNA-binding, chromatin remodelling and activation of transcription. Pho2 functions to recruit the NuA4 histone acetyltransferase complex to the *PHO5* promoter, which presets the promoter for subsequent binding by Pho4, chromatin remodelling and transcription (Nourani et al., 2004). Next, Pho2 aids in the promoter-binding of Pho4 and chromatin remodelling is induced via the action of multiple complexes including the histone acetyltransferase Gcn5 and the SWI/SNF and INO80 chromatin remodelling complexes (Barbaric et al., 1998; Barbaric et al., 1996; Fascher et al., 1990; Haswell and O'Shea, 1999; Steger et al., 2003). Finally, transcription is activated through direct interaction of the Pho4-Pho2 complex with components of the basal transcription machinery (Magbanua et al., 1997b).

Pho4 is concentrated in the nucleus when yeast cells are grown in low-Pi medium while being predominantly cytoplasmic under high-Pi conditions (O'Neill et al., 1996). Addition of phosphate to Pi-starved cells results in rapid exclusion of Pho4 from the nucleus that is dependent on its phosphorylation by the nuclear Pho85-Pho80 kinase complex (Kaffman et al., 1994; O'Neill et al., 1996). Nuclear export of Pho4 depends on the importin-β-family member Msn5 which binds only to the phosphorylated form of Pho4 in the presence of Gsp1-GTP (Kaffman et al., 1998a). This ternary complex is then exported to the cytoplasm where it is disassembled through GTP hydrolysis by Gsp1, stimulated by Rna1. Import of cytoplasmic Pho4 into the nucleus requires association with its import receptor Pse1/Kap121, another member of the importin-β-family (Kaffman et al., 1998b). In contrast to its association with Msn5, phosphorylation of Pho4 inhibits the association with Pse1. In the nucleus, binding of Gsp1-GTP to Pse1 stimulates the release of Pho4. The phosphatase responsible for

dephosphorylation of Pho4, perhaps in response to nutrient conditions, has not been identified yet.

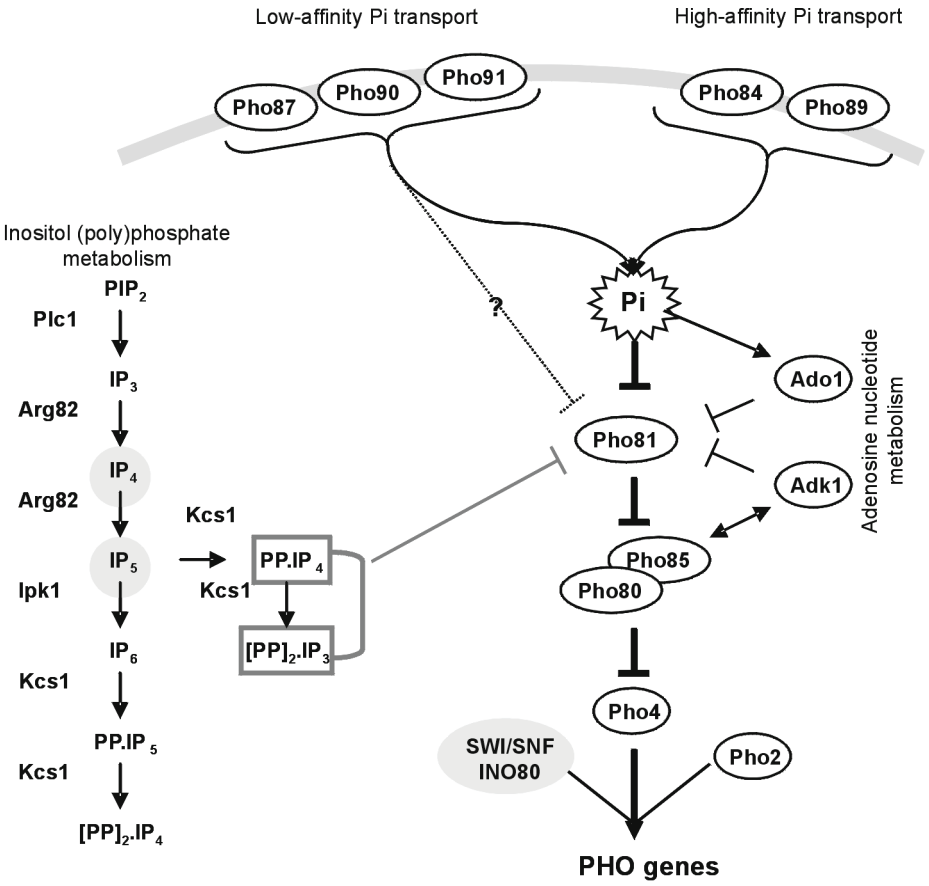


Figure I.4: The yeast PHO pathway and its interaction with inositol (poly)phosphate and adenosine nucleotide metabolism. Expression of yeast PHO genes requires the Pho4 transcription factor, its Pho2 co-activator and the SWI/SNF and INO80 chromatin remodelling complexes. Pho4 activity is inhibited through phosphorylation by the Pho85-Pho80 kinase complex, with Pho81 regulating Pho85-Pho80 activity in response to intracellular phosphate levels. Phosphate transporters mainly regulate PHO signalling by supplying the cell with intracellular phosphate, though the low-affinity phosphate transporters may regulate Pho81 function independently of intracellular phosphate. $PP.IP_4$ and $[PP]_2.IP_3$ are also implicated in the regulation of Pho81 activity, while IP_4 and IP_5 are required for PHO gene induction through stimulation of the activities of the SWI/SNF and INO80 complexes. Finally, the Ado1 and Adk1 enzymes also regulate PHO signalling. Ado1 activity is stimulated by Pi, while Adk1 is found to interact with Pho85. The grey bar represents the plasma membrane, I: inositol, P: phosphate. See list of abbreviations and gene names for the different metabolites and enzymes involved. Further details are explained in the text.

A common feature of cyclin-dependent kinases, like Pho85, is that they phosphorylate their substrates on serine (Ser) or threonine (Thr) residues which are directly followed

by a proline (Pro) residue. Within Pho4, five Ser-Pro motifs (designated as SP1 to SP4 and SP6) are phosphorylated by Pho85 *in vivo* (O'Neill et al., 1996). These multiple phosphorylation sites play distinct and separable roles in the regulation of activity of Pho4 (Komeili and O'Shea, 1999). Phosphorylation on sites 2 and 3 is necessary and sufficient to promote binding to Msn5, while phosphorylation on site 4 disrupts the association of Pho4 with Pse1. Phosphorylation on site 6 finally, was shown to be necessary and sufficient to inhibit interaction of Pho4 with its co-activating partner Pho2. A physiologically relevant role of SP1 phosphorylation has not been found yet. Kinetic analysis revealed that Pho4 phosphorylation is semi-processive and with a site preference of SP6>SP4>SP3>SP2>SP1 (Jeffery et al., 2001). This predicts that nuclear Pho4 will be phosphorylated on SP6 first, resulting in immediate repression of several Pho4-Pho2 dependent genes. Secondly, Pho4 proteins that are exported from the nucleus by phosphorylation of sites 2 and 3 will almost always be also phosphorylated on site 4, so that exported Pho4 will not be able to quickly re-enter the nucleus, avoiding futile cycling. Interestingly, these multiple levels of regulation of Pho4 activity allow the yeast to differentially express a subset of phosphate-responsive genes in intermediate-Pi conditions (Springer et al., 2003), enabling yeast cells to take up appropriate amounts of phosphate over a wide extracellular concentration range at a minimal energetic cost. Additionally, the cell's phosphate reserve, polyP, functions as a buffer that can filter transient fluctuations in extracellular phosphate levels (Neef and Kladde, 2003; Thomas and O'Shea E, 2005), further fine-tuning the response and minimizing unnecessary synthesis of genes that encode energetically expensive molecules, such as secreted enzymes that cannot be recovered or recycled.

The activity of Pho4 is mainly adjusted via phosphorylation by the Pho85-Pho80 kinase. This kinase activity, in turn, is regulated in response to phosphate levels by means of the Pho81 CKI protein. Pho81 is mainly localised in the nucleus, as is the Pho85-Pho80 complex, and this localisation is not regulated by phosphate levels but depends on the Pho80 cyclin (Huang et al., 2001; Kaffman et al., 1998a). The Pho81 protein consists of 1179 amino acids, yet a region of Pho81 containing 80 amino acids C-terminal to its ankyrin repeats was shown to be necessary and sufficient to bind to Pho85-Pho80 *in vitro* and to induce *PHO5* expression *in vivo* in response to phosphate starvation (Huang et al., 2001). It was therefore concluded that this 'minimum domain' of 80 amino acids is necessary and sufficient for Pho81 inhibition

of Pho85-Pho80 *in vivo*. However, other parts of the Pho81 protein may contribute to inhibition of Pho85-Pho80 kinase activity towards other, non-Pho4, targets (Flick and Thorner, 1998; Sopko et al., 2006; Swinnen et al., 2005), or may be necessary for functions of Pho81 which do not involve the Pho80 cyclin (Lee et al., 2000; Swinnen et al., 2005). Pho81 physically interacts with the Pho85-Pho80 kinase, mainly through interaction with Pho80, both under low- and high-Pi conditions, though it only inhibits the kinase complex under low-Pi conditions (Hirst et al., 1994; Schneider et al., 1994). This implies that Pho81 activity is controlled in response to phosphate by a posttranslational mechanism. In addition, expression of *PHO81* is also controlled by the PHO system (Creasy et al., 1993; Ogawa et al., 1993), providing a positive feedback loop wherein both enhanced *PHO81* expression and a low-Pi signal from the medium are necessary for proper regulation of the PHO pathway (Ogawa et al., 1995). Interestingly, Pho81 is phosphorylated by the Pho85-Pho80 kinase at several residues, and this phosphorylation appears to mediate the formation of a stable interaction with the Pho85-Pho80 complex, allowing it to function as an active inhibitor of the complex when the cell is starved for Pi (Knight et al., 2004; Waters et al., 2004). Therefore, in order to function, Pho81 has to be phosphorylated by the same kinase which it inhibits. In addition, the Pho80 cyclin is also phosphorylated by Pho85 and this phosphorylation is required for *in vivo* functioning of Pho80 (Waters et al., 2004). Thus, Pho85 seems to be the key molecule controlling the function of the PHO pathway by phosphorylating several vital components within the pathway.

4.1 The PHO regulon

The PHO pathway functions to make optimal use of the available Pi in the medium and/or to switch to alternative phosphorous compounds for growth in the absence of Pi. For this, a multitude of genes are induced under phosphate limitation by activation of the Pho4 transcription factor (Ogawa et al., 2000).

When Pi becomes scarce, the genes encoding the high-affinity phosphate transporters *PHO84* and *PHO89* are induced (Bun-Ya et al., 1991; Martinez and Persson, 1998). This will enable the yeast to take up the remaining phosphate of the medium with high affinity across a broad range of pH conditions and growth phases. The *PHO86* gene,

encoding the ER-resident chaperone assisting the Pho84 in its transport towards the plasma membrane, is co-ordinately induced (Yompakdee et al., 1996a).

Furthermore, several acid phosphatases, encoded by the *PHO5*, *PHO11* and *PHO12* genes, as well as an alkaline phosphatase (*PHO8*) are induced (Persson et al., 2003). The acid phosphatases (rAPase) are secreted into the periplasmic space where they will act on a wide variety of phospho-ester substrates, liberating free Pi and thus increasing the Pi-availability for the cell. The alkaline phosphatase Pho8 (rALPase) is localised in the vacuole and may serve to liberate Pi residues from several phospho-containing substrates localised in this organelle.

Activation of the PHO pathway also leads to an increased expression of the components involved in vacuolar polyphosphate accumulation; *PHM1-5* (Ogawa et al., 2000). This may seem contradictory since yeast cells consume their polyphosphate reserves under low-Pi conditions. However, this increase in the capacity to synthesise polyP primes the yeast cell for a subsequent Pi-availability. It is known that *S. cerevisiae* accumulates large amounts of polyP in the vacuole under conditions where a period of Pi starvation is followed by Pi abundance. This is referred to as the 'polyphosphate overplus' phenomenon. It is shown that polyP synthesis is required for optimal Pi-transport after Pi-readdition to the starved cells. When polyP synthesis is slow, intracellular free Pi levels become high, which in turn acts as a direct negative feedback on the Pho84 membrane transporter. Thus, polyP accumulation is required, presumably by acting as a sink, to sustain a high rate of Pi-uptake, indicating that a major physiological role of polyP may be to promote long-term uptake and accumulation of Pi.

When inorganic phosphate from the medium is exhausted, the yeast cell can switch to the use of alternative phosphorous sources. Indeed, *S. cerevisiae* can grow on media containing glycerophosphoinositol or glycerophosphocholine as the sole phosphate source (Almaguer et al., 2004; Fisher et al., 2005). These substrates enter the cell as intact molecules via the Git1 permease. Catabolism of these compounds inside the cell yields glycerol-3-phosphate, which can then generate free phosphate through the action of the glycerol-3-phosphatases Gpp1/Rhr2 and Gpp2/Hor2. Furthermore, the Gde1 protein is necessary for the use of glycerophosphocholine, not glycerophosphoinositol, as the sole Pi source, probably by acting as a glycerophosphocholine phosphodiesterase. Under low-Pi conditions the *GIT1*, *GDE1/YPL110c* and *HOR2* are all induced (Almaguer et al., 2003; Ogawa et al.,

2000), indicating a coordinated induction of genes required for the usage of these alternative phosphorous sources.

Finally, an important gene induced under Pi-limiting conditions is *PHO81*. This induction comprises an important positive feedback loop allowing for continued activation of the PHO pathway when phosphate limitation persists. However, besides keeping the Pho4 transcription factor active, the Pho81 protein is also involved in the regulation of several other stress responses during phosphate starvation (Swinnen et al., 2005), strengthening the importance of the induction of *PHO81* under these conditions.

In addition the abovementioned genes, several other genes have been identified that are induced under low-Pi conditions (Ogawa et al., 2000). For most of them, however, the precise function in the phosphate starvation response is still unclear.

4.2 Sensing phosphate

Although many components and the molecular mechanism of the PHO pathway are well understood, the fundamental question as to how the cell senses phosphate availability still remains unanswered.

Since *pho84Δ* cells exhibit constitutive acid phosphatase activity ('Pho^c phenotype'), it was originally thought that Pho84 may be a crucial part of the cell's phosphate-sensing complex (Bun-Ya et al., 1992; Bun-Ya et al., 1991; Ogawa et al., 1995). However, subsequent analysis revealed that a *pho84Δ* strain is defective in Pi-uptake even under high-Pi conditions (Wykoff and O'Shea, 2001), and has lowered intracellular levels of free phosphate and polyP (Auesukaree et al., 2004), resulting in a state of continued phosphate starvation. Furthermore, overexpression of other phosphate transporter genes, such as *PHO87*, *PHO90* and *PHO91* and even the glycerophosphoinositol permease *GIT1*, increases Pi-uptake ability and suppresses the Pho^c phenotype of the *pho84Δ* strain (Wykoff and O'Shea, 2001). Because Pho87, Pho90 and Pho91 share no significant amino acid similarity with Pho84, it seems doubtful that these transporters contain common signal-responsive domains similar to those of Pho84. More likely, the signalling defect of *pho84Δ* cells is suppressed by the increase in intracellular phosphate level, suggesting that the phosphate sensor is intracellular. Indeed, additional deletion of components of vacuolar polyP

accumulation, in a *pho84Δ* background can largely suppress the phenotype of the *pho84Δ* strain by increasing cytoplasmic phosphate concentrations (Auesukaree et al., 2004). Thus, Pho84 seems to affect phosphate signalling largely by functioning as a transporter. Although it was concluded that PHO signalling is under the regulation of intracellular orthophosphate, this compound is not the sole signalling molecule (Auesukaree et al., 2004). Other phosphate signals could be polyP (Auesukaree et al., 2004), and components of inositol phosphate and adenine nucleotide metabolism (see below).

Interestingly, the low-affinity phosphate transporters are also involved in regulating PHO gene expression (Auesukaree et al., 2003; Pinson et al., 2004). Mutations in the low-affinity Pi-carrier genes (*PHO87*, *PHO90* and *PHO91*) cause deregulation of phosphate-repressed genes, leading to enhanced expressing of PHO genes in high- or intermediate-Pi conditions. The effect was most pronounced for the *pho87Δ pho90Δ pho91Δ* triple mutant, indicating some functional redundancy between the low-affinity Pi-transporters. Strikingly, the deregulation in the *pho87Δ pho90Δ pho91Δ* mutant was neither correlated to impaired Pi-uptake capacity nor to a decrease of the intracellular orthophosphate or polyP pools (Pinson et al., 2004). Thus, the low-affinity Pi-transporters affect phosphate regulation independently of intracellular phosphate concentration through a new signalling pathway that was found to strictly require the CKI protein Pho81. Hence, phosphate-regulated gene expression is under the control of two different independent regulatory signals: the sensing of internal phosphate by a yet unidentified protein and the sensing of extracellular phosphate by the low-affinity phosphate transporters (Fig. I.4); the former would be required to maintain phosphate homeostasis while the latter would keep the cell informed on the medium phosphate richness. As both sensing pathways seem to converge on the Pho81 protein, this situation displays marked similarities to the dual intra- and extracellular detection of glucose in the Ras-cAMP pathway, converging on the adenylate cyclase protein (Rolland et al., 2000).

Cellular phosphate is required in numerous reactions and pathways. Hence, it is conceivable that phosphate signalling is coupled to other cellular processes.

Phosphate signalling seems to be tightly connected to inositol (Ins) phosphate metabolism (fig. I.4). The inositol pyrophosphates PP-$InsP_4$ and/or $(PP)_2$-$InsP_3$ are

required for repression of PHO genes in high-Pi conditions (Auesukaree et al., 2005; El Alami et al., 2003). Indeed, *plc1Δ*, *arg82Δ* and *kcs1Δ* mutants, all lacking PP-$InsP_4$ and $(PP)_2$-$InsP_3$, exhibit constitutive rAPase activity. This Pho^c phenotype is dependent on the presence of Pho81 (Auesukaree et al., 2005). Intriguingly, all these mutants display higher intracellular orthophosphate levels than a WT strain under high-Pi conditions, indicating that regulation of the PHO gene transcription by PP-$InsP_4$ and/or $(PP)_2$-$InsP_3$ is independent of intracellular phosphate levels. Surprisingly, the *plc1Δ*, *arg82Δ* and *kcs1Δ* mutants had significantly reduced intracellular polyP levels, suggesting that enzymes involved in inositol pyrophosphate synthesis are also required for polyP accumulation. In contrast to the requirement of the inositol pyrophoshates PP-$InsP_4$ and/or $(PP)_2$-$InsP_3$ for repression of PHO genes under high-Pi conditions, full induction of PHO genes under low-Pi conditions requires the inositol polyphosphates $InsP_4$ and/or $InsP_5$. The latter appear to function in modulating the ability of the SWI/SNF and INO80 chromatin remodelling complexes to induce transcription of some phosphate-responsive genes, perhaps by affecting the ability of these complexes to interact with Pho4, Pho2 and/or chromatin (Steger et al., 2003). Thus, inositol polyphosphates and inositol pyrophosphates control the expression of PHO genes at different levels and in opposite ways. As it is very likely that the amounts or ratios of inositol polyphosphates and pyrophosphates are altered under certain physiological conditions, such as phosphate starvation, this change may be used by the cell as a signal for global regulation of transcription. The Kcs1 enzyme may be crucial in this regulation as alterations in its activity will affect the levels of inositol polyphosphates and inositol pyrophosphates in opposite direction. Interestingly to note is that Kcs1 seems to interact with Bmh1 and Bmh2 (Ho et al., 2002; Uetz et al., 2000), two cytoplasmic anchor proteins involved in the transcriptional regulation of multiple pathways.

Genome-wide screening for components involved in phosphate signalling revealed that *adk1Δ* and *ado1Δ* mutants exhibit constitutive rAPase activity (Auesukaree et al., 2005; Huang and O'Shea, 2005). *ADK1* encodes an adenylate kinase, which catalyses the interconversion of nucleotides between AMP and ADP (Abele and Schulz, 1995; Konrad, 1988); *ADO1* encodes an adenosine kinase, which catalyses the salvage synthesis of adenine monophosphate from adenosine and ATP (Lecoq et al., 2001). In

both *adk1Δ* and *ado1Δ* mutants, Pho4 is localised in the nucleus under high-Pi conditions, dependent on the presence of Pho81 (Huang and O'Shea, 2005). This indicates that to Adk1 and Ado1 may act upstream of Pho81 in the PHO pathway (Fig. I.4). Since inorganic phosphate is essential for nucleotide synthesis, it is plausible that nucleotide regulation is connected to the PHO signalling pathway to coordinate these processes under different nutrient and growth conditions. Interestingly, during the initial period of phosphate starvation, intracellular phosphate levels are maintained at a significant level because of replenishment through polyP degradation. Under the same conditions, however, the ATP levels drop drastically (Martinez et al., 1998). In the *adk1Δ* mutant grown in high-Pi medium, intracellular Pi and polyP levels are similar to the WT strain (Auesukaree et al., 2005), while it is known that deletion of *ADK1* leads to a significant decrease in nucleoside triphosphates (Konrad, 1988). This indicates that Adk1 might regulate PHO gene transcription in a manner independent from intracellular phosphate, but responding to ATP-availability. As Adk1 was detected in a Pho85-associated complex, which included Pho81 (Ho et al., 2002), Adk1 might directly regulate Pho81 activity. Since there are no data available concerning intracellular (poly)phosphate levels of a *ado1Δ* mutant, it remains unclear as to which signal Ado1 responds. However, as Ado1 activity is highly dependent on the presence of inorganic phosphate (Maj et al., 2000; Maj et al., 2002), Ado1 may serve to regulate Pho81 activity in a phosphate-dependent manner.

Additional links have been made between phosphate signalling and other signalling and/or metabolic networks. For example, crosstalk between phosphate and lipid metabolism has been proposed (Karpichev et al., 2002; Lau et al., 1998), although the exact connection is still unclear. Also, the yeast casein kinase 2 (CK2) has been implicated in the regulation of PHO gene transcription in a subunit- and isoform specific manner (Glover, 1998) in which a Ckb1-Cbk2 controlled, Cka1-specific Pho4 phosphorylation might comprise an additional tuning device of PHO gene transcription (Barz et al., 2003). Importantly, the Pho2 protein seems to require phosphorylation in order to function in the PHO pathway (Liu et al., 2000). This phosphorylation appears to be critical for the Pho2-Pho4 interaction. Although this phosphorylation can be performed by the CDK Cdc28 *in vitro*, nothing is known yet about how this phosphorylation is regulated *in vivo*.

To date, the nature of the intracellular phosphate sensor is still unknown. A possible candidate would be the Pho81 CKI protein. However, given the fact that the minimum domain containing 80 amino acids has been shown to be both necessary and sufficient to regulate Pho4 activity (Huang et al., 2001), it seems unlikely that such a small region of Pho81 is responsible not only for inhibiting Pho85-Pho8 kinase activity but also for sensing phosphate directly. It is possible that the phosphate sensor is encoded by an essential gene, which will make the identification more difficult or even impossible when screenings are performed using the haploid deletion collection (Auesukaree et al., 2005; Huang and O'Shea, 2005; Winzeler et al., 1999). Alternatively, there might be multiple phosphate sensors encoded by nonessential genes that have a redundant function in phosphate sensing. In addition, it is not clear yet if yeast senses the presence of inorganic phosphate itself and/or a phosphate-derived metabolite. The fact that the nonmetabolisable phosphate analogues phosphite and methylphosphonate can efficiently repress rAPase activity (McDonald et al., 2001; Pratt et al., 2004) suggests that yeast cells can directly sense phosphate. However, as described above, several mutants have been identified in which the activity of the PHO pathway is altered independent of intracellular phosphate levels. Thus, it seems that the yeast cell is equipped with various regulatory mechanisms capable of steering PHO pathway signalling, in response to multiple signals, not only inorganic phosphate. It should be noted, however, that although through the use of mutants, PHO signalling can be affected independent of intracellular phosphate levels, in a normal yeast cell many of these signalling molecules, e.g. inositol poly- and pyrophosphates, ATP levels, … will be highly dependent on the presence of Pi, as alterations in phosphate availability will also lead to altered levels of, for instance, ATP (Martinez et al., 1998). Hence, the yeast cell can apparently detect the presence or absence of sufficient amounts of phosphate on multiple levels, creating a fine-tuned system resulting in an optimal response.

5. The pleiotropic role of Pho85 in yeast

Aside from its role in the phosphate signalling pathway, the Pho85 kinase has multiple functions in several pathways, as suggested by the pleiotropic phenotype of a *pho85Δ* strain. Cells deleted in *PHO85* not only display constitutive expression of

PHO genes, but also slow growth with G_1 delay on rich media, poor growth on non-fermentable carbon sources and on medium with proline as the sole nitrogen source (Lee et al., 2000), hyperaccumulation of glycogen (Timblin et al., 1996), abnormal morphology and irregular budding (Measday et al., 1997; Tennyson et al., 1998), sporulation defects (Gilliquet and Berben, 1993), hyperinduction of starvation-induced autophagy (Wang et al., 2001a), aberrant expression profiles in the diauxic shift (Nishizawa et al., 2004) and hypersensitivity to a large number of stress conditions (Huang et al., 2002). Furthermore, there are synthetic lethal interactions between *PHO85* and several other genes involved in the cell cycle, morphogenesis and transcription (Huang et al., 2002; Lenburg and O'Shea, 2001). Consistent with the diverse roles for Pho85, 10 genes encoding known or putative Pho85 cyclins ("Pcls") have been identified (Measday et al., 1997). They have been grouped by sequence homology into two subfamilies, each with five members: the Pcl1,2 subfamily with Pcl1, Pcl2, Clg1, Pcl5 and Pcl9; and the Pho80 subfamily with Pho80, Pcl6, Pcl7, Pcl8 and Pcl10. Binding of a cyclin partner is necessary to render the kinase catalytically active and confers substrate specificity on the kinase by modifying the inherent specificity of the catalytic site and/or providing additional high-affinity binding sites between the kinase and the substrate. We will now give an overview of cellular processes and targets known to be regulated by Pho85, as well as the Plcs involved in these regulations (see Fig. I.5 for a schematic representation). Although *in vitro* kinase studies have resulted in a plethora of possible Pho85 targets (Dephoure et al., 2005; Ptacek et al., 2005), most of these remain to be validated *in vivo*.

5.1 The role of Pho85 in cell cycle progression and morphogenesis

Several lines of evidence link Pho85 activity to the regulation of the cell cycle and cell morphology. Although deletion of *PHO85* is viable, Pho85 becomes essential in the absence of the Cdc28 G_1-cyclins Cln1 and Cln2 (Espinoza et al., 1994; Measday et al., 1994). Moreover, the Pho85 cyclins Pcl1 and Pcl2 seem to be responsible for this phenotype as a *cln1Δ cln2Δ pcl1Δ pcl2Δ* strain is also inviable. Interestingly, like *CLN1* and *CLN2*, expression of *PCL1* and *PCL2* is cell cycle regulated by the Swi4-Swi6 SBF transcription factor and peaks at the G_1 phase of the cell cycle. A major role of the Cln1 and Cln2 cyclins comprises phosphorylation of the Sic1 protein, an

inhibitor of the Cdc28-Clb kinases (Dirick et al., 1995). Phosphorylation of Sic1 promotes it for subsequent degradation which allows the yeast cells to enter the S phase and initiate DNA replication. In addition to Cdc28, Pho85 phosphorylates Sic1 *in vivo* and this Pho85-dependent phosphorylation also contributes to Sic1 degradation (Nishizawa et al., 1998). The fact that Sic1 is more stable in *pho85Δ* cells may explain the G_1 delay in this mutant. However, additional deletion of *SIC1* in the *cln1Δ cln2Δ pho85Δ* or *cln1Δ cln2Δ pcl1Δ pcl2Δ* mutant does not alleviate lethality, in contrast to a viable *cln1Δ cln2Δ cln3Δ sic1Δ* strain, suggesting that Pho85 has an additional role in cell growth, which cannot be substituted by Cdc28-Cln3. In this respect, the Pho85-Pcl1,2 kinases have been found to regulate, redundantly with the Pkc1 pathway, essential morphological aspects during the cell cycle by modulating the activities of Cdc42 and Cla4, proteins involved in the regulation of polarised growth of yeast cells and necessary for proper budding during cell division (Baetz et al., 2001; Dephoure et al., 2005; Gray et al., 1997; Gulli et al., 2000; Huang et al., 2002; Johnson, 1999; Lenburg and O'Shea, 2001; Madden et al., 1997). Detailed analysis of the defects in a *cln1Δ cln2Δ pcl1Δ pcl2Δ* strain revealed a proper G_1 expression program, but cells failed to bud and arrest at the morphogenesis/cell-size checkpoint in G_2-M transition with replicated DNA, duplicated spindle pole bodies and short spindles (Moffat and Andrews, 2004). Pcl1 and Pcl2 localise to the incipient bud site and to the nucleus. Pcl2 was also found at the bud tip and the bud neck and this localisation is dependent on septins. The G_1 cyclins were shown to be required for phosphorylation and activation of Cdc24, the Cdc42 exchange factor, establishing a link between CDK-G_1 activity and regulation of polarised growth. In addition, other components which genetically interact with Cla4 have been isolated that interact with and are phosphorylated by Pho85-Pcl1 (Keniry et al., 2004). Consequently, a build-up or burst of G_1-CDK activity through activation of the cyclin genes *CLN1,2* or *PCL1,2* is essential for correct mitotic entry through establishment of proper morphogenesis.

Pho85 is also implicated in the *in vivo* control of Swi5 activity, although the exact mechanism is not yet clear (Measday et al., 2000). Swi5 is a zinc finger transcription factor that is required for the expression of a number of genes early in the cell cycle (Aerne et al., 1998; Simon et al., 2001). One of the target genes induced by Swi5 is the *ASH1* gene. Ash1 is a daughter cell-specific repressor of HO gene transcription. Pho85 regulates the stability of Ash1 via phosphorylation, thereby regulating the

asymmetric accumulation of Ash1 between mother and daughter cells (McBride et al., 2001). Furthermore, hyperactive Ash1 may also contribute to other phenotypes of a *pho85Δ* mutant. The reduced sporulation efficiency observed in *pho85Δ* diploids is partially suppressed by additional deletion of *ASH1* and the germination delay exhibited by *pho85Δ* spores is completely suppressed by mutation of *ash1*, suggesting that the inappropriate presence of Ash1 in spores in a *pho85Δ* mutant may inhibit germination (Gilliquet and Berben, 1993; McBride et al., 2001). Consistent with this interpretation, the level of *ASH1* mRNA decreases during sporulation (Chu et al., 1998).

Cells deleted in *PHO85* or the Pcl1,2 subfamily display morphological and budding abnormalities, that include elongated buds, connected chains of cells and a random pattern of budding in diploids. (Measday et al., 1997; Tennyson et al., 1998). Regulation of cell morphology by Pho85 occurs in part via the Rvs167 protein. Rvs167 plays a role in endocytosis, organisation of the actin cytoskeleton and cell survival after starvation, and many of the phenotypes of a *rvs167Δ* mutant are similar to those of a *pho85Δ* strain (Bauer et al., 1993; Lee et al., 1998). *In vitro*, Rvs167 is a substrate of the Pho85-Pcl2 kinase and maximal phosphorylation of Rvs167 *in vivo* requires Pho85 and the Pcl1,2 subfamily of cyclins. Furthermore, phosphorylation of Rvs167 is induced *in vivo* under conditions when Pho85-Pcl2 kinase activity is also induced. Phosphorylation of Rvs167 by Pho85-Pcl1,2 kinases appears to regulate protein-protein complexes involving Rvs167 that coordinate actin dynamics, endocytosis and response to starvation (Bauer et al., 1993; Colwill et al., 1999; Lee et al., 1998).

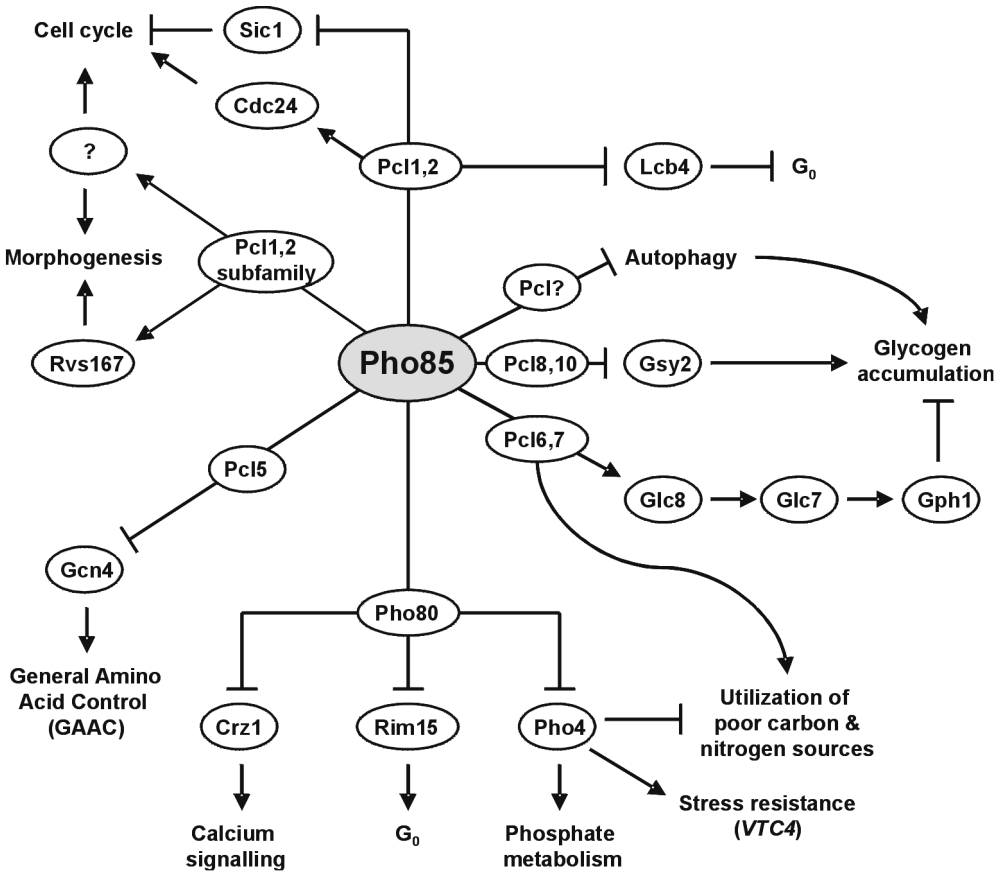


Figure I.5: The pleiotropic role of Pho85 in *S. cerevisiae*. Pho85 can associate with 10 cyclin subunits (Pcls). This figure gives an overview of the wide variety of Pho85 functions in the yeast cell, focussing on known physiological targets and the Pcls involved in their regulation. See text for details.

5.2 The function of Pho85 in signalling environmental conditions

Multiple functions of the Pho85-Pho80 kinase

As described in the previous section, the function of the Pho85-Pho80 kinase in the regulation of transcription of phosphate-responsive genes through the transcription factor Pho4 is well documented. However, starving yeast cells for phosphate induces transcription not only of PHO genes but also of stress responsive genes (Giots et al., 2003; Swinnen et al., 2005). Although the latter also requires downregulation of the kinase activity of Pho85-Pho80 by Pho81, the protein kinase Rim15 rather than Pho4 is the target of Pho85-Pho80 in this regulation (Swinnen et al., 2005; Wanke et al., 2005), implicating Rim15 as a central nutrient-integration point which allows yeast cells to properly adapt to poor nutrient conditions (Pedruzzi et al., 2003; Roosen et al.,

2005). In addition, the minimum domain of Pho81 which is sufficient to induce transcription of PHO genes (Huang et al., 2001), is not sufficient to regulate transcription of stress responsive genes via Rim15 (Swinnen et al., 2005).

Recently, the Pho85-Pho80 kinase is also implicated in the negative regulation of the Crz1 transcription factor (Sopko et al., 2006), which plays a critical role in calcium signalling (Hopper, 1999). Phosphorylation of Crz1 by Pho85-Pho80 results in its nuclear exclusion, similar to Pho4 and Rim15. Interestingly, these three Pho85-Pho80 targets all use Msn5 as their nuclear export receptor (Boustany and Cyert, 2002; Kaffman et al., 1998a; Wanke et al., 2005), indicating a common mechanism by which the Pho85-Pho80 kinase regulates its nuclear targets.

The PHO system also genetically interacts with the phospholipase C system. The temperature sensitive growth of a *plc1Δ* strain can be suppressed by overexpression of *PHO81* or a homologue *SPL2*, by deletion of *PHO85* or *PHO80*, or by growing the cells on a low-Pi medium (Flick and Thorner, 1998). Surprisingly, these suppressions are independent on Pho4, suggesting that the Pho85-Pho80 kinase has an additional target that regulates growth on elevated temperatures and nutrient uptake and/or utilisation. Rim15 may be a good candidate for this target, but this remains to be investigated.

Pho85 and sphingoid signalling

In yeast, sphingoid long-chain base 1-phosphates (LCBPs) act as bioactive lipid molecules with roles in diverse cellular processes including the progression from G_1- to S-phase of the cell cycle (Gottlieb et al., 1999; Lanterman and Saba, 1998). These LCBPs are synthesised by two LCB kinases, Lcb4 and Lcb5 (Nagiec et al., 1998). Interestingly, this kinase activity seems to be regulated by nutrients and proper levels of LCBPs are required for normal cell cycle progression or arrest in response to nutrient conditions (Gottlieb et al., 1999; Lanterman and Saba, 1998). Pho85-Pcl1 and Pho85-Pcl2 redundantly phosphorylate Lcb4 as cells enter the stationary phase, thereby targeting Lcb4 for ubiquitination and subsequent degradation (Iwaki et al., 2005). This Pho85-mediated downregulation of Lcb4 may be physiologically important for proper cellular responses to nutrient deprivation.

Pho85 activity is required for the utilisation of poor nutrient sources

Pho85 is essential for yeast cells in the utilisation of poor carbon and nitrogen sources, as *pho85Δ* cells fail to grow on non-fermentable carbon sources and on medium containing proline as the sole nitrogen source (Lee et al., 2000). Intriguingly, these growth defects can be suppressed by the additional deletion of the *PHO4* gene (Nishizawa et al., 1999b; Popova Iu et al., 2000), indicating interplay with the PHO pathway. In spite of this, growth of a *pho80Δ* strain seems to be highly dependent on the strain background, as *pho80Δ* cells can grow on non-fermentable carbon sources as well as on proline-based medium in some genetic backgrounds (Dimmer et al., 2002; Gilliquet and Berben, 1993; Nishizawa et al., 1999b; Popova Iu et al., 2000). It is thus possible that Pho85 regulates growth on poor carbon and nitrogen sources through Pho4 via the use of multiple cyclins, not only Pho80. Indeed, the Pcl6 and Pcl7 cyclins have also been implicated in the use of alternative carbon sources and proline (Lee et al., 2000), though a possible link with the Pho4 transcription factor was not investigated. Interestingly, activity of the Pho85-Pcl7 kinase was shown to be regulated by phosphate availability, through direct interaction of Pcl7 with Pho81. Moreover, the activity of the Pho85-Pcl6 kinase is also reduced in response to a fall in extracellular phosphate levels. However, Pcl6 does not interact with Pho81 and the Pho85-Pcl6 complex is regulated normally in response to changes in phosphate levels in the absence of Pho81. Finally, micro-array data show an impaired induction of many mitochondrial genes when a *pho85Δ* strain approaches the diauxic shift (Nishizawa et al., 2004). This role of Pho85 in maintaining proper mitochondrial function may partly explain the inability of a *pho85Δ* strain to grow on non-fermentable carbon sources.

Pho85 and the yeast stress response

Using micro-array approaches, it was shown that Pho85 has a significant function in the regulation of the expression of a plethora of genes (Carroll et al., 2001; Huang et al., 2002; Nishizawa et al., 2004). Many of the genes that are induced by a rapid loss of Pho85 activity are involved in the environmental stress response (ESR) (Carroll et al., 2001; Gasch et al., 2000; Gasch and Werner-Washburne, 2002). Most of them,

however, are not constitutively expressed in a strain deleted for *PHO85*, suggesting that activation of the ESR is eventually repressed in a *pho85Δ* strain. At present, the Pho85 cyclin partners and transcription factor(s) involved in this response are still largely unknown. This transcriptional response mediated by Pho85 is, at least in part, independent from the STRE promoter element (Enjalbert et al., 2004; Timblin and Bergman, 1997) and many, but not all, effects seem to be independent of the Pho4 transcription factor (Carroll et al., 2001; Huang et al., 2002; Nishizawa et al., 2004; Nishizawa et al., 2001). Pho85 may regulate some of these targets via the Rim15 protein kinase (Swinnen et al., 2005), as Rim15 regulates stress responsive genes via the transcription factors Msn2/4 and Gis1 (Cameroni et al., 2004).

Cells deleted in *PHO85* display sensitivity to a wide variety of stress conditions (Huang et al., 2002) and this sensitivity can be largely attributed to two effects. First, a significant cell integrity defect that is connected to the Pcl1,2 subfamily of cyclins. As discussed before, this subfamily is implicated in the regulation of processes such as yeast morphology in concert with the cell integrity Pkc1 pathway. Secondly, *pho85Δ* cells display a vacuole-related defect that can be assigned to the Pho80 cyclin (Huang et al., 2002). It appears that appropriate Pho85-Pho80 mediated regulation of *VTC4*, a key target of Pho4, is required for proper vacuole function and for yeast survival under a variety of suboptimal conditions. Vtc4/Phm3 encodes a putative membrane-associated protein that is involved in vacuolar polyP accumulation (Cohen et al., 1999; Ogawa et al., 2000). Deletion of *VTC4* as well as *PHO4* in a *pho80Δ* background rescues the sensitivity to various stress conditions such as high Ca^{2+} and sorbitol (Huang et al., 2002). The severe abnormalities in vacuole structure and function of *pho80Δ* and *pho85Δ* mutants are also suppressed by additional deletion of *VTC4* (or *PHO4*). Hyperinduction of *VTC4* thus leads to a failure of proper functioning of the yeast vacuole, resulting in the sensitivity to various stress conditions.

Pho85 is involved in nitrogen signalling through the Gcn4 protein

Gcn4, a member of the AP-1 family of transcription factors, is involved in diverse responses to starvation and stress signals and is induced by different signals (Hinnebusch and Natarajan, 2002). The best known function of Gcn4 is the induction

of many amino acid biosynthesis genes in response to amino acid deprivation, commonly termed the general amino acid control (GAAC) (Irniger and Braus, 2003). When cells are deprived for amino acids, general translation is impeded. However, translation of *GCN4* mRNA is selectively activated leading to an increased number of Gcn4 molecules. Secondly, nuclear Gcn4 is stabilised under amino acid deprivation, leading to a further induction of Gcn4 target genes.

In the nucleus, degradation of Gcn4 is induced by its phosphorylation and subsequent ubiquitination, after which Gcn4 is degraded by the 26S proteasome (Kornitzer et al., 1994; Meimoun et al., 2000; Pries et al., 2002). Two cyclin-dependent kinases, Srb10 and Pho85, are involved in the phosphorylation of Gcn4, thereby priming it for subsequent degradation (Chi et al., 2001; Meimoun et al., 2000). Both kinases regulate Gcn4 independently and are differentially regulated *in vivo*.

Phosphorylation of Gcn4 by Srb10, in conjunction with the Srb11 cyclin, is thought to by a constitutive process, independent of the presence or absence of amino acids. Srb10 and Srb11 are components of the mediator complex associated with the C-terminal domain of the largest subunit of RNA polymerase II (Hengartner et al., 1998; Malik and Roeder, 2000). It is proposed that Srb10 specifically phosphorylates promoter-bound Gcn4 which has recruited RNA polymerase II to the promoter region (Chi et al., 2001). This process may function to limit the lifetime of a promoter-bound Gcn4 that is actively engaged in promoting transcription, thereby restricting the number of transcripts that a promoter-bound Gcn4 molecule can produce before being destroyed, allowing cells to rapidly turn off gene expression.

The Pho85-mediated phosphorylation of Gcn4, on the other hand, is regulated by amino acid availability, and may be responsible for degradation of excess, non-chromatin bound, Gcn4 molecules (Meimoun et al., 2000). Gcn4 stability increases upon amino acid starvation because of a decrease in Pho85-dependent phosphorylation. Pcl5 is the cyclin that is specifically required for Gcn4 phosphorylation and degradation (Shemer et al., 2002). In starved cells, stabilised Gcn4 activates transcription of its target genes, including *PCL5*. Still, Pcl5 fails to accumulate to high levels due to its intrinsic instability and slow protein synthesis under these conditions. Therefore, Pho85 activity against Gcn4 is low and the protein is stabilised. Upon recovery from amino acid starvation, translation of *PCL5* mRNA occurs is boosted, leading to higher Pho85-Pcl5 kinase activity, which in turn

phosphorylates Gcn4 and thereby triggering its degradation. It is apparent that this represents a highly sophisticated mechanism to quickly respond to environmental changes. By virtue of its constitutive metabolic instability, Pcl5 may act as a sensor of cellular protein biosynthetic capacity, regulating Gcn4 stability in response to amino acid availability.

The role of Pho85 in controlling reserve carbohydrate metabolism

Pho85 is part of an elaborate network of signal transduction cascades regulating glycogen biosynthesis, including also the Tor, PKA and Snf1 kinases (Francois and Parrou, 2001; Roosen et al., 2004). Glycogen biosynthesis is regulated at the level of transcription as well as through posttranslational modifications of key enzymes. *Saccharomyces cerevisiae* has two isoforms of glycogen synthase, encoded by *GSY1* and *GSY2*. Although both genes are induced as yeast approaches the stationary phase, Gsy2 represents the predominant activity responsible for glycogen accumulation in the diauxic shift (Enjalbert et al., 2004; Unnikrishnan et al., 2003). Transcriptional induction of *GSY2* is a result of a combinatorial control by the protein kinases PKA, Snf1 and Pho85, each affecting *GSY2* transcription independently (Enjalbert et al., 2004). Pho85 represses transcription of *GSY2* independent of the STRE promoter element and the Pho4 transcription factor (Timblin and Bergman, 1997). Instead, a 14bp-G/C rich sequence in the *GSY2* promoter seems to be the target of Pho85 action (Enjalbert et al., 2004). The required cyclin(s) for this action remain obscure. In addition, Pho85 has also been shown to be involved in the transcriptional regulation of *UGP1* (Nishizawa et al., 2001), encoding the UDP-glucose pyrophosphorylase, the enzyme that provides UDP-glucose for the biosynthesis of glycogen, trehalose and β-glucan. Interestingly, this regulation occurs via Pho4, although Pho85 may use a cyclin different than Pho80 in this regulation.

In addition to transcriptional regulation, the activities of glycogen synthase and glycogen phosphorylase is dependent on their phosphorylation state and thus on the relative activity of protein kinases and phosphatases. Phosphorylation of Gsy2 impairs glycogen synthase activity (Hardy and Roach, 1993), whilst phosphorylation of Gph1 activates glycogen phosphorylase activity (Lin et al., 1995). How the dynamic process of (de)phosphorylation is regulated *in vivo* is not yet very clear, but several reports

indicate a major role of the metabolite glucose-6-phosphate (Glc-6-P). Glc-6-P, aside from being a substrate in glycogen biosynthesis, acts as a potent stimulator of the dephosphorylation and as an inhibitor of the phosphorylation processes (Francois and Hers, 1988; Huang et al., 1997; Lin et al., 1996). Furthermore, Glc-6-P also non-competitively inhibits glycogen phosphorylase (Lin et al., 1995) and allosterically activates glycogen synthase (Pederson et al., 2000; Pederson et al., 2004).

In cooperation with the cyclins Pcl8 and Pcl10, Pho85 phosphorylates Gsy2 *in vivo*, leading to the inactivation of the enzyme (Huang et al., 1996b; Huang et al., 1998; Wilson et al., 1999). Both cyclins appear to be partially redundant in this function and Gsy2 is the sole physiological target for Pho85-Pcl8,10 identified to date. Deletion of *PHO85* as well as *PCL8* and *PCL10* thus leads to an increased activity of glycogen synthase. However, *pho85Δ* cells also display an increased glycogen phosphorylase activity independent of the Pcl8 and Pcl10 cyclins (Wilson et al., 2002), indicating that Pho85 has multiple inputs in glycogen metabolism, using different cyclin partners and acting antagonistically. This may be an explanation for the fact that the hyperaccumulation of glycogen, which was considered to be a typical phenotype of a *pho85Δ* strain (Timblin et al., 1996), is strongly dependent on the genetic background (Lee et al., 2000). Indeed, the cyclins Pcl6 and Pcl7 were additionally implicated in the regulation of glycogen levels in yeast (Lee et al., 2000), apparently regulating both glycogen synthase activity as well as glycogen phosphorylase activity (Wang et al., 2001b). The physiological target connecting Pho85-Pcl6,7 kinase activity to glycogen metabolism appears to be Glc8. *GLC8* was originally discovered in a screen for glycogen-deficient mutants (Cannon et al., 1994) and encodes for an *in vivo* activator of the protein phosphatase Glc7 (Nigavekar et al., 2002). Glc8 activity is regulated by at least two known mechanisms. First, *GLC8* expression is induced in stationary phase (DeRisi et al., 1997). Second, Glc8 requires phosphorylation for *in vivo* function, and Pho85 was identified as the sole Glc8 kinase *in vivo*, with Pcl6 and Pcl7 as the major contributing cyclins (Tan et al., 2003). Glc8 acts to promote dephosphorylation and inactivation of glycogen phosphorylase (Gph1) by the Glc7 protein phosphatase, possibly in concert with the Shp1 regulatory subunit (Wilson et al., 2005). Thus, in a *pho85Δ* or *pcl6Δ pcl7Δ* strain, loss of Glc8 function leads to elevated glycogen phosphorylase activity.

As cells approach stationary phase, autophagy is induced and this is required to maintain glycogen levels in stationary phase (Wang et al., 2001a). Compared to wild-type cells, a *pho85Δ* strain displays a hyperinduction of autophagy when approaching the stationary phase, indicating another Pho85-dependent mechanism in regulating glycogen levels. The identities of the Pcls involved in this regulation, as well as the molecular connection with the process of autophagy are still unknown.

Several data exist that also link Pho85 with the control of trehalose biosynthesis. Rapid inactivation of Pho85 activity results in an increased expression of several genes involved in trehalose metabolism (Carroll et al., 2001). Furthermore, regulation of Pho85 activity via the Pho81 protein is crucial for proper regulation of the dynamics of trehalose metabolism in response to phosphate levels (Swinnen et al., 2005). One physiological target in this regulation is the trehalase enzyme, as Pho85 affects trehalase activity, in conjunction with cyclin(s) other than Pho80.

5.3 Regulation of Pho85 activity

As discussed above, Pho85 is a multifunctional kinase involved in the regulation of numerous processes in yeast through interaction with different cyclin partners. It is therefore of vital importance that the activities of the various Pho85 kinase complexes are tightly regulated in response to the proper stimuli. Modulation of the kinase activity may occur via several means, including regulation of the Pho85 kinase itself, its associating cyclin subunits or through additional proteins.

Pho85 appears to have a regulation mechanism different from CDKs functioning throughout the cell cycle (Nishizawa et al., 1999b). More specifically, phosphorylation of Pho85 in its T-loop region is dispensable for kinase activity, while phosphorylation of tyrosine-18 (Y18) may function as a signal which discriminates various Pho85 cyclins, allowing a switch in function (Nishizawa et al., 1999b; Wilson et al., 1999).

Another important mode for controlling Pho85 activity occurs through regulation at the cyclin level. First, expression of several cyclins is cell cycle regulated. Expression of *PCL1* and *PCL2* peaks in the G_1-phase of the cell cycle and is dependent on the Swi4 transcription factor (Baetz et al., 2001; Madden et al., 1997; Measday et al.,

1994; Measday et al., 1997; Ogas et al., 1991). *PCL9* transcription depends on the Swi5 transcription factor and peaks at the late M to early G_1 phase (Aerne et al., 1998; Tennyson et al., 1998). Finally, expression of *PCL7*, as well as Pho85-Pcl7 activity, is maximal in mid to late S phase (Lee et al., 2000). Secondly, expression of several Pho85 cyclin genes is also regulated by the available carbon source, the growth phase and the Pho85 kinase itself (Nishizawa et al., 2001). The latter implicates the existence of several regulatory loops, wherein Pho85 activity may regulate the expression of some of its own regulatory subunits. For instance, Pho85 has been implicated in the regulation of the Swi5 transcription factor (Measday et al., 2000), which in turn is responsible for expression of the *PCL9* gene (Tennyson et al., 1998). Regulating the expression of cyclin genes provides a manner to ensure that certain cyclins, and thus certain Pho85 activities, are only present when required, avoiding the existence of unwanted or even harmful activities. Finally, as shown for the Pcl5 cyclin, regulation of Pho85 activity may occur by posttranslational modulation of cyclin activity, as the inherent instability of the Pcl5 protein allows regulation of the Pho85-Pcl5 kinase in response to amino acid availability (Shemer et al., 2002).

A last mode of regulation of Pho85 activity comprises the interaction with additional proteins. This is exemplified by the Pho81 CKI protein. Regulating the Pho85-Pho80 kinase activity in response to phosphate levels occurs through a phosphate-dependent inhibition of the kinase complex by Pho81. However, besides the well known Pho4 transcription factor, the Pho85-Pho80 has two additional targets; Crz1 and Rim15 (Sopko et al., 2006; Swinnen et al., 2005; Wanke et al., 2005). Intriguingly, the Pho81 minimum domain (Huang et al., 2001) is insufficient for regulating Pho85-Pho80 activity towards the Rim15 target (Swinnen et al., 2005). Thus, different domains of Pho81 may be involved in regulating the activity of the Pho85-Pho80 kinase towards different target proteins, which may constitute a fine-tuning mechanism allowing proper integration of the phosphate starvation response with other nutrient signals. In addition to Pho81, Spl2 is also thought to act as a Pho85 CKI and may be involved in the regulation of the Plc1 pathway (Flick and Thorner, 1998).

For some Pho85-Pcl kinases, there are no indications concerning the mode of regulation. For instance, although the Pho85-Pcl8,10 kinases have been demonstrated to phosphorylate Gsy2 *in vivo*, nothing is known about how the activities of these kinases are regulated. The same holds true for the Pho85-Clg1 kinase, which has been

implicated in the regulation of sporulation (Deutschbauer et al., 2002; Enyenihi and Saunders, 2003). Concerning the Pho85-Pcl6 kinase, it has been reported that its activity is also responsive to phosphate levels (Lee et al., 2000). Intriguingly, this seems to occur in a Pho81-independent manner.

Many cellular targets and modes of regulation of Pho85 activity have thus been described, shedding light on the way this kinase integrates multiple signals allowing it to perform its pleiotropic function and aiding the yeast cell to respond properly to changing environmental conditions.

5.4 Cdk5 – the mammalian orthologue of Pho85

Although Pho85 exerts many functions in budding yeast, few reports have been made about functional homologues in other organisms. Besides the *Candida albicans* Pho85 homologue (Gildor et al., 2005; Miyakawa, 2000), only the mammalian Cdk5 has been described. Cdk5 plays a pivotal role in neuronal development, with major roles in the regulation of cytoskeletal, membrane and adhesion systems. Moreover, Cdk5 is a regulator of neuronal death and survival, linking Cdk5 to the etiopathology of neurodegenerative diseases such as Alzheimer's disease (Cheung and Ip, 2004; Cruz and Tsai, 2004a; Cruz and Tsai, 2004b; Dhavan and Tsai, 2001; Smith and Tsai, 2002). Cdk5 displays 56% identity and 72% similarity with Pho85 at the primary amino acid level (Huang et al., 1999). Regulation of the two kinases appears similar: they are not further activated by Cdk-activating kinases, whereas phosphorylation of a tyrosine residue appears to regulate binding to their regulatory subunits (Lazaro et al., 1996; Nishizawa et al., 1999b; Poon et al., 1997). Like Pho85, activation of Cdk5 requires binding to one of its regulatory cyclin subunits, p35/25 and p39/29. Additionally, Cdk5 can form a functional kinase by binding to several Pho85 cyclins and conversely, Pho85 can form a functional kinase by binding to the Cdk5 p35/25 subunit (Huang et al., 1999). As expression of *CDK5* in yeast cells complements most phenotypes associated with *pho85Δ* mutant, Cdk5 and Pho85 are regarded as true functional homologues (Huang et al., 1999; Nishizawa et al., 1999a). Some functions of Pho85 and Cdk5 seem to be conserved between yeast and mammalian cells. For instance, both Cdk5 and Pho85 have been implicated in the regulation of the actin

cytoskeleton, in part through phosphorylation of the mammalian amphiphysin/yeast Rvs167 orthologues. (Floyd et al., 2001; Rosales et al., 2000; Tomizawa et al., 2003), (Lee et al., 1998). Interestingly, regulation of the Cdk5-p35 kinase may be very similar to that of the yeast Pho85-Pho80 kinase. C42, a protein isolated in a two-hybrid screen with p35, displays an inhibitory effect on Cdk5-p35 activity (Ching et al., 2002). Moreover, the inhibitory domain of C42 shows a significant homology to the inhibitory region of Pho81.

Given the functional homology of Cdk5 and Pho85, studies of Pho85 and Cdk5 function in yeast and neuronal cells are likely to be mutually informative. In addition, this functional homology allows yeast to serve as a model system to study relevant Cdk5-dependent human disease features (Vandebroek et al., 2005; Zabrocki et al., 2005).

Outline of this study

Since inorganic phosphate is an essential nutrient, it is critical for the yeast cell to be able to sense phosphate availability and to integrate this information with the general nutrient status of the cell. The yeast PHO pathway constitutes a well-characterised signalling pathway by which inorganic phosphate regulates its own uptake and metabolism (Persson et al., 2003). However, Pi also acts as a nutrient signal regulating general growth characteristics. Specifically, phosphate has been shown to act in concert with glucose as a nutrient signal regulating so-called PKA properties, indicated as phosphate-induced FGM signalling (Giots et al., 2003). Yet, the mechanisms and components involved in this Pi-induced signalling pathway are still elusive. However, as Pi signalling impinges on targets also regulated by other nutrient-induced signalling pathways, some components may be shared between different nutrient signalling pathways, thereby acting as integration points for different nutritional signals.

In this study, we decided to analyse the role of the PHO pathway in the phosphate-dependent regulation of some typical PKA targets in yeast. As phenotypic readouts, we chose to study trehalose metabolism and the expression of stress responsive genes, as both these properties are tightly regulated by PKA (Winderickx et al., 2003). We followed these properties both during phosphate starvation and after re-addition of KH_2PO_4 to Pi-starved yeast cells. Two major goals were set at the beginning of our study:

- Define the role of the PHO pathway in the regulation of the specified PKA targets and determine the function of each component of the PHO pathway within this regulation.
- Identification of components integrating the phosphate signal derived from the PHO pathway with other nutrient signalling pathways.

Since the components of the PHO pathway are known, the activity of this pathway can be modulated by targeted deletion of particular components, allowing us to assign possible new functions to these proteins. In Chapter II we initiated our study by comparing the response to Pi-availability of a wild-type yeast strain with several

pho mutant strains in which the activity of the Pho85-Pho80 kinase is affected in a specific known way. Combined with further epistasis analysis our results validate the involvement of the PHO pathway in the phosphate-dependent regulating of

1. trehalose metabolism
2. the expression of PDS-controlled stress responsive genes.

As the latter is regulated by the Sch9 and Rim15 protein kinases in a glucose-dependent manner, we focussed our attention to these kinases as possible points of integration of the phosphate signal with other nutrient-induced signalling pathways. We show that while Sch9 seems to be functioning largely independent of Pho85, Rim15 regulates G_0-properties in response to Pi by functioning downstream of the Pho85-Pho80 kinase. During the course of these studies, Rim15 was identified as a physiological target of the Pho85-Pho80 kinase (Wanke et al., 2005), thereby validating our genetic data and placing Rim15 in a central position in the integration of nutrient signals.

The yeast response to the re-addition of Pi to cells previously starved for this nutrient is closely related to its ability to take up Pi. Although it is assumed that Pho84 represents the major Pi-uptake system in Pi-starved yeast cells, we observed clear phosphate signalling in the *pho4Δ* and *pho81Δ* strains, which lack detectable *PHO84* expression. Therefore, in Chapter III, we investigated the requirement of specific phosphate transporters in the phosphate signalling process. We established that rapid phosphate transport and signalling are specifically mediated by

1. the high-affinity phosphate transporter Pho84, when Pho4 is active
2. the Pho87 low-affinity phosphate transporter in a strain lacking Pho4

Furthermore, we demonstrate that the regulation of the activity of Pho87 occurs at a posttranscriptional level.

In search for a physiological function for this phenomenon, we discovered that Pho4 is inactivated upon glucose removal from Pi-starved yeast cells, indicating that Pho87 might be responsible for phosphate transport and/or signalling when yeast cells are exposed to a combination of phosphate and glucose limitation. Interestingly, we determined Rim15 as an essential component in the glucose-dependent regulation of Pho4 activity, further emphasizing the role of Rim15 in phosphate signalling and the integration of nutrient signals.

Chapter II

The involvement of the yeast PHO pathway in phosphate starvation-induced stress responses

Abstract

The PHO pathway in yeast is regulated by inorganic phosphate (Pi) to control expression of genes involved in acquisition of phosphate from the medium. This pathway was also reported to contribute to other nutritional responses and as such it affects several phenotypic characteristics known also to be regulated by protein kinase A (PKA), including trehalose metabolism and transcription of genes involved in the general stress response. We now demonstrate that transcription of PDS-controlled stress responsive genes is solely regulated by the Pho85-Pho80 complex whereas regulation of trehalose metabolism apparently involves several Pho85 cyclins. Interestingly, both read-outs depend on Pho81 but while the previously described minimum domain of Pho81 is sufficient to sustain phosphate-regulated transcription of PHO genes, full-length Pho81 is required to control trehalose metabolism and the PDS-targets. Consistently, neither expression control of stress regulated genes nor trehalose metabolism relies directly on Pho4. Finally, we present data supporting that the PHO pathway functions in parallel to the Sch9-controlled pathway and that both pathways share the protein kinase Rim15, which was previously reported to play a central role in the integration of glucose, nitrogen and amino acid availability.

Introduction

Inorganic phosphate (Pi) is an essential nutrient for all organisms, being required for the biosynthesis of nucleic acids, phospholipids and cellular metabolites as well as for energy metabolism and signal transduction. Microorganisms often encounter limiting concentrations of this essential nutrient and have evolved complex signal transduction networks that enable them to make optimal use of the scarce available phosphate sources. When Pi in the medium becomes limiting, the budding yeast, *Saccharomyces cerevisiae*, activates a regulatory mechanism known as the PHO pathway, which results in an increased expression of multiple genes involved in the acquisition, uptake and storage of phosphate, which enables the yeast to adapt its growth to low concentrations of Pi and scavenge the environment for alternative phosphate sources (Almaguer et al., 2004; Fisher et al., 2005; Ogawa et al., 2000; Persson et al., 2003).

When all phosphate sources from the medium are depleted, yeast cells enter the quiescent G_0-phase and acquire typical G_0-traits, such as the accumulation of the reserve carbohydrates trehalose and glycogen, and the induction of a multitude of stress responsive genes (Giots et al., 2003; Lillie and Pringle, 1980). These properties will lead to a general increase in stress resistance of the yeast cell allowing it to survive the starvation period. Yeast protein kinase A (PKA) is known to be a master regulator of G_0-traits (Thevelein et al., 2000; Thevelein and de Winde, 1999) and it has been shown that inorganic phosphate acts in concert with glucose as a nutrient signal regulating these PKA-dependent properties (Giots et al., 2003). In general, readdition of any essential nutrient to yeast cells starved for this nutrient, on a glucose-containing medium, elicits similar responses that mimic the addition of glucose to derepressed cells (Winderickx et al., 2003). However, cAMP does not act as a second messenger. Since this type of nutrient signalling depends on the presence of a rapid fermentable sugar, such as glucose, and all other nutrients essential for growth, the pathway involved was named the FGM (Fermentable Growth Medium induced) pathway (Thevelein, 1994).

In this chapter, we decided to investigate cross-talk between the PHO pathway and phosphate-induced FGM signalling. The central kinase of the PHO pathway, the

CDK Pho85, is known to modulate several stress responses which are also targets for PKA-dependent regulation, making a possible connection between phosphate-induced PHO- and FGM-signalling plausible. As the PHO pathway is the best characterised phosphate-dependent signalling pathway in yeast, its activity can be modulated with great precision. This permits us to assign possible new functions of this pathway to specific components. In addition,

Results and Discussion

1. Pho81-mediated inhibition of the Pho85-Pho80 complex is required for the correct regulation of stress-related phenotypes

To investigate the role of the PHO pathway in phosphate-induced signalling towards PKA targets, we start our study by comparing the response to Pi-availability of a wild type yeast strain with that of specific *pho* mutant strains. For this, yeast cells growing exponentially on glucose, in which nutrient-induced stress responses are minimal, are transferred to medium lacking Pi and supplemented with 4% glucose. Therefore, any stress responses induced are specifically due to the lack of Pi.

The activity of the Pho85-Pho80 kinase is modulated in response to Pi levels via the Pho81 protein (Lenburg and O'Shea, 1996). Therefore, we decided to investigate the following mutant strains:

- *pho85Δ* and *pho80Δ*. These mutants will reveal which phenotypes are dependent on an active Pho85 kinase (*pho85Δ*). Furthermore, by comparing the *pho85Δ* and *pho80Δ* strains we can discriminate between processes regulated specifically by the Pho85-Pho80 kinase and processes which may require other or additional Pho85-Pcl kinases.
- *pho81Δ*, *pho81Δ pho85Δ* and *pho81Δ pho80Δ*. The *pho81Δ* mutant contains a constitutively active Pho85-Pho80 kinase, which is not inactivated during Pi starvation, and will reveal the role of Pho81 in the Pi-signalling process. If certain phenotypes are affected in a *pho81Δ* mutant, the *pho81Δ pho85Δ* and *pho81Δ pho80Δ* strains will tell us if this is specifically due to an active Pho85-Pho80 kinase or due to another Pho85-Pcl kinase which is also regulated by Pho81.

Transferring exponentially growing yeast cells to Pi-depleted medium will induce growth arrest (Fig. II.1) and the cells enter the G_0 phase due to Pi starvation..

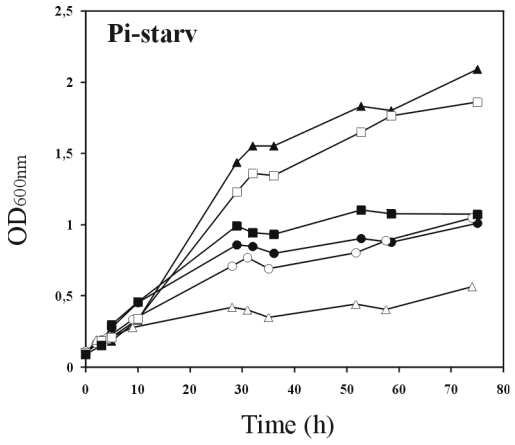


Figure II.1: Growth profile of phosphate-starved yeast cells.

Mid-exponential growing cells were harvested and transferred to Pi-starvation medium containing 4% glucose. Growth arrest was monitored for 3 days. Strains: wild type (●), *pho85Δ* (○), *pho80Δ* (▲), *pho81Δ* (△), *pho81Δ pho85Δ* (■), *pho81Δ pho80Δ* (□).

During starvation, these cells acquire stress resistance as they accumulate the stress-protectant trehalose and induce the expression of stress-responsive genes (Fig. II.2). The latter include genes that contain solely PDS-elements in their promoter, such as *SSA3* and *GRE1*, allowing transcriptional control by the transcription factor Gis1 and genes that contain STRE-elements in their promoter, such as *HSP26*, which constitute the binding sites for the transcription factors Msn2 and Msn4 (Fig. II.2.B) (Cameroni et al., 2004; Pedruzzi et al., 2000; Roosen et al., 2005). The deletion of *PHO80* or *PHO85* did not prevent the starvation-induced responses, which was expected since the Pho85-Pho80 complex is inhibited by Pho81 under conditions of phosphate limitation. Deletion of *PHO81*, on the other hand, impaired the accumulation of trehalose (Fig. II.2.A) and it displayed a delayed and reduced induction of the PDS-driven genes *SSA3* and *GRE1* while effects on the STRE-driven gene *HSP26* were minimal (Fig. II.2.B, II.2.C). The defects observed in the *pho81Δ* strain cannot be ascribed to a retarded entry into stationary phase, as the *pho81Δ* mutant arrests growth even sooner than a wild-type strain (Fig. II.1), thus pointing to a true signalling defect.

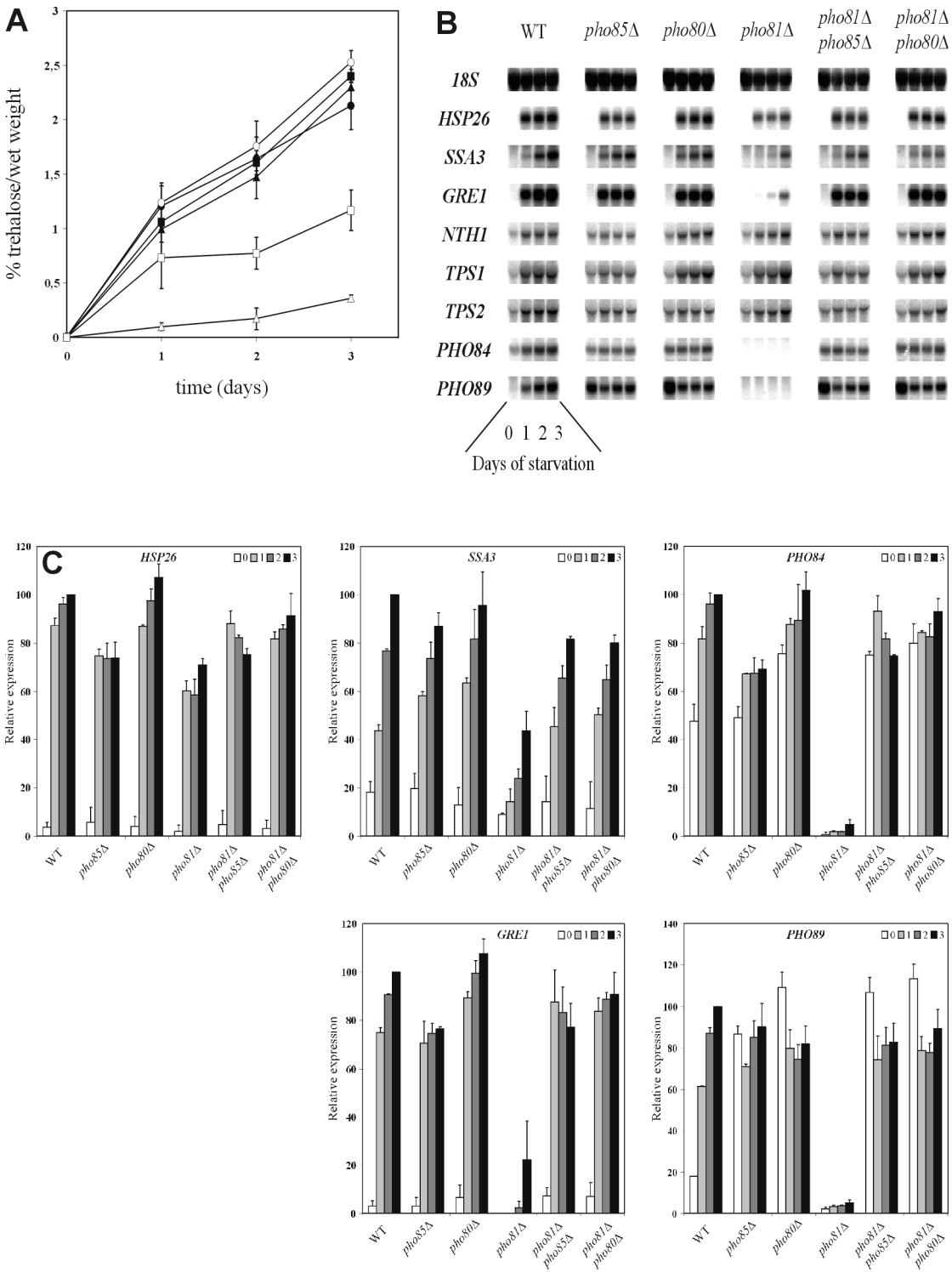


Figure II.2: **The PHO-pathway mediates phosphate starvation-induced effects on known PKA targets.**

Exponentially growing cells of the wild type (BY4741) and isogenic *pho* mutants were harvested and transferred to phosphate-depleted medium. The trehalose content in the different strains **(A)** was determined each day of the starvation period. Strains: wild type (●), *pho85Δ* (○), *pho80Δ* (▲), *pho81Δ* (△), *pho81Δ pho85Δ* (■), *pho81Δ pho80Δ* (□). Mean values from three independent experiments are

shown, with standard deviation (SD) (error bars). Samples of wild type strain and isogenic *pho* mutants were also taken during the starvation period for Northern blot and gene expression analysis **(B)** of stress responsive genes, i.e. *HSP26*, *SSA3* and *GRE1*, genes encoding proteins involved in trehalose metabolism, i.e. *NTH1*, *TPS1* and *TPS2*, and known phosphate responsive genes, i.e. *PHO84* and *PHO89*. *18S* mRNA levels are shown as a standard. Expression levels for the stress responsive genes and PHO genes were quantified **(C)** to demonstrate the significance of the difference between the wild type and different *pho* mutants. Average values from three independent experiments are shown, with SD (error bars). The wild type level of each gene after 3 days of starvation was set to 100%.

Subsequent analysis of the *pho81Δ pho85Δ* and *pho81Δ pho80Δ* double deletion strains showed that the defect in PDS-controlled gene expression of the *pho81Δ* mutant was due to a constitutive active Pho85-Pho80 complex. However, additional deletion of *PHO80* in the *pho81Δ* mutant only partially suppressed the defect in trehalose accumulation of the *pho81Δ* strain, in contrast to the full suppression obtained by additional deletion of *PHO85* (Fig. II.2.A). This result indicates that other Pho85-associated cyclin(s) are required to maintain increasing trehalose levels during phosphate starvation. Examination of the expression of *NTH1*, *TPS1* and *TPS2*, encoding respectively the trehalase enzyme, the trehalose-6-phosphate synthase and phosphatase subunit of the trehalose synthase complex, during phosphate starvation revealed no significant defects in the induction of these genes in any of the *pho* mutants compared with the wild type strain (Fig. II.2.B). This suggests that the defect in trehalose accumulation of the *pho81Δ* mutant is likely due to impaired post-transcriptional regulation of the enzymes involved in trehalose metabolism.

Re-addition of phosphate to phosphate-starved wild type cells reversed the starvation responses and triggered a rapid activation of trehalase (Fig. II.3.A) allowing for mobilization of the trehalose reserves (Fig. II.3.B). The single and combined *pho85Δ* and *pho80Δ* mutants, however, did not (efficiently) mobilize their trehalose reserves despite the observation that there was still a considerable activation of trehalase, albeit lower than in the wild-type. This uncoupling of trehalose degradation and trehalase activation was even more pronounced in the *pho81Δ* strain where trehalose levels appeared to increase upon phosphate re-addition despite clear activation of trehalase. The *pho81Δ* mutant also displayed a significant higher basal

trehalase activity before phosphate re-supplementation, which is consistent with the lower trehalose content found in starved cells. Interestingly, the basal trehalase activity was only restored by the additional deletion of *PHO85* but not by deletion of *PHO80*. The latter again indicates that besides Pho80, other Pho85-associating cyclins should be involved to maintain proper trehalose metabolism.

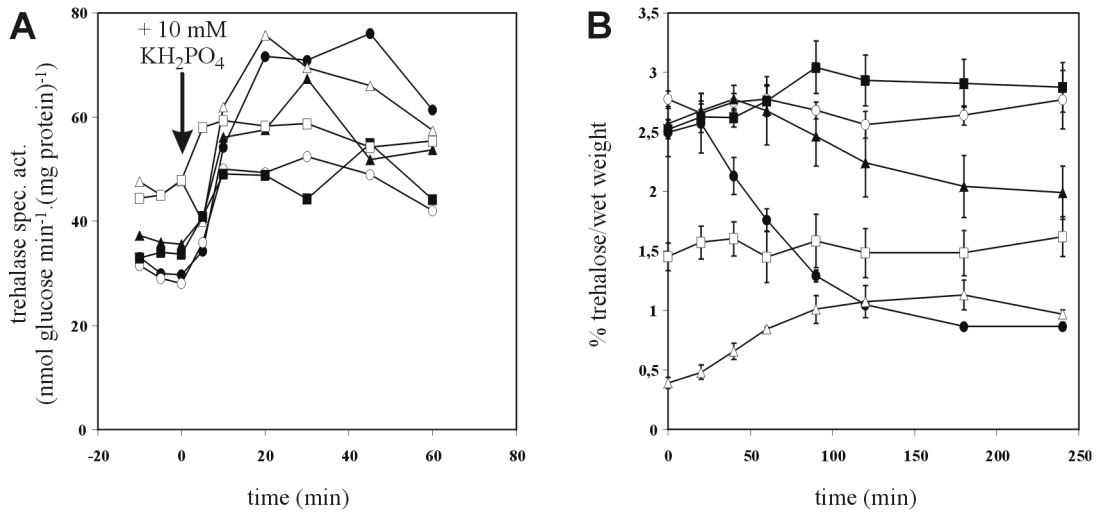


Figure II.3: The effect on trehalose metabolism of phosphate re-supplementation to phosphate-starved cells.

Trehalase activation **(A)** and mobilization of trehalose **(B)** after the addition of 10 mM KH_2PO_4 to phosphate-starved cells of the wild type strain and isogenic *pho* mutants. Strains: wild type (●), *pho85Δ* (○), *pho80Δ* (▲), *pho81Δ* (△), *pho81Δ pho85Δ* (■), *pho81Δ pho80Δ* (□). For trehalose levels, mean values from three independent experiments are shown, with SD (error bars).

Re-supplementation of phosphate to the starved cells also triggered a rapid repression of both PDS- and STRE-controlled genes in the wild type strain as well as in the single *pho81Δ* mutant (Fig. II.4.A, II.4.B). In the mutants lacking *PHO85* or *PHO80*, however, this repression was significantly delayed and for some genes even absent, indicating that phosphate-induced repression of the PDS- and STRE-driven genes is dependent on the Pho85-Pho80 complex. Surprisingly, repression of some or all of the genes involved in trehalose metabolism (*NTH1*, *TPS1* and *TPS2*) was defective in all *pho* mutants tested, even in the *pho81Δ* strain, and may in part account for the lack of proper trehalose mobilization after the addition of phosphate, despite activation of trehalase under the same conditions. As noted previously, this result indicates that these genes do not behave as typical STRE-controlled genes and that

other but yet unknown mechanisms and factors contribute to the regulation of their expression (Winderickx et al., 1996; Zahringer et al., 1998; Zahringer et al., 2000).

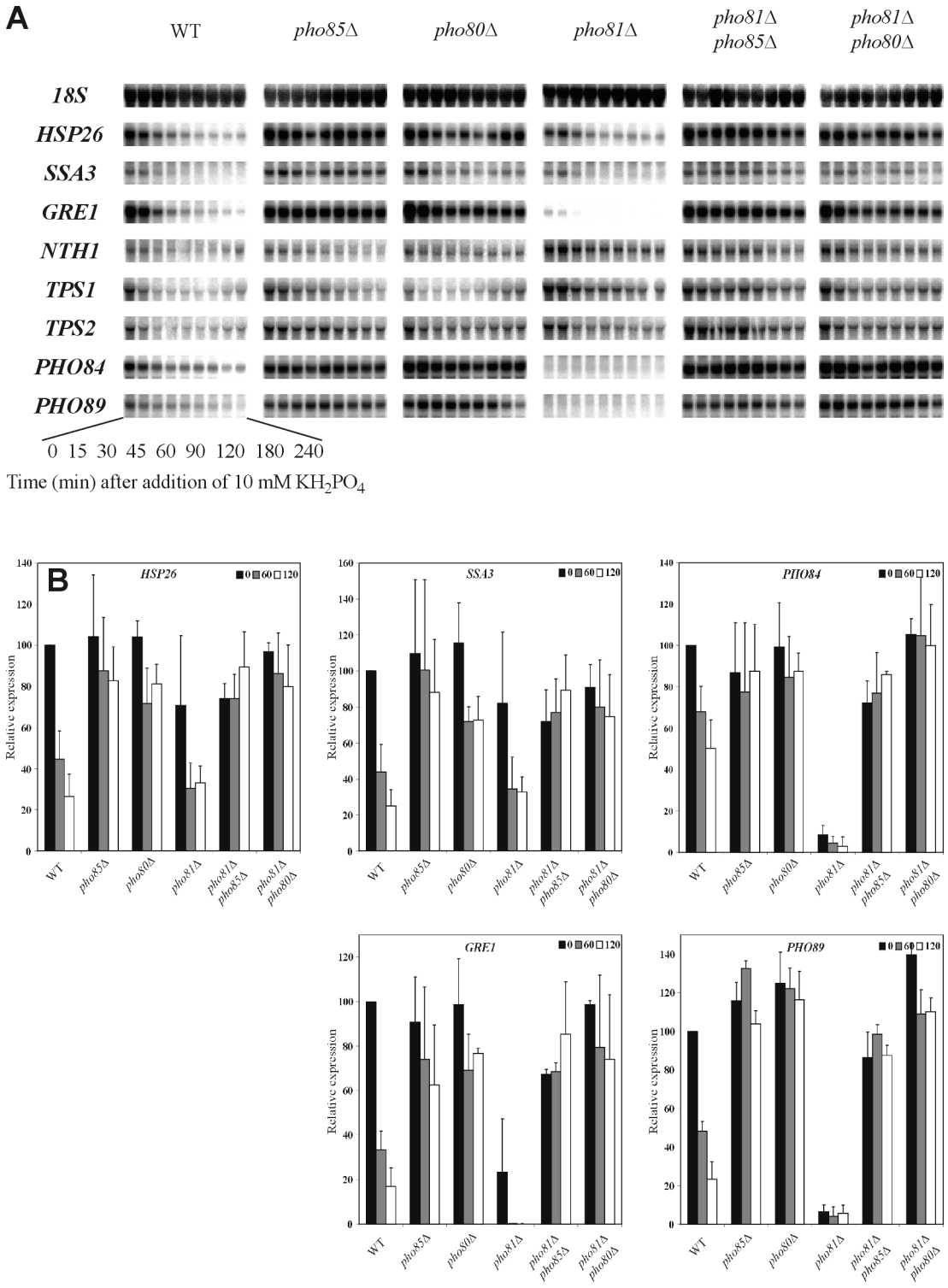


Figure II.4: The effect on gene expression of phosphate re-supplementation to phosphate-starved cells.

Northern blot analysis **(A)** for stress responsive genes, i.e. *HSP26*, *SSA3* and *GRE1*, genes encoding proteins involved in trehalose metabolism, i.e. *NTH1*, *TPS1* and *TPS2*, and phosphate responsive genes, i.e. *PHO84* and *PHO89*, after the addition of 10 mM KH_2PO_4 to the phosphate-starved cells of the wild type strain and isogenic *pho* mutants. Samples were taken before and several minutes after phosphate re-supplementation as indicated. *18S* mRNA levels are shown as a standard. Expression levels for the stress responsive genes and PHO genes before phosphate re-addition and 60 and 120 minutes after the addition of 10 mM KH_2PO_4 to the phosphate-starved cells were quantified **(B)** to demonstrate the significance of the difference in repression between the wild type and different *pho* mutants. Average values from three independent experiments are shown, with SD (error bars). The initial wild type level of each gene was set to 100%.

As a control for the experiments described above, the expression of typical Pho4 targets such as *PHO84* and *PHO89*, was monitored during phosphate starvation (Fig. II.2.B, II.2.C) and after re-supplementation of phosphate to the starved cells (Fig. II.4.A, II.4.B). Consistent with previously reported data (Lenburg and O'Shea, 1996), the Pho4 target genes are induced in wild type strain upon starvation and repressed when phosphate is again available. Their expression is completely abolished in the *pho81Δ* mutant, while being constitutive in the single *pho85Δ* and *pho80Δ* mutants as well as the double *pho81Δ pho85Δ* and *pho81Δ pho80Δ* mutants.

2. The Pho81 minimum domain is not sufficient for the Pi-dependent regulation of trehalose metabolism and the expression of PDS-driven genes.

Pho81 contains a minimum domain that appeared to be necessary and sufficient for its CKI function (Huang et al., 2001). This minimum domain comprises a region of 80 amino acids C-terminal to the ankyrin repeats (amino acids 645-724, see Fig. II.5.A) and is required for binding of Pho81 to the Pho85-Pho80 kinase complex. So far, only the regulation of the Pho5 acid phosphatase activity in a *pho81Δ* strain was used as readout to demonstrate the importance of this minimum domain. Therefore, different *PHO81* truncations (depicted in Fig. II.5.A) were tested for their ability to complement the defects related to trehalose metabolism and expression of the PDS genes *SSA3* and *GRE1* in the *pho81Δ* mutant.

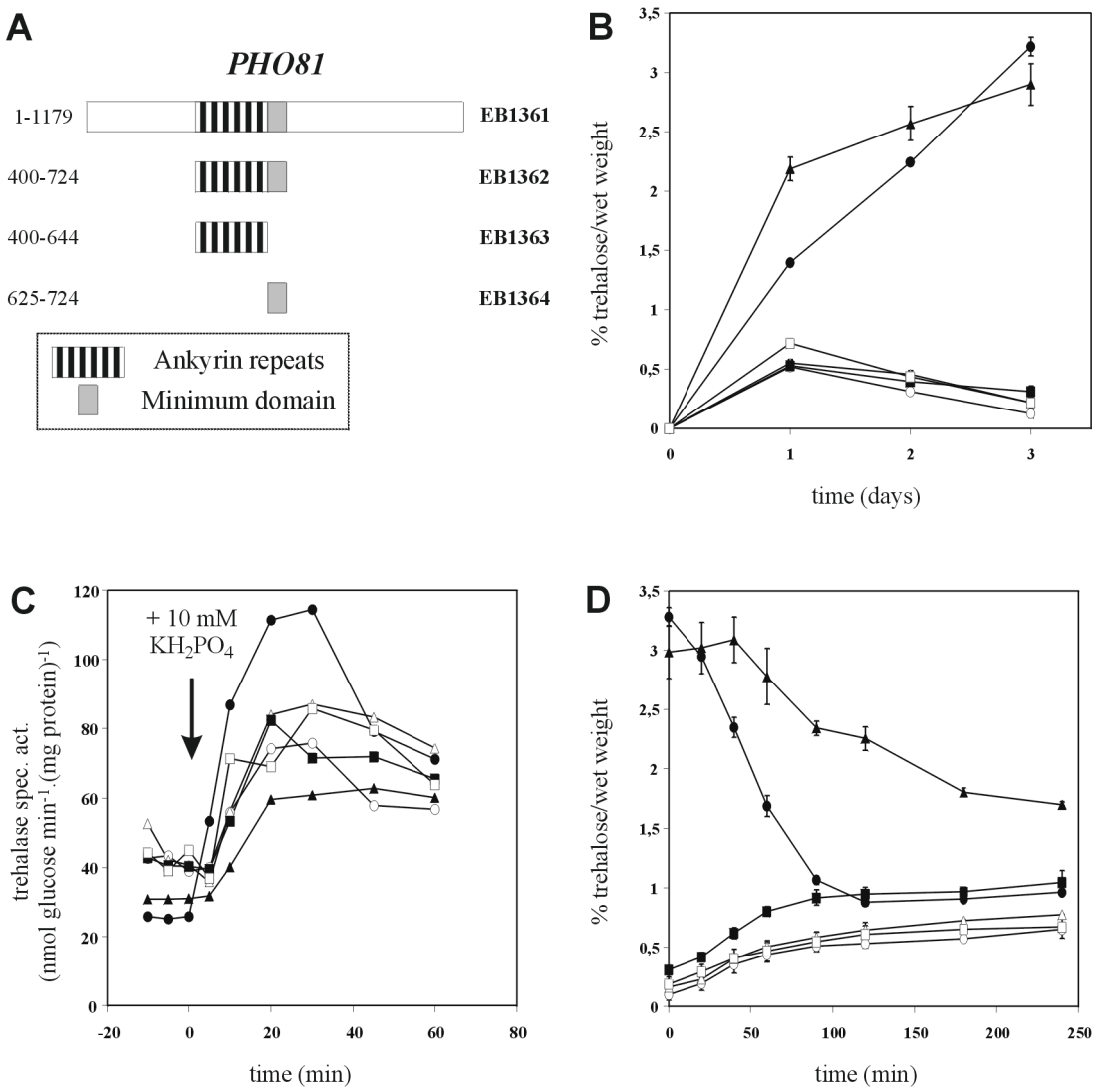


Figure II.5: Analysis of different *PHO81* constructs for their ability to complement the *pho81Δ* strain in trehalose metabolism.

(A) Schematic representation of the different *PHO81*-constructs used (adapted from (Huang et al., 2001)). Plasmid EB1361 contains full-length *PHO81*. All constructs are expressed from the *ADH1*-promoter. See materials and methods for the detailed description of the different Pho81-encoding plasmids. Exponentially growing cells of the wild type strain and *pho81Δ* mutant strains transformed with different Pho81-encoding constructs were harvested and transferred to phosphate-depleted medium. The accumulation of trehalose **(B)** was followed each day of the starvation period for the different strains. The same strains were analyzed after the addition of 10 mM KH_2PO_4 to the phosphate-starved cells in order to monitor the trehalase activation **(C)** and trehalose mobilization **(D)**. Strains: wild type (BY4741) + EB0730 (●), *pho81Δ* + EB0730 (○), *pho81Δ* + EB1361 (▲), *pho81Δ* + EB1362 (△), *pho81Δ* + EB1363 (■), *pho81Δ* + EB1364 (□). For trehalose levels, mean values from three independent experiments are shown, with SD (error bars).

The *pho81Δ* mutant transformed with an empty plasmid (construct EB0730) or with constructs encoding only the ankyrin repeats (construct EB1633), the ankyrin repeats and the minimum domain (construct EB1362) or only the minimum domain (construct EB1364) behaved similar and failed to accumulate trehalose when starved for phosphate (Fig. II.5.B), restore the enhanced basal trehalase activity during starvation (Fig. II.5.C) or to mobilize the carbohydrate upon re-supplementation of phosphate (Fig. II.5.D).

Expression of truncated *PHO81* alleles did also not restore the delayed and reduced induction of the PDS-driven genes upon phosphate starvation although the overexpression of at least the *PHO81* minimum domain (EB1362 and EB1364) in the *pho81Δ* strain enabled the strain to induce again the Pho4 target genes *PHO84* and *PHO89* under the same conditions (Fig. II.6.A). Only re-introduction of full-length *PHO81* (construct EB1361) partially restored all phosphate starvation defects in the *pho81Δ* mutant. Thus, while the minimum domain is able to regulate the activity of the Pho85-Pho80 kinase complex towards the Pho4 target, it is ineffective in regulating the activity of the same kinase complex towards trehalose metabolism and expression control of the stress responsive genes in response to phosphate starvation. In addition, overexpression of full-length *PHO81* resembles the phenotypes of the *pho85Δ* and *pho80Δ* mutants after re-supplementation of phosphate. Indeed, the *pho81Δ* mutant overexpressing construct EB1361 displays slower mobilization of trehalose and repression of stress responsive genes when compared to the wild-type strain (Fig. II.5.D and Fig. II.6.B), indicating that efficient downregulation of Pho81 function, in part through downregulation of *PHO81* transcription, is necessary to achieve an optimal response to phosphate availability.

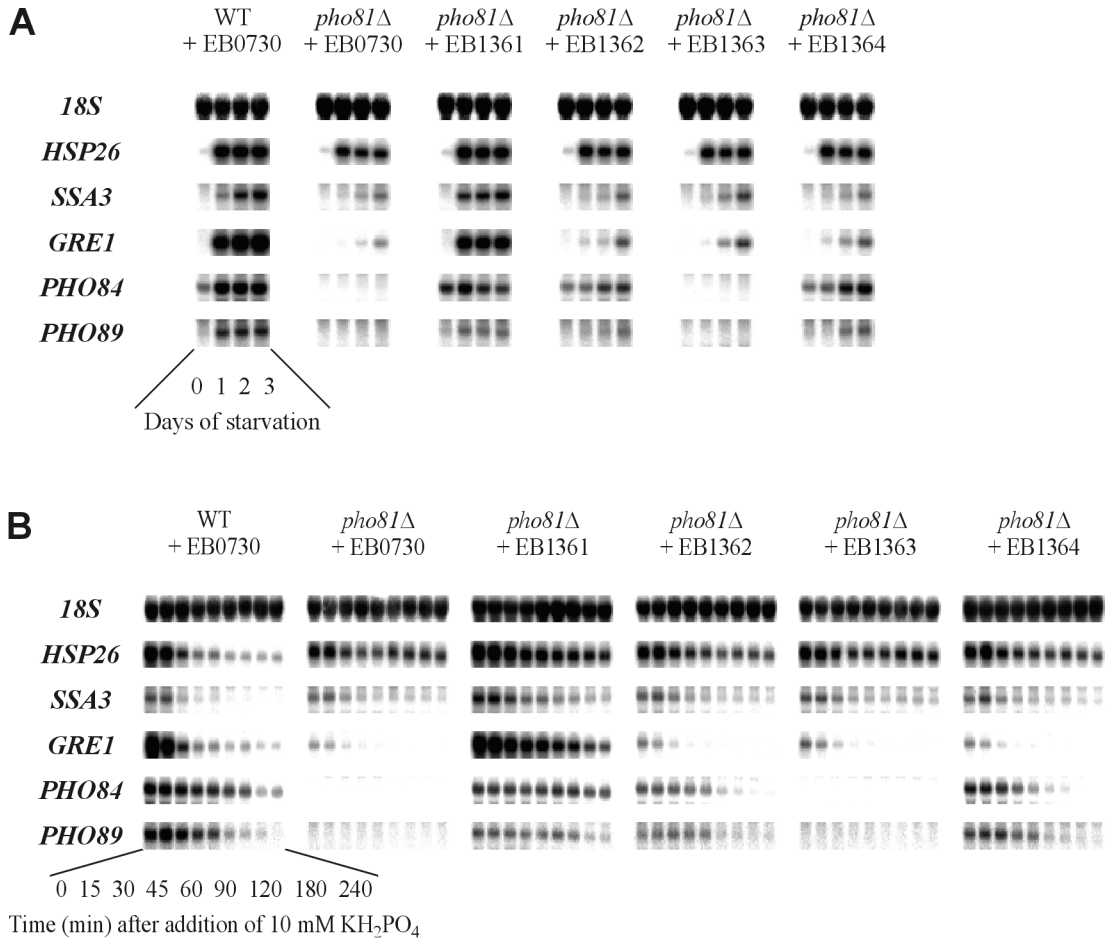


Fig. II.6: Analysis of different *PHO81* constructs for their ability to complement the *pho81Δ* strain in the expression of stress responsive genes.

Samples of the wild type strain and the different *pho81Δ* mutant strains were taken during phosphate starvation **(A)** and after addition of 10 mM KH_2PO_4 to the phosphate-starved cells **(B)** for Northern blot and gene expression analysis of the stress responsive genes *HSP26*, *SSA3* and *GRE1*, and the phosphate responsive genes *PHO84* and *PHO89*. *18S* levels are shown as a standard.

3. The defect of a strain lacking a functional Pho4 can be suppressed by overexpression of full-length *PHO81*

During phosphate starvation Pho81 inhibits the kinase activity of the Pho85-Pho80 complex, leading to an activation of PHO gene transcription via the Pho4 transcription factor (Komeili and O'Shea, 1999; Lenburg and O'Shea, 1996). One of the target genes of the Pho4 transcription factor is *PHO81* itself, and it was shown that this positive feedback loop is required for proper signalling through the PHO pathway (Creasy et al., 1993; Ogawa et al., 1995; Ogawa et al., 1993). Consistently,

cells lacking Pho4 failed to accumulate trehalose during phosphate starvation and, similar as cells deficient for Pho81, this defect was only restored by overexpression of full-length *PHO81* and not by overexpression of the minimum domain of *PHO81* (Fig. II.7.A). Also the abnormal mobilization profile of trehalose after re-supplementation of phosphate to the starved *pho4Δ* cells could only be corrected by overexpressing full-length *PHO81* (Fig. II.7.B). Note that the trehalose levels initially tended to increase in the *pho4Δ* mutant upon re-supplementation of phosphate even though this strain displayed an almost wild-type curve for the activation of trehalase (Fig. II.7.C). Thus, both the *pho4Δ* and the *pho81Δ* mutants share the uncoupling of trehalose degradation and trehalase activation. Furthermore, the overexpression of the full-length *PHO81* not only reduced the phosphate-induced trehalase activation in the *pho4Δ* mutant but also partially restored the increased basal trehalase activity before addition of phosphate to the phosphate-starved cells. With respect to the induction of the PDS-driven *SSA3* and *GRE1* genes during phosphate starvation, the *pho4Δ* mutant was clearly delayed when compared to the wild-type strain and again this delay was only restored by overexpression of full-length *PHO81* (Fig. II.7.D). Re-addition of phosphate to *pho4Δ* cells expressing full-length *PHO81* again reveals a slower response compared to the wild-type in both trehalose mobilisation (Fig. II.7.C) and repression of stress responsive genes (Fig. II.7.E). As the only difference we see between *pho81Δ* and *pho4Δ* strains expressing the *PHO81* constructs is limited to the expression of PHO genes, we can conclude that Pho4 is not affecting the targets studied directly but indirectly via the positive feedback loop for expression of *PHO81*. In line with this hypothesis, expression of a constitutively active form of Pho4, containing 5 serine-to-alanine substitutions at its sites of phosphorylation by Pho85-Pho80 (Kaffman et al., 1998a; Kaffman et al., 1998b), could not alleviate the defective trehalose accumulation or retarded induction of stress responsive genes in the *pho81Δ* mutant during phosphate starvation, despite the fact that it rescued PHO gene transcription (results not shown).

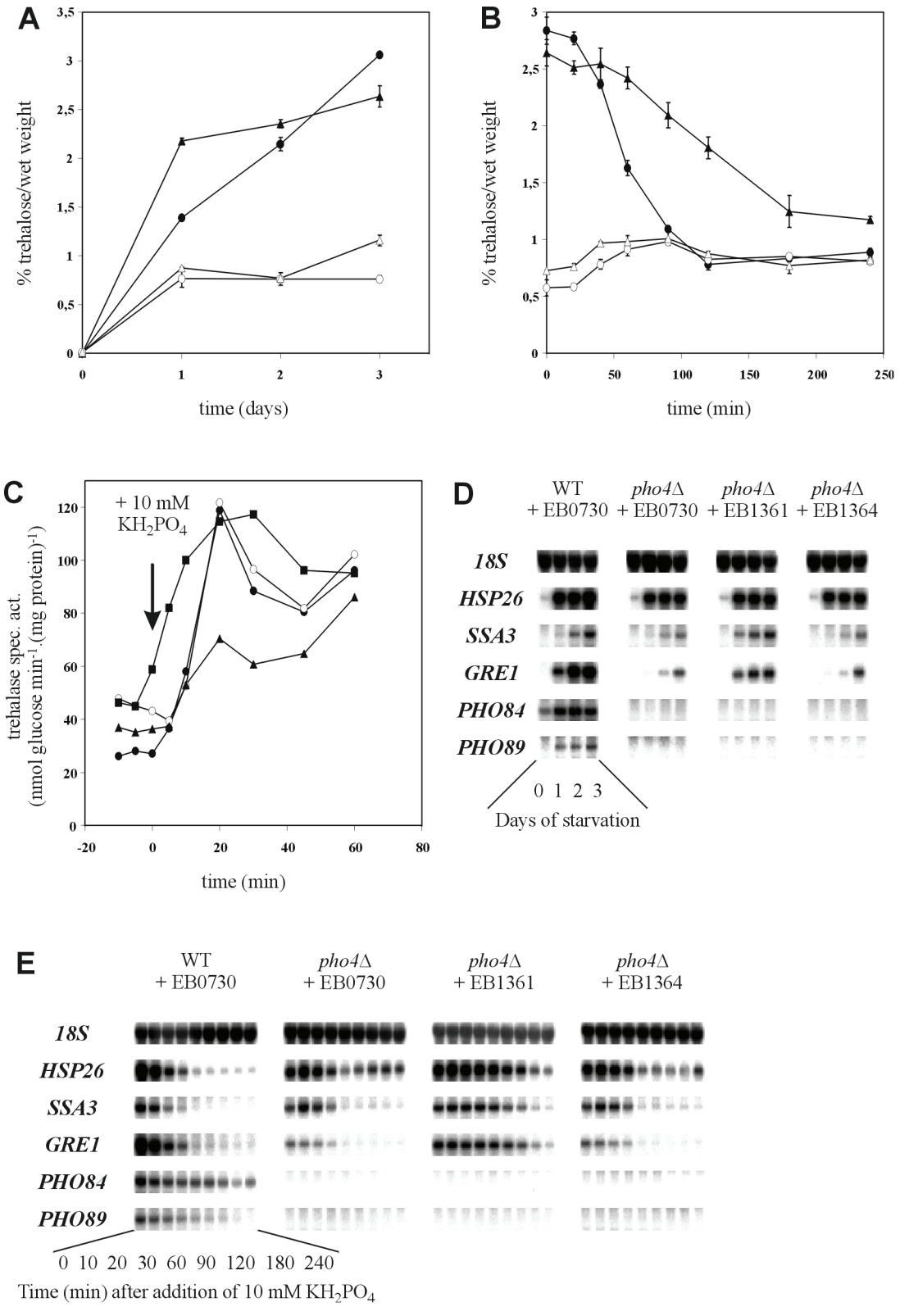


Figure II.7: Expression of full-length *PHO81*, but not the minimum domain, can suppress the defects of a *pho4Δ* strain.

Exponentially growing cells of the wild type strain and *pho4Δ* mutants transformed with various Pho81 encoding plasmids were harvested and transferred to phosphate-depleted medium. The trehalose

content **(A)** was determined for the different strains each day of the starvation period. The strains were also followed after the addition of 10 mM KH_2PO_4 to the phosphate-starved cells to monitor the trehalose mobilization **(B)** and activation of trehalase **(C)**. Strains: wild type (BY4741) + EB0730 (●), *pho4Δ* + EB0730 (○), *pho4Δ* + EB1361 (▲), *pho4Δ* + EB1364 (△). For trehalose levels, mean values from three independent experiments are shown, with SD (error bars). Samples were also taken each day of the phosphate starvation period **(D)** and after the addition of 10 mM KH_2PO_4 to the phosphate-starved cells **(E)** to follow the expression of the stress responsive genes *HSP26*, *SSA3* and *GRE1*, and the phosphate responsive genes *PHO84* and *PHO89*. *18S* levels are shown as a standard. See materials and methods for description of the different Pho81-encoding plasmids.

4. The PHO-pathway functions in parallel to the Sch9-controlled signalling to regulate Rim15 and the Rim15-effectors

The observation that aberrant signalling of the PHO-pathway affected mainly those stress-responsive genes that contain solely PDS-elements and no additional STRE-consensus sequences, incited us to investigate possible cross-talk with other nutrient-controlled pathways. As mentioned above, PDS elements are recognized by the transcription factor Gis1, which is of utmost importance to allow yeast cells to traverse the diauxic shift and adapt metabolism and growth from fermentation to respiration (Pedruzzi et al, 2000). More recent research confirmed that the transcription activity of Gis1 is in turn regulated by the PAS-like protein kinase Rim15 and the protein kinase Sch9 (Cameroni et al., 2004; Pedruzzi et al., 2000; Roosen et al., 2005). In order to illustrate the interplay of these kinases with the PHO-pathway and pinpoint a likely candidate to transmit signals derived from the Pho85/Pho80 complex to Gis1, we introduced the additional deletion of *SCH9* or *RIM15* in the *pho85Δ* mutant. As illustrated in Fig. II.8.A, tetrad analysis of a heterozygote diploid indicated that although spores with a combined deletion of *SCH9* and *PHO85* could germinate, the strain displays a dramatic synthetic growth defect and therefore could not be analyzed further. Nevertheless, this synthetic phenotype indicates that Sch9 and Pho85 may function in parallel but converging pathways. For *RIM15*, we found that its additional deletion did not influence the growth properties of *pho85Δ* cells when these are spotted on glucose-supplemented media. This could be expected, since Rim15 is immediately downstream and inactivated via phosphorylation by PKA during fermentative growth (Reinders et al., 1998). However, when spotted on glycerol-containing medium the additional deletion of *RIM15* gave

partial suppression of the respiratory growth defect typical for the *pho85Δ* mutant (Fig. II.8.B).

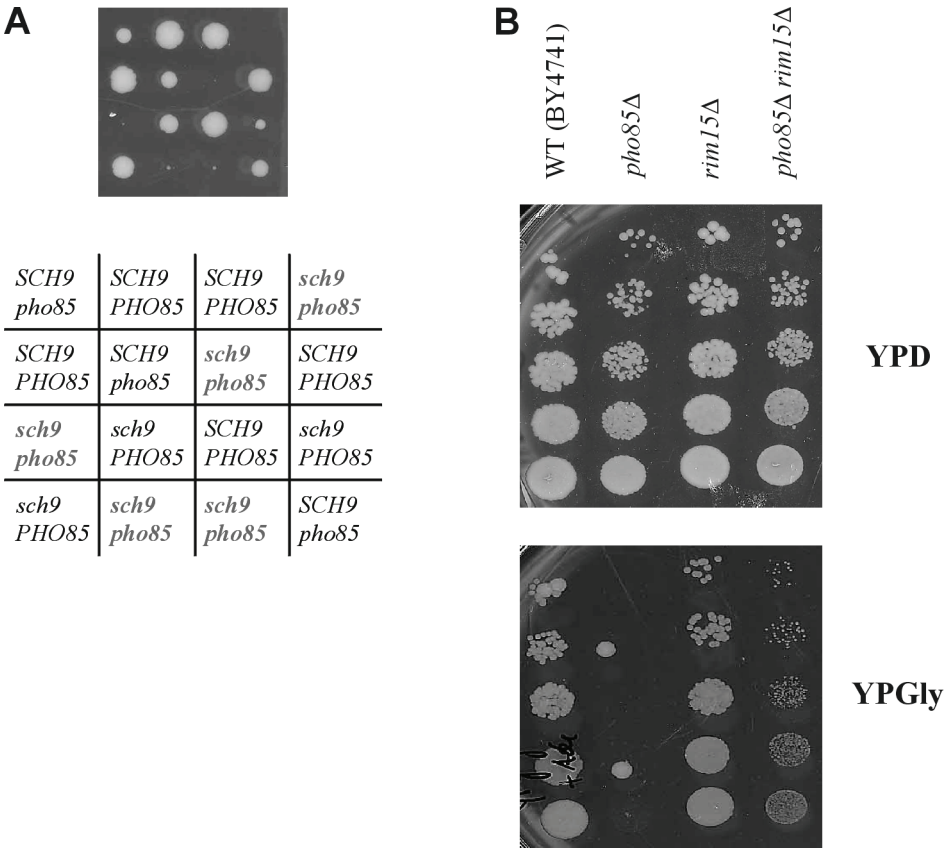


Figure II.8: Pho85 functions in parallel to Sch9 and regulates the protein kinase Rim15.
Diploid cells heterozygous for *PHO85* and *SCH9* were sporulated and subjected to tetrad analysis **(A)**. Haploid cells with a *pho85Δ sch9Δ* genotype display a severe growth defect, failing to form healthy colonies. Growth analysis of strains deleted in *PHO85* and/or *RIM15* **(B)**. Cells were spotted on solid medium for 3 to 5 days before scanning.

In line with this, the additional deletion of *RIM15* in the *pho85Δ* mutant as well as in the *pho80Δ* mutant also overruled phosphate-signalling towards the read-outs studied. Similar to the single *rim15Δ* mutant, the double mutants *pho85Δ rim15Δ* and *pho80Δ rim15Δ* failed to accumulate trehalose (Fig. II.9.A). These strains were also defective for the induction of the stress responsive genes upon phosphate starvation (Fig. II.9.B) while they gave a faster repression of these genes upon re-supplementation of phosphate to the starved cells (Fig. II.9.C). Hence, consistent with a model that Rim15 is negatively regulated by the Pho85-Pho80 kinase, the *rim15Δ*

mutants persistently displayed phenotypes that are better comparable to those of the *pho81Δ* mutant than to those of the *pho85Δ* or *pho80Δ* mutant.

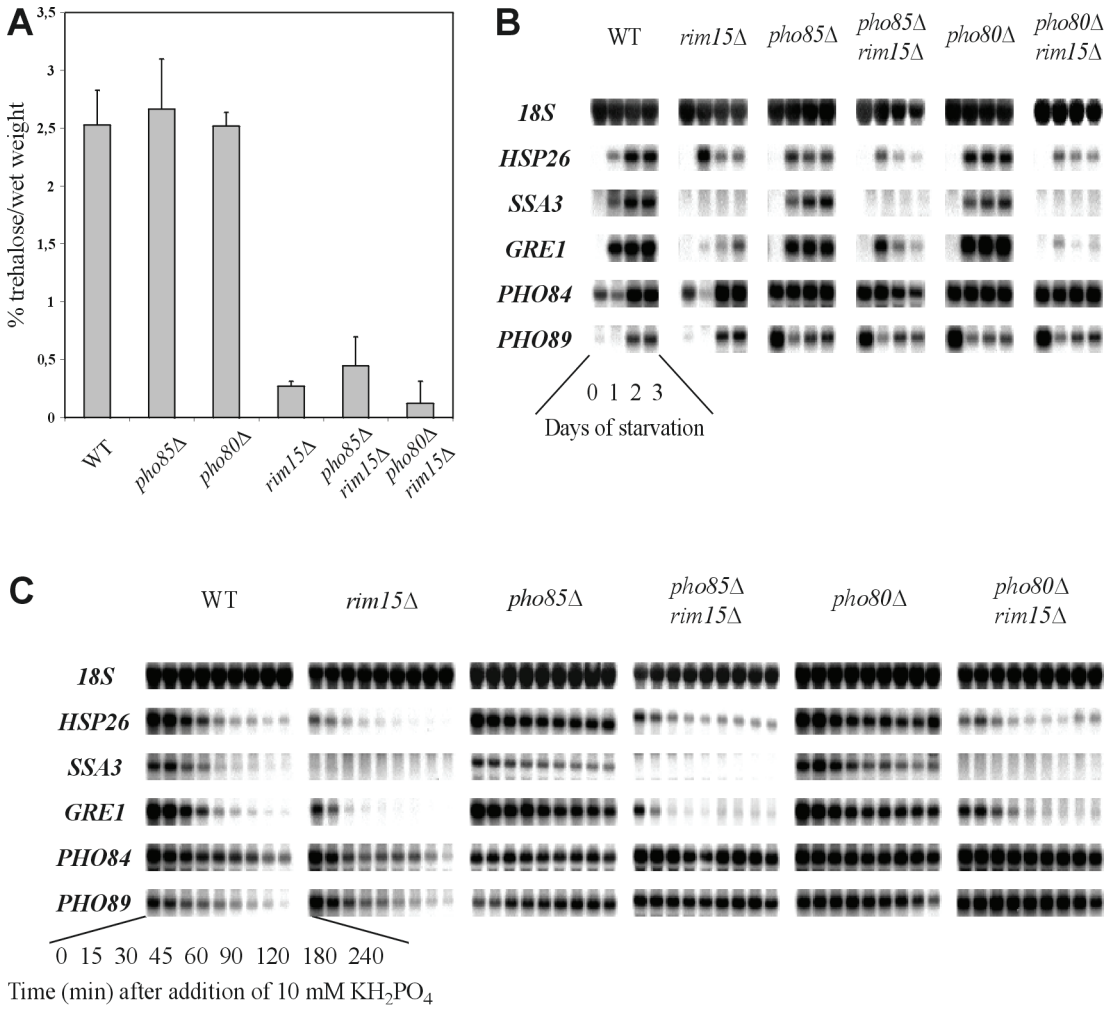


Figure II.9: The protein kinase Rim15 is essential for phosphate-dependent signalling.

The effect of a *rim15Δ* on phosphate-signalling with or without additional deletion of a component of the Pho85-Pho80 kinase was determined. Cells were starved for phosphate during 3 days, after which the trehalose content **(A)** was determined. Mean values for three independent experiments are shown, with SD (error bars). Samples were also taken during the starvation period samples **(B)** and after re-supplementation of 10 mM KH_2PO_4 to the phosphate-starved cells **(C)** in order to monitor the expression of the stress responsive genes *HSP26*, *SSA3* and *GRE1*, and the phosphate responsive genes *PHO84* and *PHO89*. *18S* levels are shown as a standard.

Concluding remarks

When microorganisms, such as *S. cerevisiae*, encounter nutrient starvation, they respond by activating several stress responses, such as the accumulation of reserve carbohydrates and the induction of a multitude of stress responsive genes (Roosen et al., 2004; Winderickx et al., 2003). These properties are known to be tightly regulated by multiple signalling pathways with a major role being played by PKA. While glucose-induced signalling is accompanied by a cAMP signal, the effects of nutrients on typical PKA targets was shown to be independent of cAMP and hence the pathway involved was called the 'Fermentable Growth Medium induced' (FGM)-pathway (Thevelein, 1994). In this chapter, we investigated the role of the well-known phosphate-responsive PHO pathway in the regulation of typical PKA targets such as trehalose accumulation and expression of stress responsive genes. We demonstrate that PHO signalling is crucial for proper regulation of trehalose metabolism and expression of certain stress responsive genes in a phosphate-dependent manner (Fig. II.10). We additionally identified the Rim15 protein kinase as a physiological Pho85-Pho80 target, thereby connecting the PHO pathway with other nutrient-induced signalling pathways converging on Rim15 (Pedruzzi et al., 2003).

The yeast PHO pathway controls typical PKA targets in a phosphate-dependent manner

Similar to the control of Pho4-mediated transcription of PHO genes, Pho81-dependent regulation of Pho85 kinase activity is required for proper trehalose metabolism in response to phosphate availability. Unregulated high activity of Pho85 due to absence of Pho81 impairs trehalose accumulation during phosphate starvation whereas the absence of Pho85 activity results in the loss of a quick trehalose mobilization after the re-addition of phosphate to the starved cells. However, in contrast to the known PHO pathway, regulation of trehalose levels apparently requires other Pho85 associating cyclins in addition to Pho80 (Fig. II.10). This could be concluded from the observations that the *pho81Δ pho80Δ* mutant still displays lower levels of trehalose than a wild type strain in contrast to the *pho81Δ pho85Δ* mutant and that the increased basal activity of trehalase in the *pho81Δ* mutant can only be

restored by the additional deletion of *PHO85* but not by the additional deletion of *PHO80*. Concerning the rapid phosphate-induced trehalase activation, our results indicate that proper functioning of the PHO pathway is important for maximal signalling. However, other signalling systems are involved as trehalase activation is not abolished in all the *pho* mutants tested. For instance, it has been shown that PKA plays an important role in the phosphate-induced activation of trehalase (Giots et al., 2003). Several Pho85 cyclins are already implicated in the regulation of the reserve carbohydrate glycogen and one may argue that they may also be required for correct regulation of trehalose levels. These cyclins include Pcl6, Pcl7, Pcl8 and Pcl10 (Huang et al., 1996; Huang et al., 1998; Lee et al., 2000; Wilson et al., 1999; Wilson et al., 2005). Of them, Pcl7 might be a very interesting candidate, since this cyclin has been shown to directly interact with Pho81 *in vivo* as to regulate the kinase activity of the Pho85-Pcl7 complex in response to phosphate levels (Lee et al., 2000). However, other Pcl proteins cannot be excluded. The role of the Pho4 transcription factor in the regulation of trehalose levels is limited to its positive action on Pho81 function via transcriptional induction of the *PHO81* gene. This is an interesting result as it shows that the Pho4-mediated feedback on *PHO81* expression (Ogawa et al., 1995) is not only required for continued activation of Pho4 but also important to allow Pho81 to regulate other Pho85-mediated processes in order to ensure a proper response to phosphate starvation.

A second common response of yeast cells to nutrient starvation conditions is the transcriptional induction of a large set of stress responsive genes and our results confirm that this also applies to phosphate starvation (Crauwels et al., 1997; Donaton et al., 2003; Giots et al., 2003; Thevelein and de Winde, 1999) . Interestingly, only for the expression regulation of the PDS-driven genes *SSA3* and *GRE1* our data clearly demonstrated the importance of the CKI function of Pho81. For the regulation of the STRE-driven gene *HSP26* the involvement of the PHO-pathway was much less obvious as its induction during phosphate starvation seemed to be largely independent of Pho81 while its repression upon phosphate re-supplementation to the starved cells was delayed in strains lacking Pho85 or Pho80. Note that also the expression of the genes encoding trehalase and the different subunits of the trehalose synthase complex is controlled in part via STRE-elements, though they do not seem to act as other typical STRE-controlled genes (Winderickx et al., 1996; Zahringer et al., 1998; Zahringer et al., 2000). We also observed this phenomenon as repression of *NTH1*,

TPS1 and/or *TPS2* after the readdition of phosphate to the starved cells was delayed in strains lacking *PHO85* or *PHO80*, as well as *PHO81*. Hence, it is possible that the uncoupling between trehalase activation and trehalose mobilization that we and others (Giots et al., 2003) observed in mutant strains affected in phosphate signalling may be due to an overshoot of trehalose synthesis as compared to trehalose degradation during the first hours after phosphate re-supplementation to starved cells.

Interestingly, the effects on expression of the PDS-genes appeared to be solely dependent on the Pho85-Pho80 complex. However, as these PDS-controlled genes are not constitutively expressed in either a *pho85Δ* or *pho80Δ* strain, in contrast to the PHO genes, this indicates that Pho4 does not directly activate transcription of these stress responsive genes and another effector links the Pho85-Pho80 kinase activity to phosphate-dependent transcription regulation of the PDS-driven genes. Indeed, as with the regulation of trehalose metabolism, the effects of a *PHO4* deletion on the expression of stress responsive genes is mediated via the feedback loop controlling PHO81 expression. The existence of an additional Pho85-Pho80 effector has already been suggested by Flick and Thorner who noticed that the temperature-sensitive growth defect of a *plc1Δ* strain could be suppressed either by overexpression of *PHO81*, by disruption of the *PHO80* gene or by growing the cells in a low-phosphate environment (Flick and Thorner, 1998). Since these suppressive effects were independent of the Pho4 transcription factor, it was concluded that the Pho85-Pho80 kinase inhibits another target, which is also downstream of Plc1 and which is required for the proper response to nutrient levels and involved in growth at elevated temperatures (Flick and Thorner, 1998). It would be interesting to find out if this target is the Rim15 protein kinase or the Rim15 effector Gis1 (see below).

The Pho85-Pho80 kinase regulates Rim15 activity

The observation that aberrant signalling of the PHO pathway clearly affected the expression of the PDS-driven genes, pointed us to the specific involvement of Gis1, the transcription factor that binds the PDS-elements. That Gis1 would be specifically required to induce gene expression in response to phosphate starvation may not be so surprising. Indeed, this factor was previously shown to be essential to induce expression when glucose becomes limiting and cells enter the diauxic shift

upon which they acquire stationary phase characteristics. Therefore, it is feasible to assume that Gis1 is activated whenever cells encounter shortage of an essential nutrient. The activity of Gis1 is positively regulated by the protein kinases Rim15 and Sch9 (Pedruzzi et al., 2000; Reinders et al., 1998; Roosen et al., 2005) Interestingly, apart from being an activator of Gis1, Sch9 also acts as negative regulator of Rim15, indicating that Sch9 may function as a molecular buffer system that regulates the amplitude of Rim15-dependent responses. In addition, Rim 15 is also under negative control of PKA and TORC1 and thus the kinase integrates signals from at least three nutrient sensing cascades (Pedruzzi et al., 2003).

In this study, we show that combined deletion of *SCH9* and *PHO85* results in a severe growth defect, indicating that both protein kinases operate in parallel though converging pathways. Furthermore, we observed that growth of the *pho85Δ* mutant is hypersensitive to the addition of the immunosuppressant rapamycin (results not shown), as previously demonstrated (Huang et al., 2002), indicating that Pho85 and the TORC1 may also function in parallel, but converging pathways. These observations are consistent with Rim15 defining the point of convergence. In line with this, the deletion of *RIM15* in a *pho85Δ* background did not lead to an additional growth defect, but in contrast partially alleviates the growth defect of a *pho85Δ* strain on glycerol medium. This places Rim15 indeed downstream and under negative control of Pho85 and it suggests that one of the reasons why the *pho85Δ* cells fail to grow on non-fermentable carbon sources is because of an uncontrolled Rim15 activity. Consistently, a recent high-throughput protein-interaction analysis suggested that Rim15 and Pho85 may interact with each other *in vivo* (Ho et al., 2002). During the course of this work, this interaction has been confirmed and it was shown that the Pho85-Pho80 kinase directly phosphorylates Rim15, thereby promoting its cytoplasmic retention (Wanke et al., 2005). Together with our data, this indicates that the nutritional integrator function of Rim15 is even broader than previously described (Pedruzzi et al., 2003; Roosen et al., 2005) as we found that Rim15 is indeed required for trehalose accumulation as well as correct transcriptional induction of stress responsive genes during phosphate starvation. As with the *pho81Δ* mutant, the PDS-controlled genes *SSA3* and *GRE1* display a much stronger dependency on a functional Rim15 kinase than the STRE-controlled gene *HSP26*, a finding that supports our

model in which Rim15 is under negative control of the Pho85-Pho80 kinase (Fig. II.10).

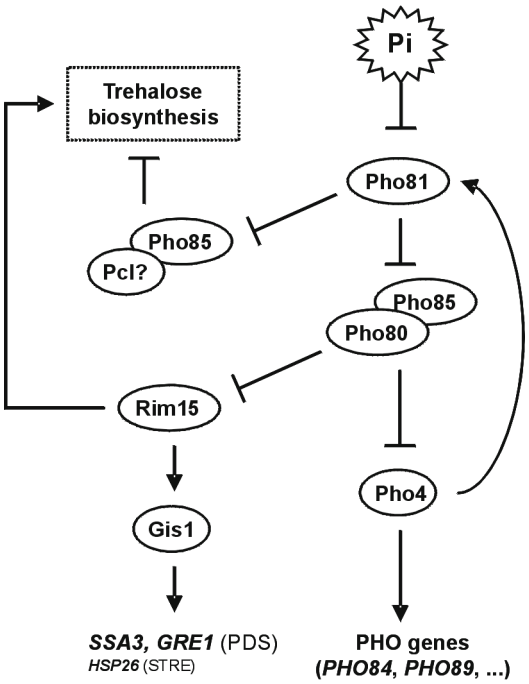


Figure II.10: Model for the involvement of the PHO regulatory pathway in phosphate-induced signalling and genetic interaction with other signalling components.
When yeast cells are starved for phosphate, the PHO pathway will activate the Pho4 transcription factor through Pho81 mediated inhibition of the Pho85-Pho80 kinase complex. This will lead to induced expression of classical PHO genes. Importantly, the *PHO81* gene is also induced, creating a positive feedback loop within the pathway. The Pho85-Pho80 kinase also appears to regulate the protein kinase Rim15, which functions downstream of PKA and Sch9 to orchestrate induction of stress-responsive genes, particularly those genes controlled by Gis1, the transcription factor known to bind the PDS elements. In addition, Pho81 controls the activity of (an) additional Pho85 complex(es), involved in the biosynthesis of the reserve carbohydrate trehalose in response to phosphate levels. See text for further details.

It should be emphasized that the induction of the PDS-genes in a *pho81Δ* strain is not totally abolished, but only severely impaired in response to phosphate depletion. Therefore, these genes are subject to additional control allowing to bypass the defect in Pho81-dependent inhibition of the Pho85-Pho80 kinase. Since this control becomes apparent only after prolonged starvation of the cells, it may be mediated by the lack of metabolic signals when cells are severely starved for phosphate, for instance depletion of ATP. Thus, yeast cells may have a biphasic

response to phosphate starvation. In a first phase, the cells rapidly adapt to the lack of phosphate through activation of the PDS-driven genes via the PHO pathway. In a second, slower phase, the lack of metabolic signals generated after prolonged phosphate starvation triggers a stress signal leading induction of the PDS-genes independent of the PHO pathway. Possibly, the second phase may depend on the stress-activated transcription factors Msn2 and/or Msn4, which are known to mediate part of the Rim15-dependent transcriptional responses redundantly to Gis1 (Cameroni et al., 2004).

Molecular analysis of Pho81 function

Regulation of the activity of the Pho4 transcription factor in response to phosphate levels is known to be accomplished through Pho81-mediated regulation of Pho85-Pho80 kinase activity, for which a region of Pho81 containing only 80 amino acids was shown to be both necessary and sufficient (Huang et al., 2001). Our results indicate that this minimum domain is insufficient in regulating trehalose metabolism, which requires additional cyclins besides Pho80. Under phosphate-limiting conditions Pho81 inhibits the activity of the nuclear Pho85-Pho80 complex (Kaffman et al., 1998a). Nuclear localization of Pho81 in this condition depends on its interaction with the Pho80 cyclin (Huang et al., 2001). As the *pho81Δ pho80Δ* mutant has a defect in trehalose accumulation when yeast cells are starved for phosphate, in contrast to the *pho80Δ* mutant, proper regulation of trehalose metabolism also requires Pho81 function in cellular locations outside the nucleus. In this respect, Pho81 localization has also been observed in the cytosol and at the plasmamembrane in phosphate-starved yeast cells (Huang et al., 2001), where it may regulate the activities of the trehalase enzyme and/or the trehalose synthase complex (Francois and Parrou, 2001; Thevelein and Hohmann, 1995).

In addition, the Pho81 minimum domain is not sufficient for proper regulation of the Rim15-dependent PDS-driven genes in response to phosphate availability and thus, other domains of Pho81 must be involved. Intriguingly, these domains must then be able to discriminate between the different effectors, Pho4 and Rim15, of the same Pho85-Pho80 kinase. The molecular mechanism behind this phenomenon is still elusive. Perhaps Pho81 has additional roles in regulating Rim15 function besides the

inhibition of the Pho85-Pho80 kinase, though this is speculation at this point. Furthermore, the Crz1 transcription factor, involved in calcium signalling (Hopper, 1999), has recently been identified as yet another physiological target of the Pho85-Pho80 kinase (Sopko et al., 2006). This apparent integration of calcium and phosphate signalling makes sense, considering the fact that a major function of vacuolar polyP, the metabolism of which is controlled by the PHO pathway (Ogawa et al., 2000), comprises the detoxification of the cytosol by trapping cations such as Ca^{2+} in vacuoles (Dunn et al., 1994). Remarkably, all three Pho85-Pho80 targets described here; Pho4, Rim15 and Crz1, use the Msn5 as their nuclear export receptor (Boustany and Cyert, 2002; Kaffman et al., 1998a; Wanke et al., 2005). Considering the differential requirement of Pho81 domains in the regulation of Pho4 and Rim15 by the Pho85-Pho80 kinase, it would be interesting to investigate if and how Pho81 is involved in the regulation of Crz1 activity. This will further elucidate the role of Pho81 protein in the integration of nutrient signals and in general may provide new, interesting insights in the general mechanisms of CKI function.

Chapter III

Regulation of phosphate signalling through the Pho84 high-affinity and the Pho87 low-affinity phosphate transporters

Abstract

When starved for phosphate, the main phosphate transport activity in yeast is performed by the Pho84 high-affinity permease, consistent with the induction of *PHO84* expression by the PHO pathway under these conditions. In addition, the Pho84 transporter is also required for rapid phosphate signalling after re-addition of Pi to starved cells. However, we clearly observed phosphate-induced signalling in strains with a deletion of either *PHO4* or *PHO81*, both of which lack expression of *PHO84*. We found that phosphate signalling in these strains is mediated specifically by the Pho87 low-affinity phosphate transporter. Interestingly, the negative regulation of Pho87 function by the PHO pathway occurs at a posttranscriptional level and Pho87 acts as a low-affinity phosphate sensor. We also show that Pho4 activity is modulated by glucose signalling which may offer a physiological relevance of phosphate signalling via Pho87. Regulation of Pho4 activity in response to glucose depends on a functional Rim15 protein kinase, a direct target of the Pho85-Pho80 kinase, indicating reciprocal regulation between glucose and phosphate signalling via Rim15. Finally, we demonstrate that optimal growth recovery after phosphate starvation depends on the interplay of rapid phosphate consumption and subsequent inactivation of the Pho4 transcription factor, and that an active Pho4 appears to have a general growth-inhibiting effect.

Introduction

As inorganic phosphate (Pi) is an essential nutrient for all organisms, maintaining proper phosphate homeostasis is critical to ensure survival of cells and organisms. A crucial step in maintaining phosphate homeostasis encompasses the transport of Pi across the plasma membrane into the cell. Since cellular membranes are impermeable to Pi, specialised integral membrane proteins are required to transport this essential compound across these barriers. In *Saccharomyces cerevisiae*, two symport systems of phosphate transport exist (Borst-Pauwels, 1981; Persson et al., 1999; Tamai et al., 1985). First of all, a low-affinity transport system with a K_m for external phosphate of approximately 1 mM satisfies the cellular need for Pi at normal or high Pi concentrations. This low-affinity Pi transport system is believed to be composed of the partially redundant Pho87, Pho90 and Pho91 H^+/Pi symporters (Wykoff and O'Shea, 2001). In addition, yeast possesses a high-affinity Pi transport system with a K_m of about 10 μM, which is encoded by the *PHO84* and *PHO89* genes, representing a high-affinity H^+/Pi and Na^+/Pi symporter, respectively (Bun-Ya et al., 1991; Martinez and Persson, 1998). Transcription of *PHO84* and *PHO89* is regulated by the Pho4 transcription factor and is induced when Pi becomes limiting, while expression of the *PHO87*, *PHO90* and *PHO91* genes was shown to be independent of phosphate concentrations (Auesukaree et al., 2003).

The ability of inorganic phosphate to act as a nutrient signal affecting PKA targets is closely correlated to its uptake into the cell, as inactivation of Pi transport by destruction of the proton gradient across the plasma membrane with the protonophore 2,4-dinitrophenol completely prevented phosphate-induced trehalase activation (Giots et al., 2003). Although the predominant phosphate transport activity present in phosphate-starved cells is represented by the Pho84 transporter (Bun-Ya et al., 1992; Bun-Ya et al., 1991), we previously observed clear activation of trehalase after addition of Pi to cells deleted for *PHO4* or *PHO81*, in which no *PHO84* transcription can be detected (Swinnen et al., 2005). The role of each of the five phosphate transporters (Pho84, Pho87, Pho89, Pho90 and Pho91) in signalling towards PKA targets has been investigated through overexpression of the corresponding genes in a *pho84Δ pho87Δ pho89Δ pho90Δ pho91Δ* strain, and only Pho84 and Pho87 seemed to be capable of rapid phosphate signalling (Giots et al., 2003). However, overexpression

may lead to unnatural high levels of gene products, thereby impairing their proper physiological regulation. Therefore, in order to shed more light on the connection between phosphate transport(ers) and signalling, we set out to thoroughly re-investigate the role of phosphate transporters in signalling, using strains in which all the genes of phosphate transporters still present are being expressed by their natural promoters.

Results and discussion

1. The high-affinity phosphate transporter Pho84 is essential for phosphate signalling

In a first step towards elucidating the role of phosphate transporters in Pi-induced signalling, we assayed typical signalling events in strains in which one of the genes encoding H^+/Pi symporters, i.e. *PHO84*, *PHO87*, *PHO90* or *PHO91*, was deleted separately. In addition, we also examined a *pho87Δ pho90Δ pho91Δ* strain, in which low-affinity Pi transport is completely abolished. In our approach, we chose not to examine the Pho89 transporter for several reasons. The maximum activity of Pho89 was shown to be at least a hundred fold lower than that of Pho84 under low-Pi conditions (Pattison-Granberg and Persson, 2000). Importantly, Pho89 functions at a pH optimum of 9.5 and was shown to be completely inactive at a pH of 4.5 (Martinez and Persson, 1998), which is the condition used in our experiments. Pho89 also appears to be incapable of signalling, even at optimal conditions for this transporter (Giots et al., 2003).

All of the strains grew equally well on rich YPD medium (not shown). When starved for Pi, the *pho84Δ* strain arrests growth somewhat sooner than the other strains (Fig. III.1.A), in agreement with the lower (poly)phosphate reserves present in this strain (Auesukaree et al., 2004). All the strains display a normal response to phosphate deprivation. Accumulation of the reserve carbohydrates trehalose and glycogen occurs normal in response to phosphate starvation in all the mutants (Fig. III.1.B). In addition, expression of the PHO genes *PHO84* and *PHO89* is induced (except for *PHO84* expression in the *pho84Δ* strain), as is the expression of the stress responsive genes *HSP12*, *HSP26*, *SSA3* and *GRE1* (Fig. III.1.C). Expression of *PHO89* is constitutive in the *pho84Δ* mutant, in agreement with previous observations stating that a *pho84Δ* strain is defective in Pi-uptake even under high-Pi conditions (Wykoff and O'Shea, 2001), resulting in a state of continued phosphate starvation leading to elevated Pho4 activity under high-Pi conditions.

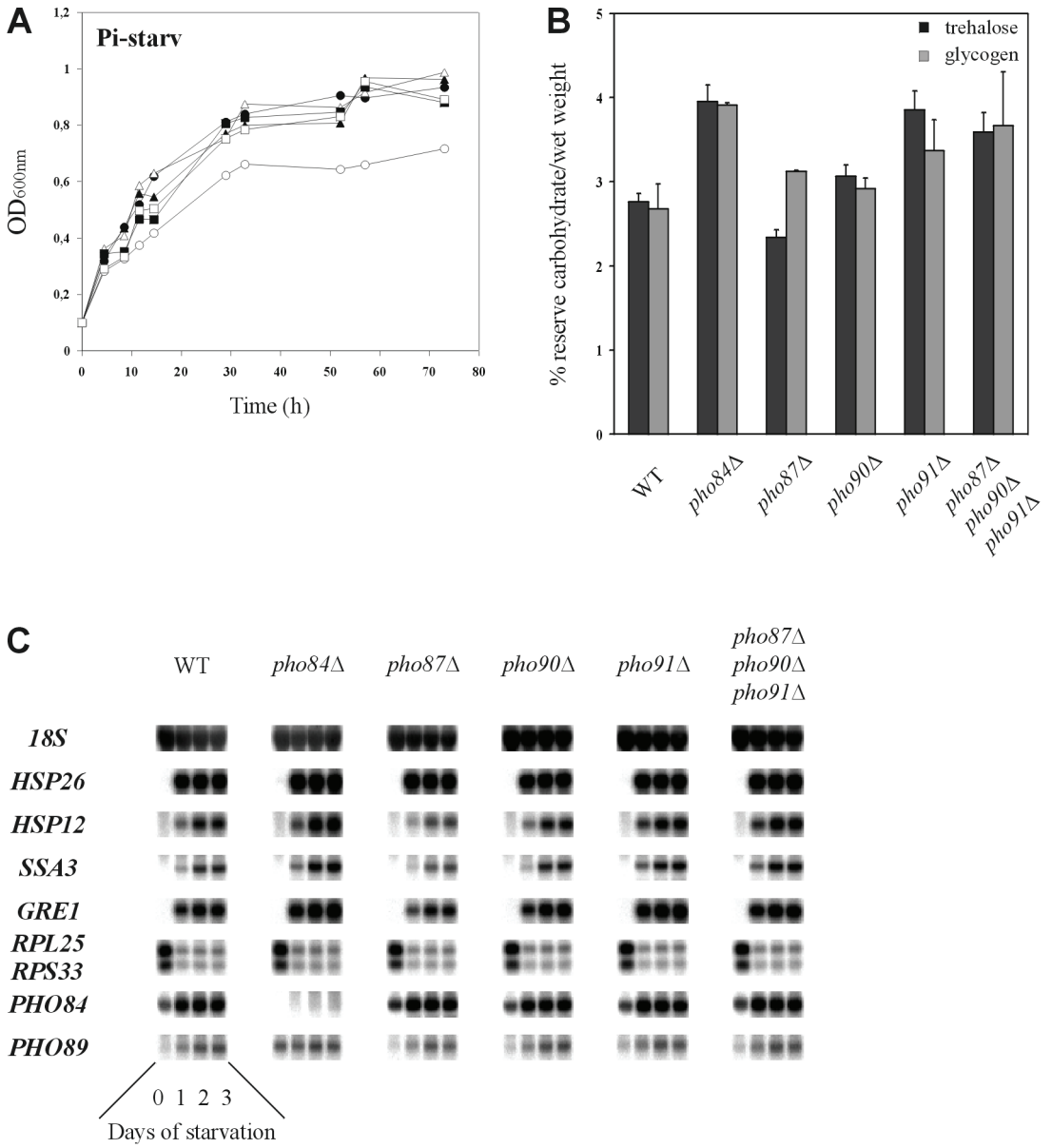


Figure III.1: Strains deleted in genes encoding phosphate transporters display a normal response to phosphate deprivation.

Indicated strains isogenic to the wild-type strain (BY4741) are grown to mid-exponential phase in YPD medium after which they are transferred to phosphate starvation medium. **(A)** Growth profiles during Pi-starvation were determined. Strains: wild-type (●), *pho84Δ* (○), *pho87Δ* (▲), *pho90Δ* (△), *pho91Δ* (■), *pho87Δ pho90Δ pho91Δ* (□). **(B)** Levels of trehalose and glycogen after Pi-starvation were determined for the different strains. Mean values from three independent experiments are shown, with SD (error bars) **(C)** Each day during the starvation period samples were taken for gene expression analysis by Northern blot. Expression was analysed of the stress responsive genes *HSP26*, *HSP12*, *SSA3* and *GRE1*, the ribosomal protein genes *RPL25* and *RPS33*, and the phosphate responsive genes, *PHO84* and *PHO89*. *18S* mRNA levels are shown as a standard.

Finally, all the strains display a repression of the ribosomal protein genes *RPL25* and *RPS33* upon phosphate starvation. Induction of stress responsive genes and trehalose accumulation is slightly lower in a *pho87Δ* strain, although this effect is minimal and is restored in the *pho87Δ pho90Δ pho91Δ* mutant.

Readdition of 10 mM KH_2PO_4 to Pi-starved wild type cells results in the rapid activation of the trehalase enzyme (Fig. III.2.A). Single gene deletion of any of the low-affinity Pi transporters does not affect this activation and even the *pho87Δ pho90Δ pho91Δ* triple mutant strain displays normal trehalase activation. Deletion of *PHO84*, on the other hand, results in a complete loss of phosphate-induced trehalase activation. The inability of the *pho84Δ* mutant to activate trehalase correlates with its inability for rapid phosphate consumption from the medium (Fig. III.2.B). The wild type strain and all the other mutants are able to rapidly take up phosphate from the medium, albeit with different affinities. Concomitant with the activation of trehalase, all the strains except the *pho84Δ* mutant rapidly degrade their trehalose reserves after the addition of 10 mM KH_2PO_4 to the starved cells (not shown). When phosphate becomes available again, wild type cells repress the expression of PHO genes as well as stress responsive genes (Fig. III.2.C). Finally, ribosomal protein genes are induced, allowing the yeast cell to restart their growth. In cells deleted specifically for *PHO84* this transcriptional response after phosphate readdition is absent (Fig. III.2.C). Interestingly, the low-affinity phosphate transport system is totally dispensable for rapid phosphate signalling as even the *pho87Δ pho90Δ pho91Δ* triple mutant displays wild-type like responses.

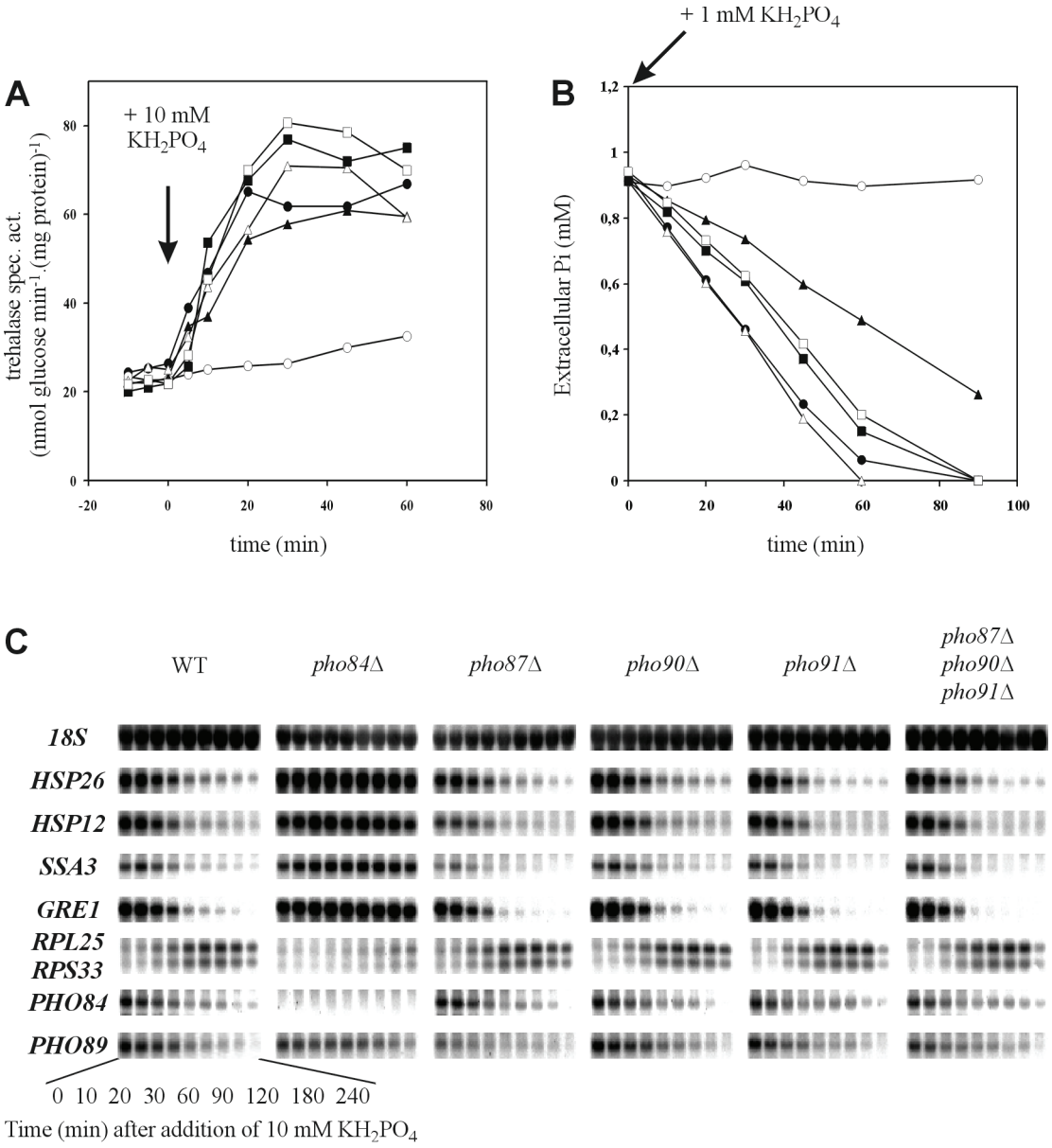


Figure III.2: The Pho84 phosphate transporter is essential for phosphate signalling.

Indicated strains isogenic to the wild-type (BY4741) were analysed for activation of trehalase **(A)** and Pi-consumption **(B)** after re-addition of KH_2PO_4 in the indicated concentrations to the Pi-starved cells. Strains: wild-type (●), *pho84Δ* (○), *pho87Δ* (▲), *pho90Δ* (△), *pho91Δ* (■), *pho87Δ pho90Δ pho91Δ* (□). In addition, the transcriptional response was monitored after re-addition of 10 mM KH_2PO_4 to the starved cells **(C)**. Expression was analysed of the stress responsive genes *HSP26*, *HSP12*, *SSA3* and *GRE1*, the ribosomal protein genes *RPL25* and *RPS33*, and the phosphate responsive genes, *PHO84* and *PHO89*. *18S* mRNA levels are shown as a standard.

2. The yeast PHO pathway inhibits the function of the Pho87 low-affinity phosphate transporter

2.1 Growth-requirement of the Pho90 low-affinity phosphate transporter in the absence of a high-affinity phosphate transport system

As described above, Pho84 is the major phosphate transporter responsible for signalling, as in its absence, rapid phosphate signalling is abolished. Intriguingly, we previously observed rapid phosphate signalling, such as the phosphate-induced activation of trehalase, in cells deleted for *PHO4* or *PHO81* (Swinnen et al., 2005). In both these strains, no expression of *PHO84* is detected. Either expression of *PHO84* below detection level is sufficient to sustain phosphate signalling or another phosphate transporter takes over the function of Pho84 in the absence of Pho4 activity. To investigate this, we deleted the *PHO84*, *PHO87*, *PHO90* or *PHO91* gene in a *pho4Δ* background and assayed the strains for rapid phosphate signalling. When constructing the mutant strains, we noticed that the *pho4Δ pho90Δ* mutant displays an extended lag phase for growth on rich YPD medium, whereas the other strains grew similar as the wild type (Fig. III.3.A). The same growth defect is observed in a *pho81Δ pho90Δ* and *pho84Δ pho89Δ pho90Δ* strain (Fig. III.3.B). Thus, the requirement for Pho90 in the absence of a functional Pho4 transcription factor appears to be due to the absence of the high-affinity phosphate transport system, encoded by *PHO84* and *PHO89*, and not due to additional regulation of Pho90 by Pho4/Pho81 under these conditions.

2.2 In the absence of the Pho4 transcription factor, the low-affinity phosphate transporter Pho87 specifically mediates rapid phosphate signalling

When transferred to Pi-starvation medium, all the mutants with a deleted *PHO4* gene arrest growth sooner than the wild type strain, regardless of the gene additionally deleted (Fig. III.4.A). This is expected as Pho4 activity is required for proper storage of (poly)phosphate in the cell (Ogawa et al., 2000) and deletion of *PHO4* will thus result in a more rapid growth arrest in the absence of Pi.

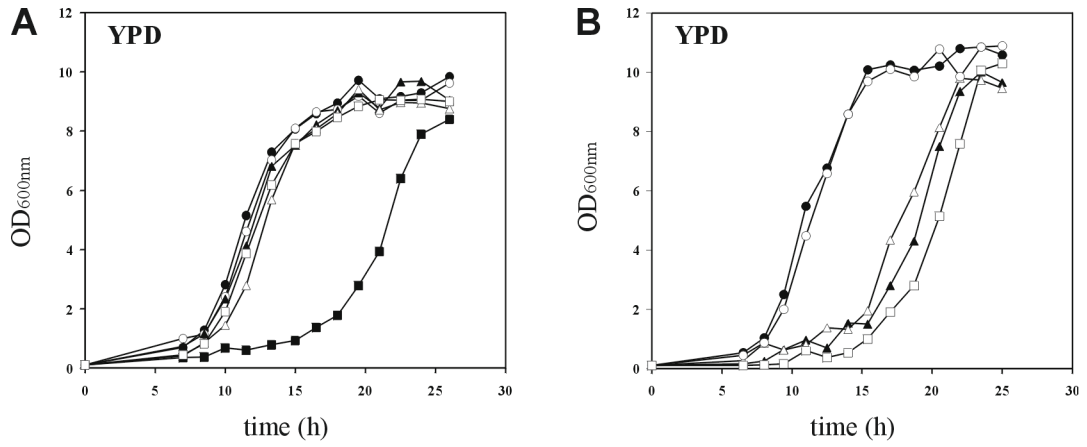


Figure III.3: The Pho90 low-affinity phosphate transporter is required for growth adaptation to rich YPD medium in the absence of the high-affinity phosphate transport system.

Growth was monitored after strains from a stationary preculture were inoculated into fresh YPD medium. **(A)** Effect of additional deletion of genes encoding H^+/Pi symporters in a *pho4Δ* background. Strains: wild-type (●), *pho4Δ* (○), *pho4Δ pho84Δ* (▲), *pho4Δ pho87Δ* (△), *pho4Δ pho90Δ* (■), *pho4Δ pho91Δ* (□). **(B)** Strains lacking both the high-affinity phosphate transport system and the Pho90 low-affinity transporter display a similar growth defect on YPD medium. Strains: wild-type (●), *pho84Δ pho89Δ* (○), *pho4Δ pho90Δ* (▲), *pho81Δ pho90Δ* (△), *pho84Δ pho89Δ pho90Δ* (■).

In line with our previous report (Swinnen et al., 2005), the *pho4Δ* strain has a defect in the accumulation of trehalose and glycogen in response to Pi starvation (Fig. III.4.B). Furthermore, induction of the stress responsive genes *HSP12*, *SSA3* and *GRE1* is impaired in the *pho4Δ* strain, while effects on *HSP26* expression are minimal (Fig. III.4.C). Additional deletion of *PHO84*, *PHO90* or *PHO91* in the *pho4Δ* strain led to phenotypes similar to the single *pho4Δ* mutant (Fig. III.4.B and III.4.C). The additional deletion of *PHO87*, however, exacerbates the defects in starvation responses when compared to the single *pho4Δ* mutant, with lower trehalose and glycogen levels (Fig. III.4.B) and lower expression of the *HSP12*, *SSA3* and *GRE1* genes (Fig. III.4.C). Overexpression of *PHO87* in a *pho4Δ* strain, on the other hand, did not suppress the starvation defects of a *pho4Δ* strain (not shown). Finally, deletion of *PHO4* leads to the inability to express typical PHO genes, such as *PHO84* and *PHO89* (Fig. III.4.C).

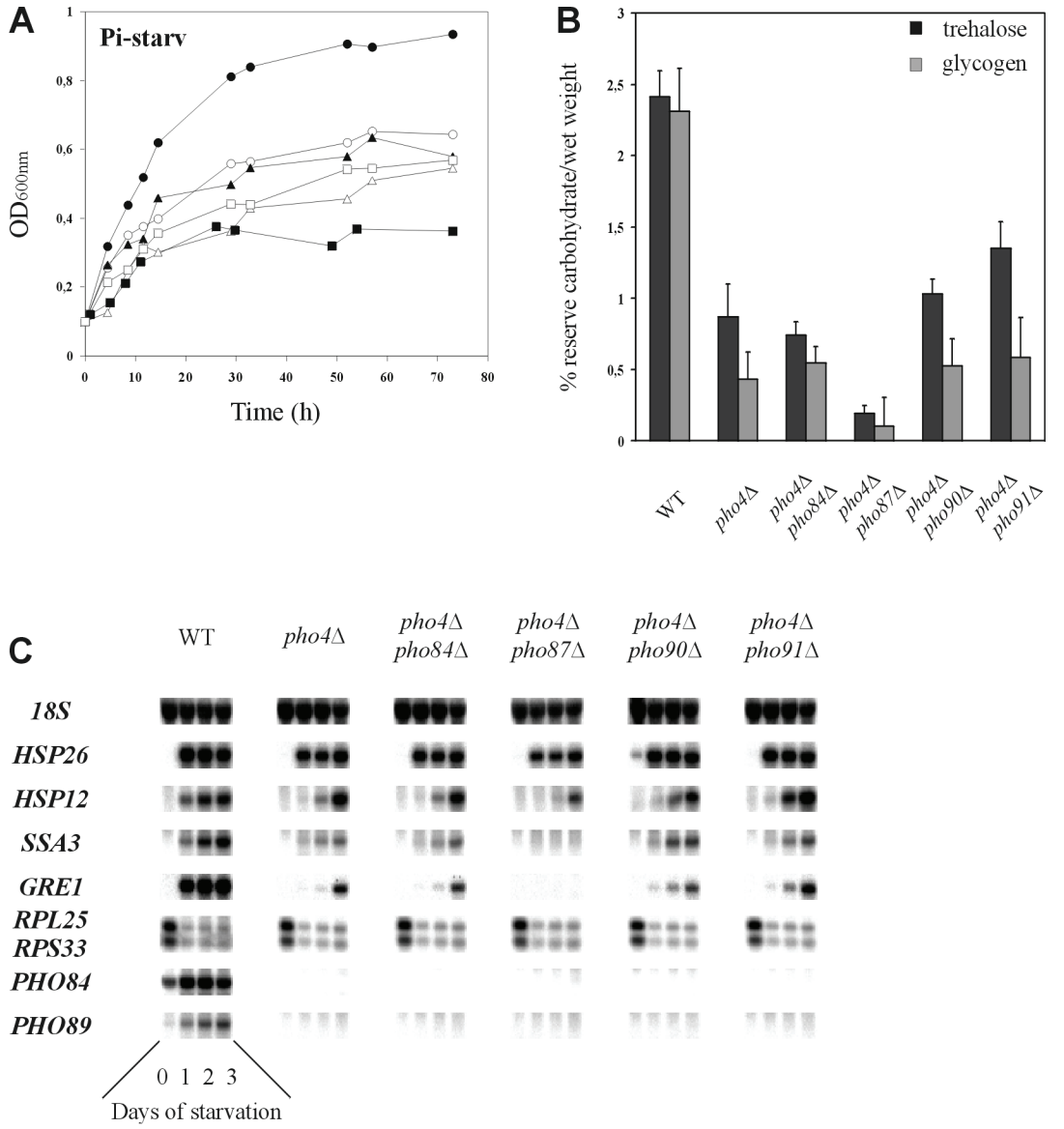


Figure III.4: Phosphate starvation response of different *pho4Δ*-containing mutants.

Exponentially growing cells of the wild type (BY4741) and isogenic mutants were harvested and transferred to phosphate-depleted medium. **(A)** Growth profiles during Pi-starvation were determined. Strains: wild-type (●), *pho4Δ* (○), *pho4Δ pho84Δ* (▲), *pho4Δ pho87Δ* (△), *pho4Δ pho90Δ* (■), *pho4Δ pho91Δ* (□). **(B)** Levels of trehalose and glycogen after Pi-starvation were determined for the different strains. Mean values from three independent experiments are shown, with SD (error bars) **(C)** Each day during the starvation period samples were taken for gene expression analysis by Northern blot. Expression was analysed of the stress responsive genes *HSP26*, *HSP12*, *SSA3* and *GRE1*, the ribosomal protein genes *RPL25* and *RPS33*, and the phosphate responsive genes, *PHO84* and *PHO89*. *18S* mRNA levels are shown as a standard.

Readdition of 10 mM KH_2PO_4 to Pi starved cells results in a rapid activation of trehalase in the wild type strain as well as in the *pho4Δ* mutant (Fig. III.5.A). Basal trehalase activity in the *pho4Δ* strain is higher than in the wild type strain, as previously reported (Swinnen et al., 2005), and correlates with the reduced trehalose levels after phosphate starvation (Fig. III.4.B). Importantly, trehalase activation in the *pho4Δ* mutant is truly independent of Pho84, as activation is similar in the *pho4Δ* and *pho4Δ pho84Δ* mutants (Fig. III.5.A). Intriguingly, additional deletion of *PHO87* in the *pho4Δ* strain, but not *PHO90* or *PHO91*, results in a complete loss of phosphate-induced trehalase activation. In the previous section, we observed that the loss of trehalase activation in the *pho84Δ* mutant is correlated to its loss in rapid Pi-consumption. When comparing Pi-consumption rates in strains deleted in *PHO4*, it is clear that only Pho87 contributes to Pi-consumption in the *pho4Δ* strain (Fig. III.5.B). Additional deletion of *PHO84*, *PHO90* or *PHO91* did not affect the Pi-consumption rate of the *pho4Δ* mutant. When looking at the repression of stress responsive genes, specifically the *pho4Δ pho87Δ* strain displays a defect in the repression of *HSP12* after Pi readdition (Fig. III.5.C). The levels of *SSA3* and *GRE1* were too low to be detected in this mutant. Finally, induction of the ribosomal protein genes *RPL25* and *RPS33* is also impaired in the *pho4Δ pho87Δ* mutant. On the contrary, all other mutant strains display responses to Pi readdition similar as those observed in the wild type strain. Thus, these data clearly indicate that in the *pho4Δ* strain Pho87 is specifically responsible for phosphate signalling. The other low-affinity phosphate transporters, Pho90 and Pho91, cannot substitute for Pho87 in this function.

2.3 Regulation of Pho87 function occurs at a posttranscriptional level

The results discussed so far indicate that the Pho87 phosphate transporter can only mediate signalling in the absence of a functional Pho4 transcription factor. Indeed, in the phosphate-starved *pho84Δ* mutant strain, Pho4 is active and Pho87 cannot substitute for Pho84 in the signalling process (Fig. III.3). Additional deletion of *PHO4* in a *pho84Δ* mutant restores phosphate signalling which then depends on the Pho87 protein, as both the *pho4Δ pho87Δ* and *pho4Δ pho84Δ pho87Δ* strain are incapable of rapid phosphate signalling (Fig. III.5 and results not shown).

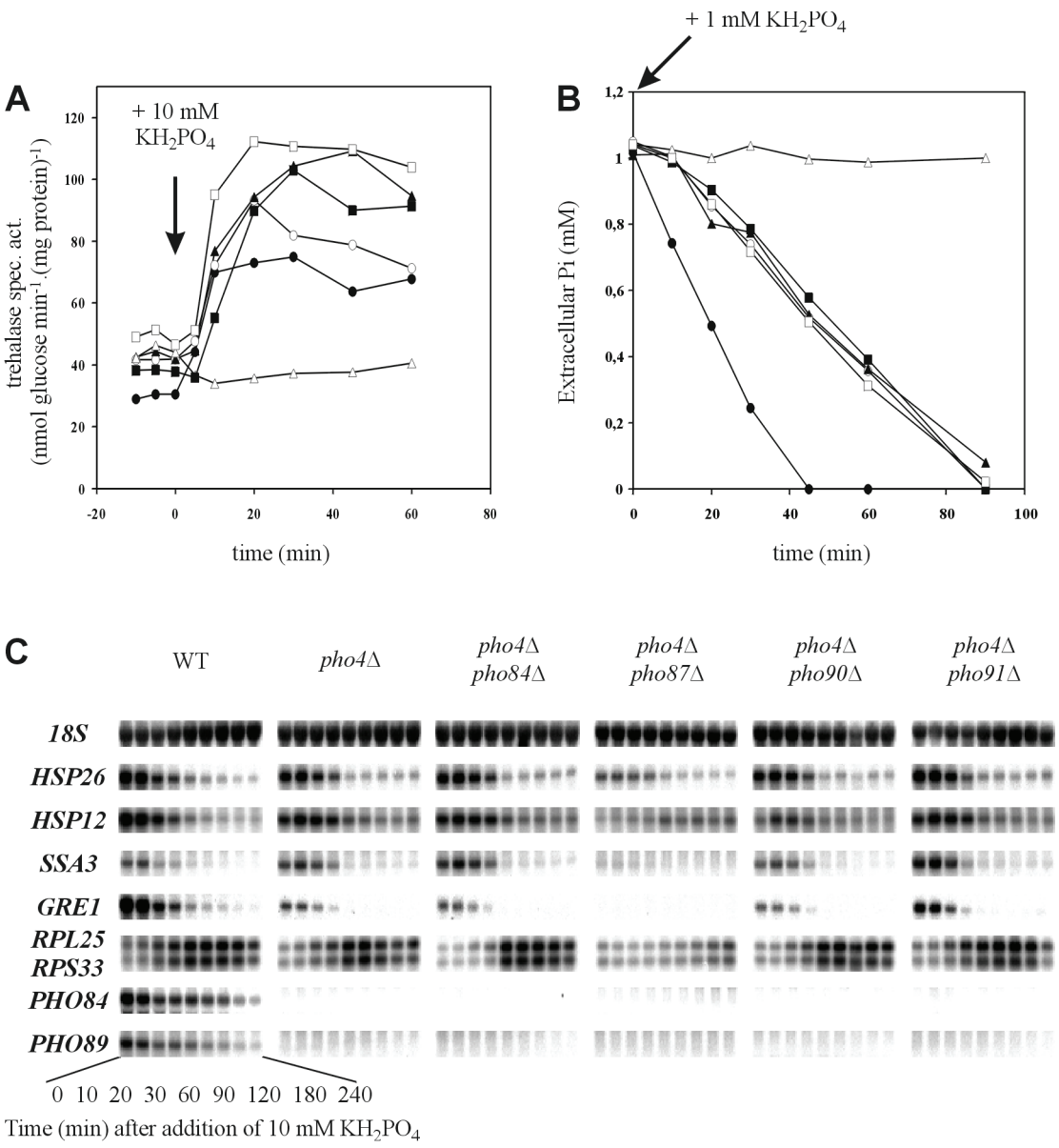


Figure III.5: The low-affinity phosphate transporter Pho87 mediates phosphate signalling in the *pho4Δ* strain.

Indicated strains isogenic to the wild-type (BY4741) were analysed for activation of trehalase **(A)** and Pi-consumption **(B)** after re-addition of KH_2PO_4 in the indicated concentrations to the Pi-starved cells. Strains: wild-type (●), *pho4Δ* (○), *pho4Δ pho84Δ* (▲), *pho4Δ pho87Δ* (△), *pho4Δ pho90Δ* (■), *pho4Δ pho91Δ* (□). In addition, the transcriptional response was monitored after re-addition of 10 mM KH_2PO_4 to the starved cells **(C)**. Expression was analysed of the stress responsive genes *HSP26*, *HSP12*, *SSA3* and *GRE1*, the ribosomal protein genes *RPL25* and *RPS33*, and the phosphate responsive genes, *PHO84* and *PHO89*. *18S* mRNA levels are shown as a standard.

Previous studies already indicated that transcription of *PHO87*, as well as that of *PHO90* and *PHO91*, is independent of both phosphate concentration and Pho4 activity (Auesukaree et al., 2003). However, these studies were performed by comparing yeast cells grown in high- or low-Pi conditions. Since the physiology and transcriptional programs of cells grown under nutrient-limited conditions vary significantly from yeast cells starved for nutrients (Boer et al., 2003; Saldanha et al., 2004; Wu et al., 2004), we re-investigated the effect of phosphate levels and Pho4 activity on the expression of the genes encoding the low-affinity phosphate transporters in our conditions. As shown in Fig. III.6 we could see no significant alterations in the expression of either *PHO87*, *PHO90* or *PHO91* when cells are starved for phosphate. In addition, expression of the low-affinity phosphate transporters is unaffected in both the *pho4Δ* and *pho84Δ* mutant. In contrast, expression of the components of the high-affinity phosphate transport system, *PHO84* and *PHO89*, is strictly depending on the presence of the Pho4 transcription factor (Fig. III.6). This demonstrates that the inability of Pho87 to signal phosphate availability in the *pho84Δ* mutant is not due to altered expression of *PHO87* and that Pho4 affects the function of the Pho87 transporter on a posttranscriptional level.

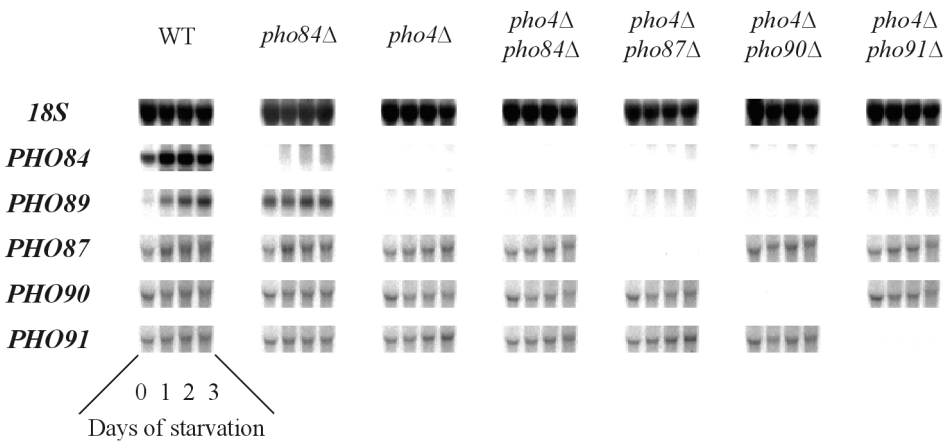


Figure III.6: Expression of *PHO87*, *PHO90* and *PHO91* is independent of phosphate concentration and Pho4 activity.

Expression of *PHO87*, *PHO90* and *PHO91* was monitored during every day of the starvation period. For comparison, expression of the phosphate-responsive genes *PHO84* and *PHO89* is shown. *18S* levels are shown as a standard.

As it was determined that overexpression of *PHO87* in a *pho84Δ pho87Δ pho89Δ pho90Δ pho91Δ* strain can mediate phosphate signalling (Giots et al., 2003), we investigated the effect of overexpression of *PHO87* in our *pho84Δ* strain. Overexpression of *PHO87* under the control of the *ADH1* promoter restored phosphate-induced activation of trehalase in the *pho84Δ* strain (Fig. III.7.A), together with the ability for rapid phosphate consumption (Fig. III.7.B). Overexpression of *PHO87* can therefore bypass the natural inhibition of Pho87 function under phosphate starvation conditions because of an unnatural high expression of this gene when compared to the expression from its native promoter (Fig. III.7.C). As a control, overexpression of *PHO84* in the *pho84Δ* strain also restored phosphate-induced trehalase activation (Fig. III.7.A) and phosphate consumption (Fig. III.7.B), although to a lesser extent. However, expression of *PHO84* from the *ADH1* promoter actually leads to less *PHO84* expression when compared to the endogenous *PHO84* expression under phosphate starvation conditions (Fig. III.7.C), highlighting the strength of the native *PHO84* promoter. Although the use of overexpression of genes can be useful tools for investigating signalling pathways, these results demonstrate that this technique may lead to the loss of knowledge concerning the normal physiological regulation of processes and must therefore be interpreted with great caution.

2.4 The Pho87 transporter functions as a low-affinity phosphate sensor

PHO87, *PHO90* and *PHO91* encode for partially redundant H^+/Pi symporters, constituting the low-affinity phosphate transport system in yeast (Auesukaree et al., 2003; Pinson et al., 2004; Wykoff and O'Shea, 2001). Unlike *PHO84*, expression of *PHO87*, *PHO90* and *PHO91* is unaffected by Pi-levels (Auesukaree et al., 2003 and Fig. III.6). In addition, Pho87, Pho90 and Pho91 are regarded as low-affinity phosphate transporters, as their K_m for Pi-uptake is much higher as that of Pho84 (Berhe et al., 1995; Wykoff and O'Shea, 2001). After addition of 10 mM KH_2PO_4 to Pi-starved cells, we observed clear activation trehalase in the *pho4Δ* strain, which depends on the Pho87 phosphate transporter (Fig. III.5.A). Moreover, the extent of trehalase activation is similar in a *pho4Δ* mutant when compared to the wild-type strain (i.e. 91.3 ± 13.2 % of wild-type activation, calculated from 3 independent experiments). As Pho87 encompasses a low-affinity phosphate transporter, we set out

to compare the trehalase activation extent between a WT and *pho4Δ* strain when lower phosphate concentrations are used for activation.

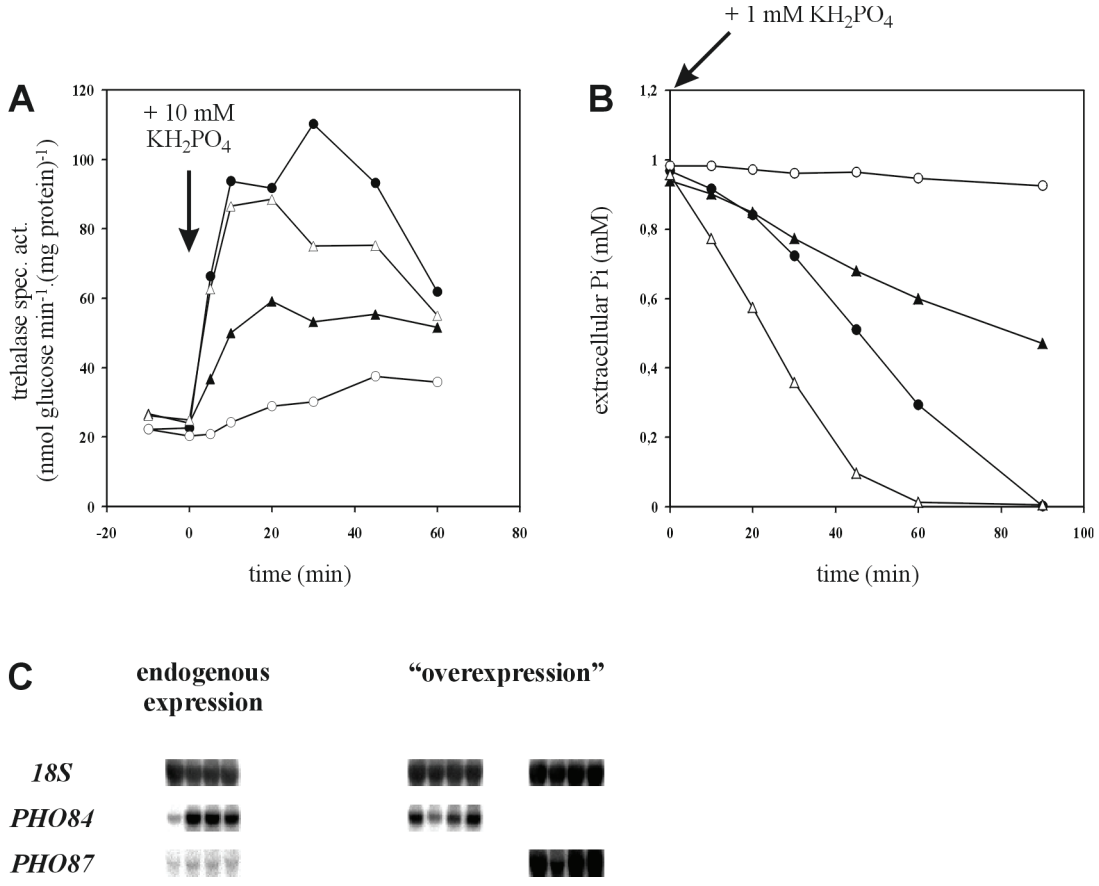


Figure III.7: Overexpression of *PHO87* can bypass the negative regulation of Pho87.

The effect of overexpression of either *PHO84* or *PHO87* in a *pho84Δ* strain was monitored for **(A)** activation of trehalase after the addition of 10 mM KH_2PO_4 to phosphate-starved cells and **(B)** phosphate consumption after the addition of 1 mM KH_2PO_4 to phosphate-starved cells. Strains: wild-type + EB0252 (empty vector) (●), *pho84Δ* + EB0252 (○), *pho84Δ* + EB1368 (*ADH1-PHO84*) (▲), *pho84Δ* + EB1247 (*ADH1-PHO87*) (△). **(C)** Endogenous expression of both *PHO84* and *PHO87* in a wild-type strain was compared with the expression of both genes from EB1368 and EB1247, resp. ("overexpression"), in the *pho84Δ* strain.

After addition of 1 mM KH_2PO_4 trehalase is rapidly activated in both the wild-type and *pho4Δ* strain, although the activation extent in the latter has lowered to 74 ± 6.7% (Fig. III.8.A). When the phosphate concentration is further decreased to 250 µM, the *pho4Δ* strain displays only a gradual increase in the activation of trehalase, while the wild-type still displays a clear, rapid activation (Fig. III.8.B). The efficiency of trehalase activation in the *pho4Δ* strain when compared to the wild-type strain thus

decreases when lower amounts of phosphate are employed, indicating that the Pho87 phosphate transporter acts as a low-affinity phosphate sensor. As expected, deletion of *PHO87* in the wild-type background did not affect phosphate-induced trehalase activation with these lower phosphate concentrations (Fig. III.8).

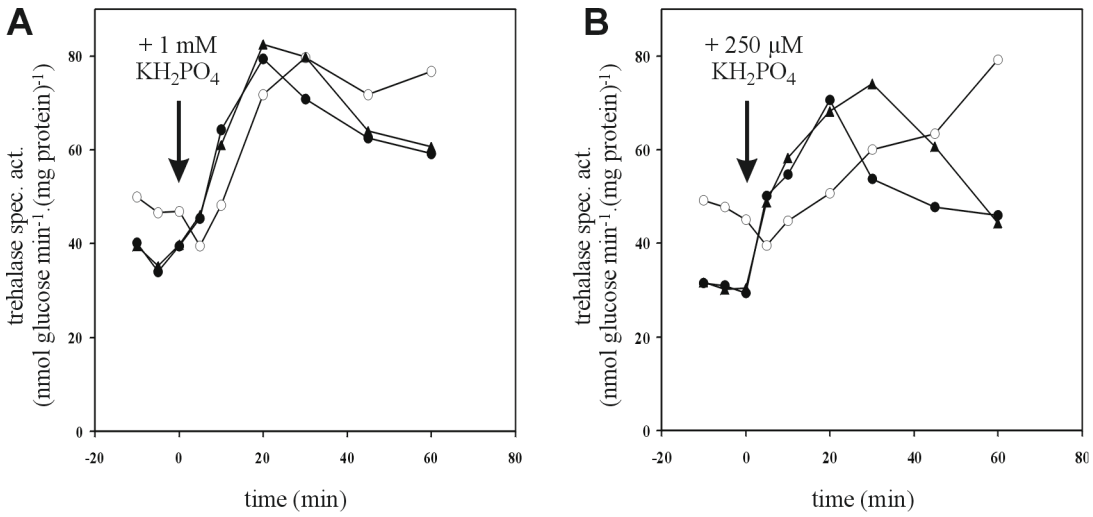


Fig. III.8: The Pho87 transporter functions as a low-affinity phosphate sensor.
The activation of trehalase was monitored after the addition of 1 mM **(A)** or 250 µM **(B)** of KH_2PO_4 to Pi-starved cells. Strains: wild-type (●), *pho4Δ* (○), *pho87Δ* (▲).

3. The phosphate starvation response is modulated by carbon source signalling

In the previous section we described how the Pho87 phosphate transporter mediates phosphate signalling in the absence of an active Pho4 transcription factor. An immediate question that arises is the significance of this phenomenon. Indeed, activation of Pho4 under phosphate starvation conditions allows the yeast cell to fully utilise all available phosphorous sources in the environment and mediates part of the stress response through stimulation of Pho81 function (Swinnen et al., 2005). Additionally, the Pho84 phosphate transporter seems more suitable in mediating phosphate signalling when compared to Pho87, as Pho84 acts to sustain high-affinity phosphate signalling, while Pho87 is only capable of rapid signalling in response to higher phosphate concentrations (Fig. III.8). However, earlier observations already pointed out that regulation of *PHO84* transcription and Pho84 abundance in the plasma membrane is subject to additional environmental conditions besides phosphate

availability. When yeast cells were grown in a low-Pi environment, it was observed that *PHO84* transcription is shut off as they approached stationary phase (Martinez et al., 1998). In addition, plasma membrane localized Pho84 was targeted to the vacuole for degradation.

To be able to follow Pho84 abundance and localization, we fused a *yEGFP3*-cassette to the chromosomal copy of *PHO84*, thereby producing a Pho84-GFP fusion protein expressed from its native promoter. We already observed a high *PHO84* expression throughout the whole period of Pi-starvation (Fig. III.1.C). When checking Pho84-GFP localization, we could see clear plasma membrane localized Pho84 in yeast cells starved for phosphate during 3 days (Fig. III.9.A). In addition to the plasma membrane, a portion of Pho84 also resides in endocytotic vesicles and the vacuole.

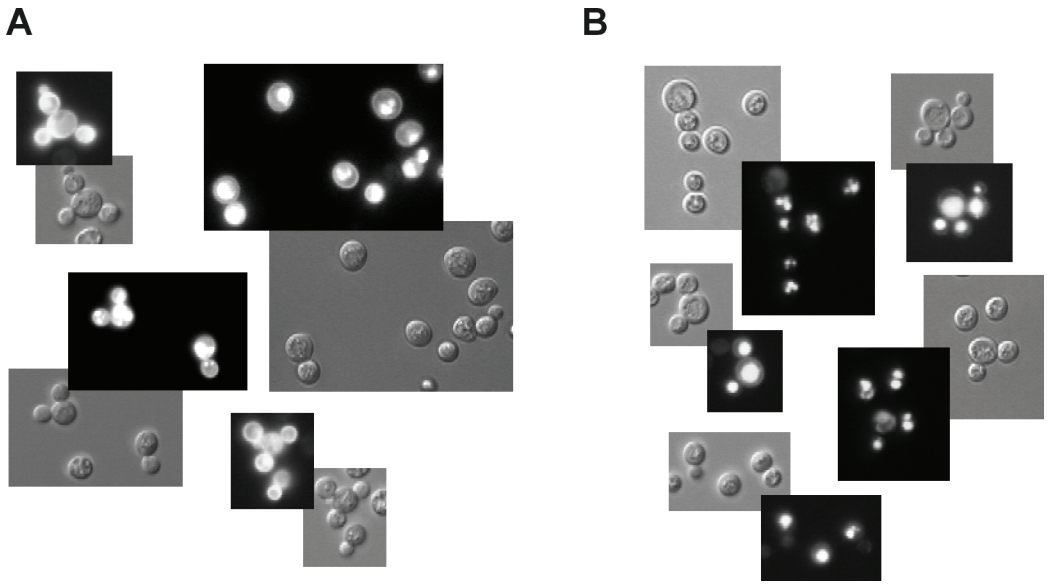


Figure III.9: The Pho84 phosphate transporter is internalized upon carbon source depletion.
Wild-type yeast cells carrying a chromosomal tagged *PHO84-yEGFP3* gene were starved for Pi during 3 days on 4% glucose. After 3 days, cells were harvested, washed and resuspended in Pi-starvation medium containing either 4% glucose **(A)** or no carbon source **(B)**. Cells were incubated for 2 hours before pictures were taken.

As it was previously observed that Pho84 is removed from the plasma membrane as cells enter the stationary phase (Martinez et al., 1998), we decided to investigate the effect of carbon source presence in our conditions. When yeast cells starved for Pi on a glucose-containing medium were resuspended in fresh Pi-starvation medium lacking a carbon source, we could see a disappearance of Pho84

from the plasma membrane and a strong fluorescence signal in the vacuoles of the cells (Fig. III.9.B). The presence of glucose is thus required to keep Pho84 at the plasma membrane in Pi-starved cells.

When yeast cells growing exponentially on glucose are transferred to glucose-containing medium lacking phosphate, Pho4 is rapidly translocated from the cytoplasm to the nucleus, allowing it to activate transcription of PHO genes, such as *PHO84* which is highly induced after 3 hours of incubation (Lenburg and O'Shea, 1996) (Fig. III.10.A and results not shown). When the same cells are transferred to Pi-starvation medium containing glycerol as a carbon source or no carbon source at all, however, Pho4 remains in the cytoplasm and *PHO84* transcription is not induced for up to 6 hours of incubation (not shown). In addition, transferring yeast cells starved for Pi during 3 days on a glucose-containing medium to medium lacking both Pi and a carbon source triggers nuclear exclusion of Pho4 (Fig. III.10.B). This nuclear exclusion of Pho4 is also observed when glucose-grown Pi-starved yeast cells are transferred to Pi-starvation medium containing glycerol or galactose as a carbon source (not shown). Hence, it appears that Pho4 localisation is regulated independently by a Pi-signal and a carbon source signal, and Pho4 is translocated out of the nucleus upon a downshift of the quality of carbon source. Nuclear accumulation upon Pi-starvation is regulated via the inhibition of the Pho85-Pho80 kinase by the Pho81 CKI (Lenburg and O'Shea, 1996), and Pho4 is constitutively nuclear in a *pho80Δ* mutant. Interestingly, deletion of *PHO80* also inhibits nuclear exclusion of Pho4 when Pi-starved yeast cells are transferred from glucose-containing medium to medium lacking a carbon source (Fig. III.10.C), indicating that the activity of the Pho85-Pho80 kinase is modulated by carbon source presence. As glucose removal is known to activate the Snf1 protein kinase (Wilson et al., 1996), we next tested localization of Pho4 in a *snf1Δ* mutant and found that nuclear exclusion of Pho4 upon a removal of carbon source is greatly impaired when *SNF1* is deleted (Fig. III.10.D). Intriguingly, we also found that carbon source-dependent nuclear exclusion depends on a functional Rim15 protein, as Pho4 remains almost completely nuclear when glucose is removed from Pi-starved *rim15Δ* cells (Fig. III.10.E).

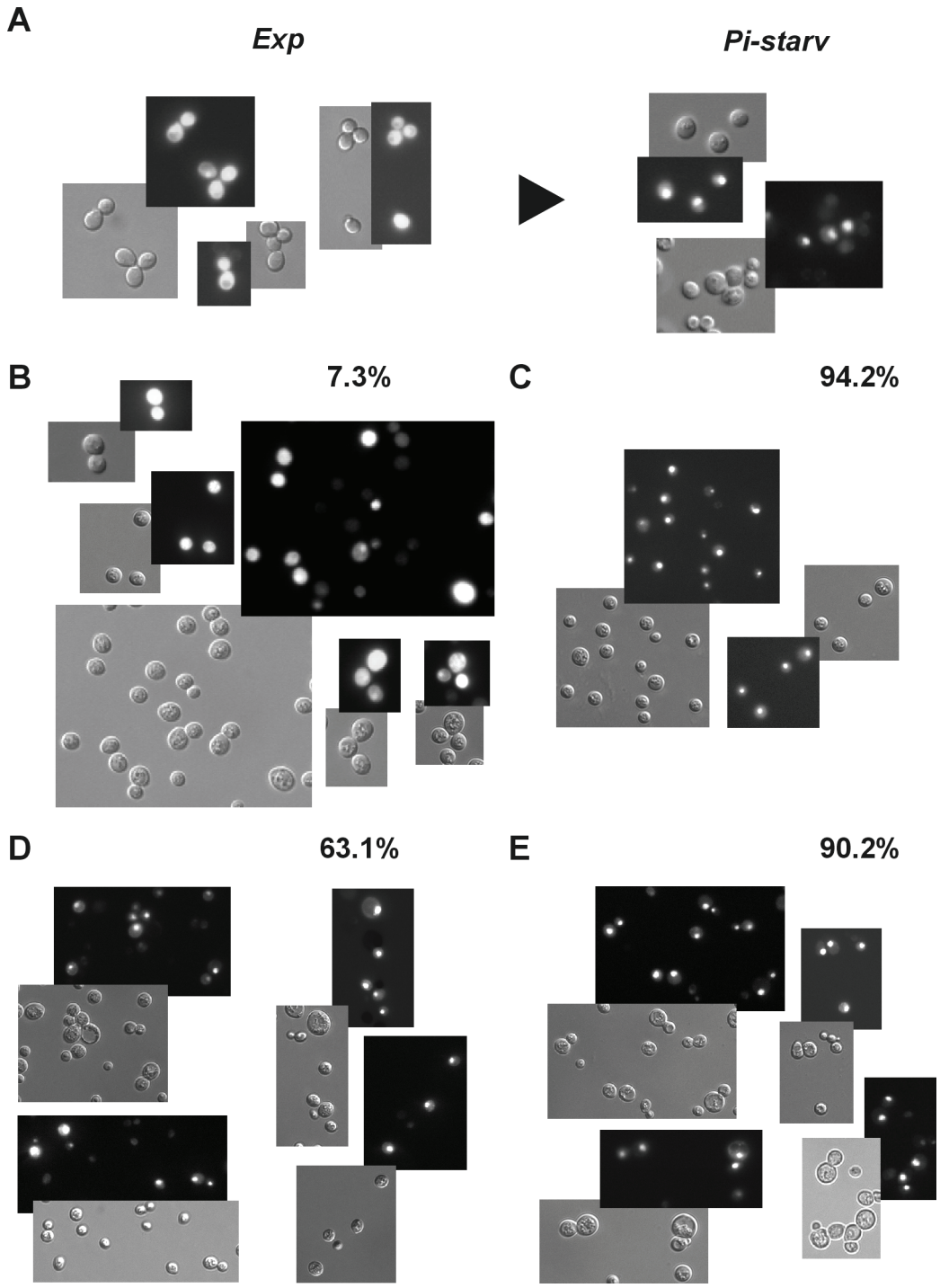


Fig. III.10: Subcellular localization of Pho4 is modulated by carbon source presence.

Strains were transformed with plasmid EB0347 (p*PHO4*pr-*PHO4-GFP*) to follow subcellular localization of Pho4. **(A)** Pho4 is cytoplasmic in cells growing exponentially on glucose ("Exp"), and translocates to the nucleus upon transfer to medium without Pi, containing 4% glucose ("Pi-starv"). **(B)-(C)-(D)-(E)** Strains were starved for Pi during 3 days on 4% glucose. After 3 days, cells were harvested, washed and resuspended in Pi-starvation medium lacking carbon source. Cells were incubated for 2 hours before pictures were taken. Subcellular localization of Pho4 was examined in the wild-type strain **(B)** and strains deleted for *PHO80* **(C)**, *SNF1* **(D)**, and *RIM15* **(E)**. Percentages of

nuclear Pho4 are indicated. Over 200 fluorescent cells were counted from two independent transformants.

As Pho87 mediates phosphate signalling in the absence of Pho4 and this transcription factor is downregulated when glucose is removed from the medium, it seems plausible that Pho87 may take over the function of Pho84 in signalling Pi availability when both carbon source and phosphate are exhausted. Therefore, we wanted to check Pho87 localization under carbon and phosphate starvation. When creating chromosomal *PHO87-yEGFP3* fusions we never obtained fluorescent signals (not shown). This is probably due to the low expression of *PHO87*, as estimated levels of Pho87 in exponentially grown cells indicate that it is present at very low levels (Ghaemmaghami et al., 2003), even about 1000 times less than the Pho84 protein, the levels of which should be minimal in this condition. In the near future, we will therefore create a *PHO87-yEGFP3* fusion gene expressed from a stronger heterologous promoter (e.g. the *ADH1*-promoter) to increase the expression of *PHO87-yEGFP3* and thereby be able to observe Pho87-GFP localization.

4. Growth recovery after phosphate starvation is a dynamic interplay between phosphate transport and Pho4 activity

Nutrient signalling pathways generally function to allow the cell to rapidly acclimatize to changing nutrient conditions, adapting metabolism and growth to the new nutrient environment. Also in our case, rapid phosphate signalling functions in part to quickly restart growth of the yeast cell as the *pho84Δ* and *pho4Δ pho87Δ* strains, which are deficient in rapid phosphate signalling, display a defect in growth resumption when compared to the wild-type strain (Fig. III.11.A and III.11.B). This correlates nicely to the defect in phosphate consumption and induction of ribosomal protein genes. Interesting to note is that the *pho4Δ pho90Δ* strain displays wild-type like growth recovery after re-addition of 10 mM KH_2PO_4 to phosphate-starved cells (Fig. III.11.B), while the same strain has an extended lag phase when cells are inoculated from a stationary preculture into fresh YPD medium (Fig. III.3.A). In contrast, the *pho84Δ pho89Δ pho90Δ* strain displays an extended lag phase in both conditions (Fig. III.3.B and Fig. III.11.C). The requirement of the Pho90 transporter in

the absence of a high-affinity phosphate transport system thus depends on the specific nutrient conditions and Pho4 activity.

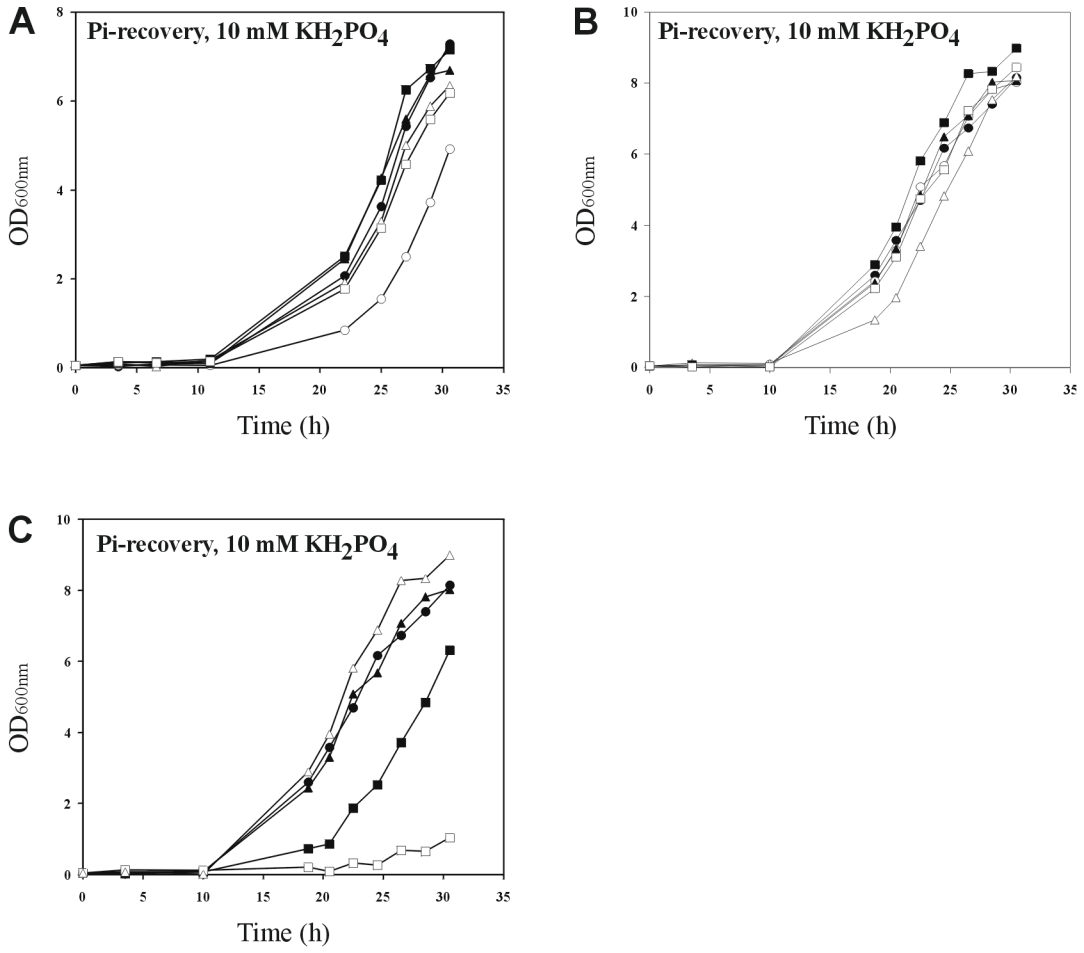


Figure III.11: Requirement of specific phosphate transporters for optimal growth recovery after phosphate starvation.

Growth recovery was monitored after the addition of 10 mM KH_2PO_4 to phosphate-starved cells. Strains: **(A)** wild-type (●), *pho84Δ* (○), *pho87Δ* (▲), *pho90Δ* (△), *pho91Δ* (■), *pho87Δ pho90Δ pho91Δ* (□);

(B) wild-type (●), *pho4Δ* (○), *pho4Δ pho84Δ* (▲), *pho4Δ pho87Δ* (△), *pho4Δ pho90Δ* (■), *pho4Δ pho91Δ* (□);

(C) wild-type (●), *pho4Δ* (▲), *pho4Δ pho90Δ* (△), *pho84Δ pho89Δ* (■), *pho84Δ pho89Δ pho90Δ* (□).

These results indicate that an active Pho4 transcription factor inhibits efficient growth recovery after phosphate starvation. Indeed, additional deletion of *PHO4* in a *pho84Δ* strain results in faster growth resumption after phosphate re-addition to phosphate starved cells, which is more apparent when the phosphate concentration is

lowered (Fig. III.12.A). This effect is due to the activation of the Pho87 transporter when *PHO4* is deleted. Yet, deletion of *PHO4* in a *pho84Δ pho87Δ* double mutant still results in an increase of growth recovery (although not to the level of the wild-type strain, Fig. III.12.A), while rapid phosphate consumption is absent in both the *pho84Δ pho87Δ* and *pho4Δ pho84Δ pho87Δ* strains (not shown). Thus, optimal growth recovery from phosphate starvation seems to depend on two interacting factors. Obviously, rapid phosphate consumption from the medium is required, which occurs via Pho84 or, when Pho4 is inactivated, Pho87. Secondly, the phosphate taken up by the cell leads to the inactivation of Pho4, allowing rapid growth recovery.

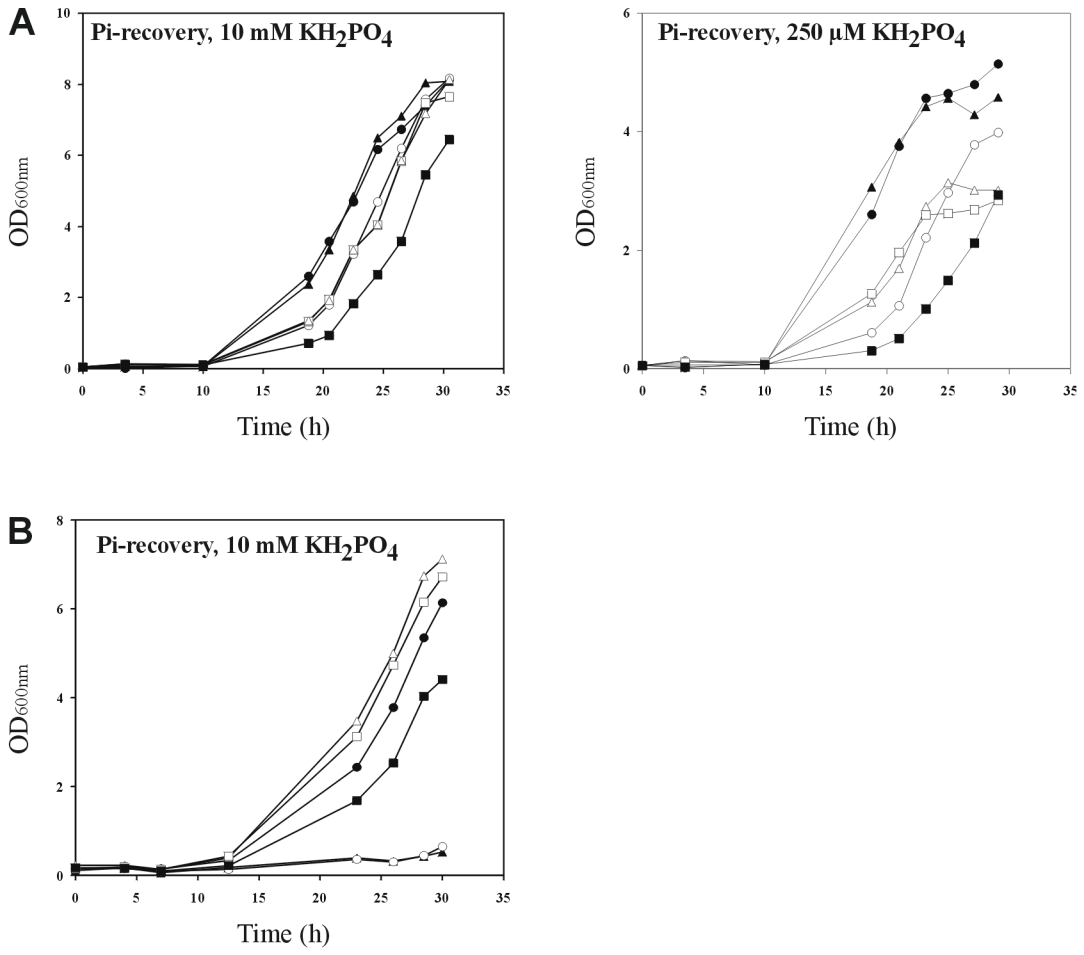


Figure III.12: Optimal growth recovery requires inactivation of the Pho4 transcription factor.
Growth recovery was monitored after the addition of KH_2PO_4 in the indicated concentrations to phosphate-starved cells. Strains: **(A)** wild-type (●), *pho84Δ* (○), *pho4Δ pho84Δ* (▲), *pho4Δ pho87Δ* (△), *pho84Δ pho87Δ* (■), *pho4Δ pho84Δ pho87Δ* (□);
(B) wild-type (●), *pho85Δ* (○), *pho80Δ* (▲), *pho4Δ* (△), *pho85Δ pho4Δ* (■), *pho80Δ pho4Δ* (□).

When Pho4 is constitutively active through deletion of either *PHO85* or *PHO80*, growth recovery from phosphate starvation is dramatically retarded, which can be largely suppressed by additional deletion of *PHO4* (Fig. III.12.B). Additional deletion of *RIM15* in a *pho85Δ* or *pho80Δ* background did not alleviate the retarded growth recovery of these strains after phosphate re-supplementation (not shown). Intriguingly, hyperactive Pho4 generally leads to impaired growth as deletion of *PHO4* in the *pho85Δ* and *pho80Δ* strains can also suppress to some extent the growth defects of these strains on rich glucose- and glycerol containing medium (Fig. III.13). Interestingly, *PHO4* was recently identified in an independent study as causing slow growth in a genome-wide screen using systematic gene overexpression (Sopko et al., 2006). Furthermore, overexpression of *PHO4* in a *pho85Δ* strain confers a synthetic lethal phenotype on galactose medium. The connection of Pho4 activity with growth regulation is not known yet, as none of the genes described in the PHO regulon has a clear connection with the cell cycle (Ogawa et al., 2000).

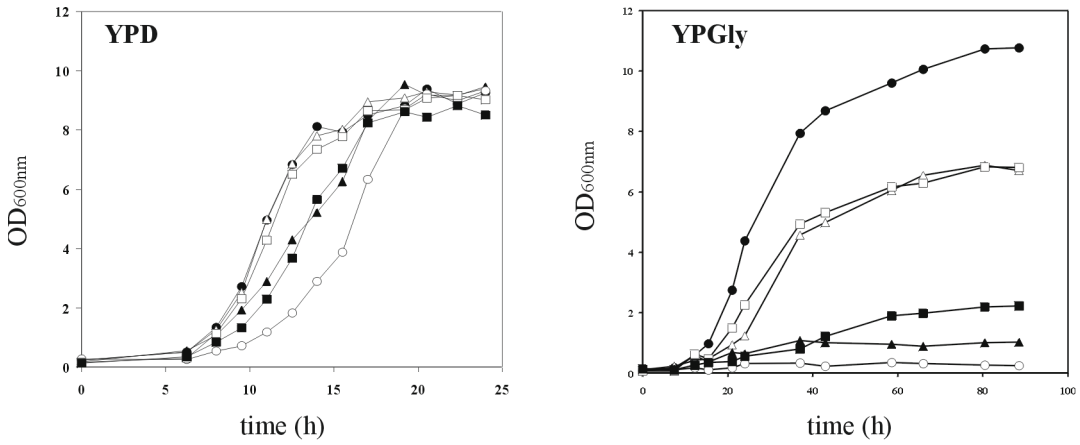


Figure III.13: The growth defect of the *pho80Δ* and *pho85Δ* strains is in part due to a hyperactive Pho4.

Growth was monitored after cells were in oculated from a stationary preculture into fresh YPD **(A)** or YPGly **(B)** medium. Strains: wild-type (●), *pho85Δ* (○), *pho80Δ* (▲), *pho4Δ* (△), *pho85Δ pho4Δ* (■), *pho80Δ pho4Δ* (□).

Concluding remarks

Transport proteins play a crucial role in maintaining proper nutrient homeostasis. Cells can respond to fluctuating levels of a certain nutrient by adjusting the transport capability for this nutrient. This includes transcriptional responses such as the induction of a high-affinity transport system and posttranslational mechanisms regulating the subcellular localization, stability and/or the affinity of the transporter.

In this chapter we investigated the involvement of yeast phosphate transporters in rapid phosphate signalling towards PKA targets. We demonstrate that two phosphate transporters, i.e. Pho84 and Pho87, can mediate rapid phosphate signalling depending on the activity of the Pho4 transcription factor. We also show that Pho4 activity is modulated by carbon source signalling which may offer a physiological relevance of phosphate signalling via Pho87 and provides additional mechanisms for the integration of different nutrient signals. Finally, while Pho4 activity generally leads to growth inhibition the dynamical balance between phosphate transport and Pho4 activity is required for optimal growth resumption after phosphate readdition.

Rapid phosphate signalling can be mediated by Pho84 or Pho87, depending on the activity of the Pho4 transcription factor

Addition of 10 mM KH_2PO_4 to Pi-starved wild-type cells leads to the activation of typical PKA targets, including activation of trehalase, mobilization of trehalose, repression of stress responsive genes and induction of ribosomal protein genes (Giots et al., 2003; Swinnen et al., 2005). All these responses are absent in a *pho84Δ* mutant, demonstrating that Pho84 is solely responsible for phosphate signalling in a wild-type strain. As deletion of *PHO84* did not abolish the yeasts response during Pi starvation, the role of Pho84 in phosphate signalling is confined to the response of yeast cells to phosphate readdition. Interestingly, the low-affinity phosphate transport system (Wykoff and O'Shea, 2001) does not contribute to Pi signalling in wild-type cells, as deletion of single genes encoding the low-affinity phosphate transporters or even the *pho87Δ pho90Δ pho91Δ* triple mutant results in normal Pi-signalling. Thus, while the low-affinity phosphate transport system can provide the yeast cell with ample amounts of Pi during growth on high-Pi medium

(typically about 7-8 mM KH_2PO_4), it cannot mediate rapid phosphate signalling when high amounts of Pi (10 mM KH_2PO_4 used in this study) are added to Pi-starved cells.

In the previous chapter we made the intriguing observation that rapid phosphate signalling occurs in *pho4Δ* and *pho81Δ* mutants despite the fact that we could not detect *PHO84* transcription in these strains (Swinnen et al., 2005). Here we demonstrate that rapid phosphate signalling in a *pho4Δ* background is truly independent of Pho84 as phosphate signalling is similar in the *pho4Δ* and *pho4Δ pho84Δ* strains. Furthermore, we show that specifically the Pho87 low-affinity phosphate transporter mediates phosphate signalling when *PHO4* is deleted and neither Pho90 nor Pho91 can take over this function.

It remains to be established if and how Pho84 and Pho87 directly activate a downstream signalling pathway, as previously suggested (Giots et al., 2003). Through their conformational change during the transport process, transport proteins may affect a signal transduction component that triggers a downstream signalling pathway. This has already been implicated for the yeast Mep2 ammonium permease and the Gap1 general amino acid transporter (Donaton et al., 2003; Holsbeeks et al., 2004; Van Nuland et al., 2006). If Pho84 and Pho87 themselves directly contribute to the phosphate signalling process, both have to be able to connect with the downstream component mediating the signal, which may be difficult to explain given the low degree of similarity between Pho84 and Pho87 (Wykoff and O'Shea, 2001). In our studies the loss of phosphate signalling in both the *pho84Δ* and *pho4Δ pho87Δ* strains closely correlates with the absence of rapid phosphate consumption. In addition, the loss of signalling in a *pho84Δ* strain can be suppressed by overexpression of *PHO87*, which also restores rapid phosphate consumption in this strain. Based solely on these observations, it is tempting to speculate that phosphate is sensed intracellular, as was previously proposed for the PHO pathway (Auesukaree et al., 2004; Wykoff and O'Shea, 2001). However, recent data suggested a model of integration of signals derived from both extracellular and intracellular Pi, regulating signalling through the PHO pathway by converging on the Pho81 protein (Pinson et al., 2004). It is thus plausible that rapid phosphate signalling controlling typical PKA targets is also controlled by signals from both extra- and intracellular phosphate, wherein the phosphate transporters might play a dual role. By full deletion of these phosphate

transporters, not only intracellular phosphate signalling would be inhibited because of the lack of transport, but also possible direct transporter-derived signals are abolished. Further research has to be performed to tackle these questions. If Pho84 and Pho87 directly mediate signalling, one would expect that specific mutant alleles could be isolated that either lack the signalling capacity and retain normal transport or have lost transport but retain signalling. In addition, to prove that transporters initiate a signalling cascade, it is crucial to identify the interacting partners that mediate the signal derived from the transport protein to the next component in the signalling cascade.

Regulation of low-affinity phosphate transport

The absence of phosphate signalling in the *pho84Δ* strain can be suppressed by an additional deletion of *PHO4*, indicating that Pho4 activity inhibits the function of Pho87. As we found that *PHO87* expression is independent of phosphate concentration as well as the presence of both Pho4 and Pho84, regulation of Pho87 function occurs at a posttranscriptional level. This implicates that Pho4 inhibits Pho87 indirectly, through transcriptional induction of an unknown component which would then inhibit Pho87 function in response to a phosphate starvation signal (Fig. III.14). In the previous chapter, we established that a critical function of Pho4 comprises the activation of a feedback loop controlling *PHO81* expression, in which Pho81-mediated control of the Pho85 CDK regulates several aspects of the phosphate starvation response independent of Pho4. However, Pho81 is unlikely to be responsible for regulating Pho87 as overexpression of *PHO81* in a *pho4Δ* strain does not inhibit phosphate-induced trehalase activation (see Chapter II, Fig. II.7.C). Factors controlling Pho87 activity are largely unknown at present. Besides phosphate concentration, *PHO87* expression also seems independent of glucose presence ((DeRisi et al., 1997) and results not shown), indicating that transcription of *PHO87* may not be influenced by the nutrient status. Nevertheless, for many nutrient transporters posttranscriptional regulation mechanisms exist regulating their intracellular localization and degradation (Hallows, 2005; Horak, 2004; Magasanik and Kaiser, 2002; Zorzano et al., 2005). Pho87 has been shown to be both phosphorylated and ubiquitinated *in vivo* (Peng et al., 2003), indicating also

posttranslational control over Pho87 activity, which may include regulated degradation via the ubiquitination process. In addition, phosphorylation of Pho87 may alter the affinity of the transporter, as has been reported for other transport proteins (Fu and Luan, 1998; Liu and Tsay, 2003). Although the proteins controlling these modifications of Pho87 are not identified yet, our results imply that Pho4 controls the expression of at least one of these proteins and future micro-array studies performed under our experimental conditions will help identify the genes involved in Pho87 regulation. In an attempt to clarify the regulation of Pho87, we created chromosomal *PHO87-yEGFP3* fusion genes. However, none of the constructs produced a visible fluorescent signal, indicating that Pho87 abundance may be too low to detect (Ghaemmaghami et al., 2003). We will proceed to clone the *PHO87-yEGFP3* fusion gene behind a stronger promoter, e.g. the *ADH1* promoter, in a further attempt to study subcellular localization of Pho87 under different environmental conditions. In addition, with the help of tagged Pho87 fusion proteins posttranslational modifications can be identified under various nutrient conditions.

While it is proposed that the low-affinity H^+/Pi transporters Pho87, Pho90 and Pho91 are largely redundant in function (Auesukaree et al., 2003; Pinson et al., 2004; Wykoff and O'Shea, 2001), we could clearly identify a specific function for Pho87 in mediating phosphate signalling when the *PHO4* gene is deleted. Curiously, neither Pho90 nor Pho91 can perform this function. Similar to *PHO87*, expression of *PHO90* and *PHO91* is unaffected by both Pi status and Pho4 (this chapter and (Auesukaree et al., 2003)). As neither Pho90 nor Pho91 can mediate phosphate signalling, either in the presence or absence of Pho4, both Pi-transporters seem to be inactivated under Pi-limiting conditions in a Pho4-independent manner.

We observed that the Pho90 phosphate transporter is most important for growth on standard rich YPD medium when the high-affinity phosphate transport system is knocked out, either by deleting both *PHO84* and *PHO89*, or by deleting *PHO4* (or *PHO81*, thereby rendering the cells unable to express *PHO84* and *PHO89*). Pho90 may therefore represent the low-affinity phosphate transporter responsible for bulk Pi-transport under conditions of sufficient Pi. However, whereas the *pho84Δ pho89Δ pho90Δ* strain also displays slow growth recovery when 10 mM KH_2PO_4 is added to Pi-starved cells, the *pho4Δ pho90Δ* strain recovers growth at a wild-type like rate under the same condition. The latter may be due to the activation of Pho87 when

PHO4 is deleted, since the *pho4Δ pho90Δ* strain shows Pi-consumption rates similar to the *pho4Δ* strain after addition of phosphate to Pi-starved cells, while in the *pho4Δ pho87Δ* strain rapid Pi-consumption is absent. This, however, also suggests that Pho87 is not (fully) activated in the *pho4Δ pho90Δ* strain when it is grown on YPD medium, indicating that both inactivation of Pho4 and a Pi-starvation signal are necessary for (full) activation of Pho87 (Fig. III.14).

In contrast to the requirement for Pho87 and Pho90 for growth and/or signalling under certain conditions, it is worth mentioning that we did not observe any effects of a deletion of *PHO91* on growth or signalling under all conditions and genetic environments tested. As with *PHO87* and *PHO90*, expression of *PHO91* is largely independent of glucose, Pi concentrations and Pho4 activity (see this chapter and (Auesukaree et al., 2003; DeRisi et al., 1997). However, Pho91 is shown to be phosphorylated *in vivo*, indicating posttranscriptional control for this protein (Peng et al., 2003). A specific physiological function for this phosphate transporter, if any, remains unknown.

Modulation of Pho4 activity by carbon source signalling

Transferring yeast cells growing exponentially on glucose to glucose-containing Pi-starvation medium results in the rapid nuclear accumulation of Pho4 and simultaneous transcriptional induction of PHO genes. However, when the same exponentially growing cells are transferred to Pi-starvation medium containing a poor carbon source, such as glycerol, or no carbon source at all, nuclear translocation of Pho4 is inhibited. In addition, nuclear Pho4 in yeast cells completely starved for Pi during 3 days on glucose-containing medium is exported from the nucleus when glucose is removed from the medium or replaced with a carbon source of a lesser quality. The latter is accompanied by a removal of Pho84 from the plasma membrane, after which it is targeted to the vacuole for degradation. Induction of PKA activity after the addition of phosphate to Pi-starved cells has been implicated in the stimulation of rapid Pho84 degradation (Mouillon and Persson, 2005). However, PKA is unlikely to mediate Pho84 degradation upon glucose removal as this condition leads to lower PKA activity (Roosen et al., 2004). The removal of Pho84 from the plasma membrane may be a rather passive process, in which continued recycling of Pho84

completely shifts to the vacuole because no new Pho84 is produced as Pho4 is inactivated under these conditions.

These results indicate that the PHO pathway is not only regulated by phosphate availability but also carbon source signalling impinges on this pathway. Moreover, it seems that the activity of the Pho85-Pho80 kinase is regulated by carbon source signalling, as Pho4 remains nuclear under all conditions in a *pho80Δ* mutant. One major signalling pathway involved in the sensing of carbon source quality is the main glucose repression pathway, in which the Snf1 kinase plays a central role (Gancedo, 1998; Schuller, 2003), and we found that Snf1 is indeed required for nuclear export of Pho4 upon removal of glucose from medium lacking Pi. Intriguingly, the Rim15 protein kinase is also essential for regulating Pho4 subcellular localization in response to glucose presence as Pho4 remains almost exclusively nuclear in *rim15Δ* cells (to the same extent as in a *pho80Δ* mutant) when glucose is removed from the Pi-starvation medium. The way in which Snf1 and/or Rim15 regulate the activity of the Pho85-Pho80 kinase remains to be established (Fig. III.14). Our preliminary data already indicate that the *snf1Δ* and *rim15Δ* mutants do not affect PHO gene transcription upon Pi starvation in the presence of glucose (Chapter II and results not shown). Thus both kinases specifically affect the PHO pathway upon removal of glucose from the medium. As Rim15 is a direct target of the Pho85-Pho80 kinase and is localized to the nucleus upon Pi-starvation (Wanke et al., 2005), this places Rim15 in an ideal environment for feedback control on the Pho85-Pho80 kinase in response to glucose presence, as Pho85-Pho80 is constitutively localized to the nucleus (Kaffman et al., 1998). Rim15 may directly regulate the Pho85-Pho80 kinase or exert its effect through Pho81. The fact that full-length Pho81 is required for regulating Rim15 activity via Pho85-Pho80 (Swinnen et al., 2005) suggests that Pho81 may interact directly with Rim15 through domains other than those required for binding to the Pho85-Pho80 kinase (Huang et al., 2001), positioning Rim15 in a appropriate position for reciprocal regulation of Pho81 function in response to glucose.

Our results imply that although Rim15 is activated by Pi-starvation conditions via the PHO pathway, it still remains responsive to glucose signals allowing a fine-tuning of the nutrient response and emphasizing the role Rim15 plays in the integration of nutrient signalling (Pedruzzi et al., 2003; Roosen et al., 2005; Swinnen et al., 2005; Wanke et al., 2005). The requirement of Snf1 in the glucose-dependent

regulation of Pho4 localization is elusive at this moment. One possibility would be that Snf1 is required for efficient expression of *RIM15*, as this gene is known to be glucose-repressed (Reinders et al., 1998). Interestingly, Snf1 has already been implicated in the phosphate starvation response (Thompson-Jaeger et al., 1991). In addition, we observed similar defects in the phosphate starvation response when comparing *rim15Δ* and *snf1Δ* mutants, including an impaired accumulation of trehalose and induction of the PDS-controlled *SSA3* gene (not shown), which validates a possible concerted requirement of Snf1 for Rim15 function.

Physiological function of Pho87 in phosphate signalling?

The observation that PHO signalling is impaired when cells are confronted with a downshift in carbon source quality makes sense as the induction of a large battery of genes involved in phosphate scavenging is likely to be energetically unfavourable under these conditions. Additional amounts of phosphate taken up by the cell will not permit further growth when no carbon sources are present or when cells still have to adjust their transcriptional and metabolic program to a downshift from rich to poor carbon sources. We postulate that under these conditions Pho87 may take over the function of Pho84 in transporting and/or signalling extracellular phosphate, and that this allows the yeast cell to rapidly stimulate growth when these carbon sources are again present and cells have adjusted their metabolism for the utilization of these carbon sources. This hypothesis is supported by our observation that Pho87 mediates phosphate signalling when Pho4 is inactive. We demonstrated that Pho87 acts as a low-affinity phosphate transporter/sensor, further indicating that the yeast cell does not invest in high-affinity phosphate uptake when other nutritional conditions are unfavourable for growth. It will be interesting to investigate whether PHO signalling is also impaired in response to depletion of other nutrients, e.g. nitrogen depletion, and which components would be involved in this cross-talk.

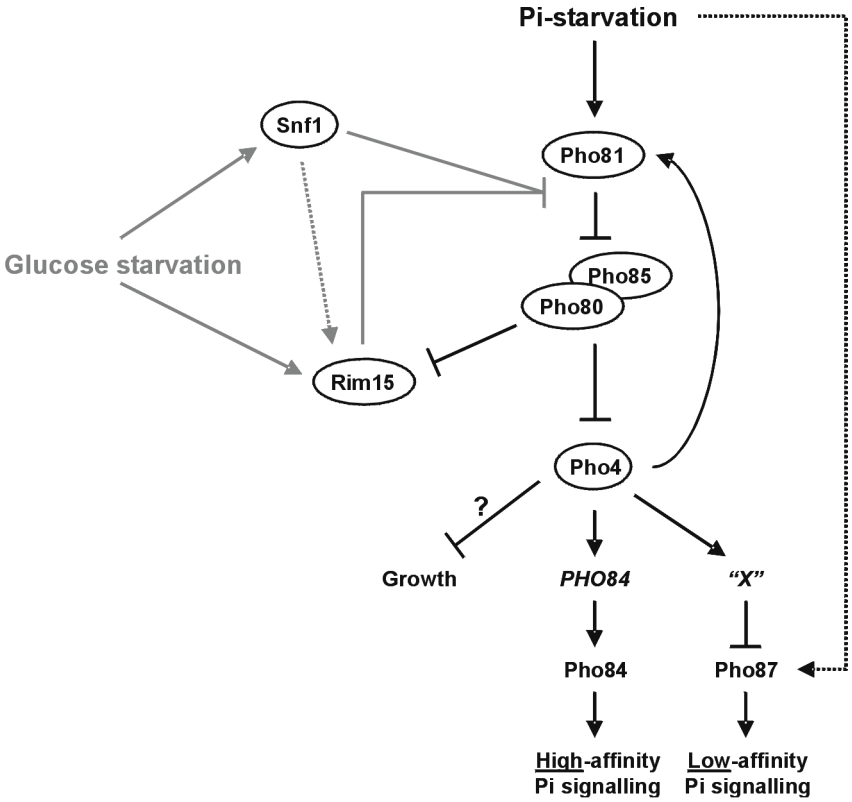


Figure III.14: The PHO pathway controls the activity of the Pho87 low-affinity phosphate transporter and PHO signalling is modulated by glucose.

In the continued presence of glucose, induction of *PHO84* transcription by Pho4 during phosphate starvation permits high-affinity phosphate signalling. Additionally, Pho4 controls the expression of an unknown gene (designated "X") whose protein product inhibits Pho87 function. In the absence of *PHO4*, Pho87 allows for Pi-signalling, though with a lower affinity than Pho84. In addition to X, an independent Pi-starvation signal is required for (full) Pho87 activity. Upon glucose removal from Pi-starved cells (shown in grey), Pho4 is exported from the nucleus, depending on the Snf1 and Rim15 protein kinases. Although depicted here as inhibiting Pho81 function, Snf1 and/or Rim15 may alternatively act directly on the Pho85-Pho80 kinase. The Snf1 requirement may result from a stimulation of Rim15 function, perhaps by inducing *RIM15* transcription. Finally, an active Pho4 hampers growth of yeast cells, though the mechanism(s) involved are still elusive. See text for further details.

Pho4 activity and growth control

We have shown that optimal growth recovery after readdition of phosphate to Pi-starved cells depends on the interplay between rapid phosphate transport and parallel the inactivation of the Pho4 transcription factor. Moreover, an active Pho4 appears to be a general inhibitor of growth as deletion of *PHO4* in either a *pho85Δ* or

pho80Δ background, both of which display a constitutively active Pho4, leads to an increase of growth rate and/or capacity. These results are in agreement with previous findings indicating that several of the growth defects displayed by a *pho85Δ* strain are due to a hyperactive Pho4 transcription factor (Dimmer et al., 2002; Gilliquet and Berben, 1993; Lee et al., 2000; Nishizawa et al., 1999; Popova Iu et al., 2000). As only some of these growth phenotypes were reproduced by a *pho80Δ* strain, the genetic background used may affect these phenotypes and in addition other Pho85 cyclins may control Pho4 activity under some nutritional conditions. In general, most transcription factors are present in very low levels (Ghaemmaghami et al., 2003), and hyperactivation of transcription factors leads to a growth defect in several occasions (Sopko et al., 2006). For Pho4, however, it remains to be determined how this activation triggers an inhibiting effect on yeast growth.

Chapter IV

Concluding discussion and perspectives

1. Integration of nutrient signalling pathways in yeast

In recent years, the general understanding of nutrient sensing and signalling, as well as the knowledge about responses triggered by altered nutrient availability, have greatly advanced. While initial studies were directed to top-to-bottom elucidation of single nutrient signalling pathways, recent investigations place the individual signalling pathways into signalling networks and pursue the identification of converging effector branches that orchestrate the dynamical responses to nutritional cues. The budding yeast, *Saccharomyces cerevisiae*, has proven to be an important model organism for studies related to nutrient-signalling events and numerous nutrient signalling pathways have been described allowing the yeast cell to sense and respond to the presence of various nutrients (Roosen et al., 2004; Wilson and Roach, 2002).

When extracellular Pi becomes limiting, yeast activates a signalling pathway known as the PHO pathway which ultimately results in the activation of the Pho4 transcription factor (Persson et al., 2003). Pho4, in turn, induces transcription of genes involved in the acquisition, high-affinity uptake and storage of Pi. When all available Pi-sources are exhausted, cellular growth ceases and yeast cells enters the stationary phase (G_0) due to Pi starvation. Entry into G_0 constitutes a general response to nutrient depletion and is accompanied by many transcriptional and metabolic rearrangements directed to the optimal survival during the starvation period (Winderickx et al., 2003). Typical G_0-characteristics include the accumulation of the reserve carbohydrates trehalose and glycogen and the transcriptional induction of stress responsive genes. All these phenotypes are under strict control of the protein kinase PKA and are often annotated as “PKA targets”.

The PHO pathway regulates typical G_0-characteristics in response to phosphate

In this study we examined the role of the PHO pathway in the regulation of typical PKA targets in response to Pi-availability. Our results demonstrate a clear involvement of the PHO pathway in regulating trehalose metabolism in response to Pi levels as well as the transcription of PDS-element controlled genes.

Detailed analysis of the functions of each component of the PHO pathway in the regulation of PKA targets revealed that:

- During Pi starvation, Pho81 mediated inhibition of Pho85 kinase activity is required for proper accumulation of trehalose and expression of PDS-driven genes, whereas an active Pho85 kinase is required for the rapid response upon re-addition of Pi to the starved cells.
- While the expression of PDS-driven genes is solely regulated by the Pho85-Pho80 kinase, proper control of trehalose metabolism requires additional Pho85-Pcl kinases which are involved in the posttranscriptional control of the activity of the trehalase enzyme. These additional Pho85-Pcl kinases are also subject to Pho81-mediated control.
- The Pho4 transcription factor participates in the control of these PKA properties solely through its positive action on Pho81 function. It does not directly activate the transcription of PDS-driven genes.
- The previously defined minimum domain of Pho81, sufficient for the Pi-dependent control of Pho4 activity, is insufficient in regulating both trehalose metabolism and expression of PDS-driven genes in response to Pi levels.

This study therefore expands the role of the PHO pathway in mediating the response to Pi starvation, revealing new physiological targets regulated by this signalling pathway. Our results also highlight the function of the positive feedback loop between Pho4 and Pho81 as this feedback loop is not only required for continued activation of Pho4 (Ogawa et al., 1995) but is also crucial for Pho81-mediated control of the PKA targets described. In addition, a physiological function is provided for the previously observed extra-nuclear localization of Pho81 (Huang et al., 2001) as we demonstrate that this pool of Pho81 is involved in the regulation of trehalase activity via the Pho85 CDK associated with cyclins other than Pho80.

As described in this study, the Pho81 protein plays a crucial role in regulating Pi signalling. In the absence of Pi, Pho81 inhibits multiple Pho85 kinase complexes, and this process must be quickly reversed when Pi becomes available again in order to allow optimal signalling. We studied the function of Pho81 in some detail using various truncated versions of Pho81. We could confirm that the previous defined

minimum domain of Pho81, comprising 80 amino acids C-terminal to the ankyrin repeats (Huang et al., 2001), is indeed sufficient to direct Pho4-dependent transcription of typical PHO genes, such as *PHO84* and *PHO89*, in response to Pi levels. However, we demonstrate that yeast cells expressing only the Pho81 minimum domain, or even the minimum domain along with the flanking ankyrin repeats, are deficient in the proper regulation of trehalose levels and the expression of PDS-driven genes. Therefore, caution has to be made when referring to the "minimum" domain of Pho81 as we show that this is highly dependent on the readout studied. Our results imply that additional or other domains within the Pho81 protein besides the ankyrin repeats and minimum domain are required for:

- regulation of cytoplasmic Pho81 involved in the regulation of trehalase activity. As this regulation involves Pho85 kinases other than Pho85-Pho80, diverse Pho81 domains may be involved in the regulation of different Pho85-Pcl kinases.
- controlling Rim15 function. Rim15 constitutes a second target of the Pho85-Pho80 kinase (as will be discussed below), though expression of the minimum domain is not sufficient to regulate Rim15 properties, indicating that Pho81 contains (a) domain(s) that allow to discriminate between two targets, Rim15 and Pho4, of the same kinase, namely Pho85-Pho80.

Rim15 constitutes an integration point for nutrient signalling

Regulation of G_0-entry is controlled by multiple nutrients, indicating that some signalling components may be shared between different nutrient-induced signalling pathways (Roosen et al., 2004). In this study we identified the protein kinase Rim15 as an essential component in the phosphate-induced signalling pathway, playing a dual role in the signalling process:

- Rim15 constitutes a downstream target of the Pho85-Pho80 mediating entry into G_0 in response to Pi starvation.
- Rim15 is involved in a signalling pathway modulating the activity of the PHO pathway in response to glucose.

We demonstrate that Rim15 is essential for the accumulation of trehalose and the expression of PDS-driven genes upon depletion of Pi and that Rim15 performs this function by operating downstream of the Pho85-Pho80 kinase. Independent studies established that Rim15 is a direct target of the Pho85-Pho80 kinase *in vivo*, whereby phosphorylation of Rim15 by Pho85-Pho80 promotes its cytoplasmic retention (Wanke et al., 2005). Therefore, Rim15 functions downstream of at least four nutrient-regulated protein kinases, i.e. PKA, TORC1, Sch9 and Pho85-Pho80, thereby controlling G_0 entry in response to starvation for different nutrients, including glucose, nitrogen sources and phosphate (Pedruzzi et al., 2003; Reinders et al., 1998; Roosen et al., 2005; Swinnen et al., 2005; Wanke et al., 2005).

In another aspect of our study we observed that PHO signalling in Pi-starved yeast cells is restrained upon glucose starvation, causing nuclear export of the phosphate-responsive transcription factor Pho4 depending on an active Pho85-Pho80 kinase. We are the first to demonstrate that glucose signalling is able to modulate the activity of Pho85-Pho80, either by directly acting on this kinase or impinging on its upstream regulator, the Pho81 CKI. Hence, in the presence of glucose, PHO signalling is allowed since Pi is the sole limiting nutrient and any Pi additionally taken up by the yeast cell permits further growth. Upon glucose starvation however, yeast cells must first adapt to this new nutrient stress and PHO signalling is blocked by inhibiting Pho4 activity. This system allows the yeast cell to conserve precious energy as Pho4 activation comprises an energetically expensive induction of a large battery of genes involved in high-affinity phosphate acquisition while additional Pi does not permit yeast growth when a carbon source is absent or the cell has not yet adjusted its metabolism to a carbon source of poorer quality.

Intriguingly, the glucose-dependent regulation of Pho4 depends on the Rim15 kinase. Thus, while Rim15 function is directly modulated by the Pho85-Pho80 kinase in response to Pi availability (Swinnen et al., 2005; Wanke et al., 2005), Rim15 reciprocally regulates the activity of the Pho85-Pho80 kinase in response to glucose. In addition, work in our laboratory implies that while Rim15 is a direct downstream target of PKA (Reinders et al., 1998), Rim15 is also involved in the feedback regulation of cAMP synthesis (Pardons, 2005), indicating the existence of a feedback loop wherein PKA-mediated control of Rim15 activity also leads to regulation of the activity of PKA itself. Thus, Rim15 also plays a central role in regulating the

activities of its own upstream signalling cascades allowing feedback control mechanisms and cross talk between different signalling pathways, thereby further fine-tuning of the yeasts response to the nutrient environment.

Rapid phosphate signalling is mediated via the Pho84 high-affinity or Pho87 low-affinity phosphate transporter depending on the activity of the PHO pathway

One of the functions of the PHO pathway is to provide the yeast cell with a high-affinity phosphate uptake system and Pho84 is regarded as the major Pi-uptake system in Pi starved yeast cells (Persson et al., 2003). In our study, we show that the Pho84 high-affinity Pi transporter is essential for Pi-induced signalling in wild type yeast cells. However, we demonstrate that in the absence of the Pho4 transcription factor, rapid Pi signalling is mediated specifically by the Pho87 low-affinity Pi transporter. In addition, regulation of Pho87 function occurs at a posttranscriptional level, indicating that Pho4 controls the expression of a gene which is involved in the inhibition of Pho87 function.

Thus, the yeast cell seems equipped with multiple phosphate sensing systems operating under different physiological circumstances. We postulate that a model wherein the high-affinity transport/sensing system Pho84 is induced when Pi is the sole limiting nutrient, while under conditions of simultaneous glucose and Pi limitation, Pho4 is inactivated to conserve energy but the yeast cell is still able to detect the presence of extracellular phosphate via the Pho87 low-affinity phosphate transporter.

2. In search for the phosphate signal

Although many physiological responses to phosphate availability are shared among the biological kingdoms, the actual sensing event for phosphate must still be defined (Silver and Dranitzki-Elhalel, 2003). Research in yeast, predominantly focussed on the PHO pathway, has stated that phosphate is sensed primarily inside the cell (Mouillon and Persson, 2006). The use of metabolically inert phosphate analogues indicate that Pi itself may act as a signal (McDonald et al., 2001; Pratt et al., 2004). However, other Pi-derived metabolites have been implicated in directing PHO signalling. Polyphosphates may contribute to phosphate signalling by buffering cytoplasmic Pi concentrations (Auesukaree et al., 2004; Thomas and O'Shea E, 2005). Furthermore, inositol (poly)phosphate and adenine nucleotide metabolism contribute to the regulation of the PHO pathway independent of intracellular Pi (Auesukaree et al., 2005; El Alami et al., 2003; Huang and O'Shea, 2005; Steger et al., 2003).

Phosphate transporters play a crucial role in maintaining cytoplasmic Pi homeostasis. Altering the Pi transport capacity can therefore have pronounced impacts on phosphate signalling by affecting intracellular concentrations of Pi and its metabolites. In addition, evidence is growing that nutrient transporters themselves may also directly contribute to nutrient signalling by stimulating downstream signalling cascades upon the transport process (Holsbeeks et al., 2004). The low-affinity phosphate transporters have already been implicated in the regulation of the PHO pathway independent of intracellular Pi concentrations (Pinson et al., 2004). In our work concerning the involvement the Pho84 and Pho87 phosphate transporters in rapid signalling, we could see a strict correlation between the absence of signalling (e.g. Pi-induced trehalase activation) and rapid Pi-consumption. Although this favours the idea of intracellular Pi sensing, contributions of transporter-derived signals cannot be excluded as discussed in chapter III. Finally, considering the sensing of extracellular phosphate, it is worth mentioning that to date no reports have been made about the existence of membrane proteins functioning solely as phosphate receptors, favouring the idea that the sensing of extracellular phosphate is mediated by transport proteins.

3. Phosphate signalling in higher eukaryotes

Mechanisms involved in phosphate signalling in many eukaryotic organisms are still elusive in contrast to the well-characterized phosphate signalling pathway in *Saccharomyces cerevisiae*. Due to economic or clinical motives, large efforts are being performed to elucidate phosphate signalling mechanisms in plants and mammals. We will briefly discuss these topics and discuss the potential value of studying phosphate signalling in yeast.

Phosphate signalling in plants

The concentration of inorganic phosphate in the soil solution is often low (2 to 10 μM) and limiting for plant growth. Plants respond to Pi limitation via alterations in root morphology to increase soil surface exploration, changes in metabolism and physiology to conserve precious P and the induction of systems which increase Pi acquisition (Hammond et al., 2004; Plaxton, 2004; Ticconi and Abel, 2004). The latter part includes a transcriptional induction of high-affinity Pi-transporters and secretion of acid phosphatases, similar to the situation seen in yeast. Several plant hormones such as auxins, ethylene and cytokinins, have been implicated in controlling the plants response to Pi deprivation, creating a complex system composed of local Pi sensing coupled to systemic responses. In addition, some plants are capable to form symbiotic associations with arbuscular mycorrhizal (AM) fungi, in which the fungus aids in phosphate delivery to the plant while receiving carbon (Karandashov and Bucher, 2005; Requena, 2005).

Because soil Pi often restrains plant growth, crops are supplied with inorganic P fertilizers. However, the non-renewable nature of inorganic P fertilizers means that cheap sources of P, such as phosphate rocks, will be exhausted within the next century (Hammond et al., 2004). In addition, excess P added to crops can pollute local watercourses, contributing to the process of eutrophication. Therefore, P fertilization should be minimized and a major goal of research on plant phosphate signalling is the development of crops which can use P-sources more efficiently and to create more precise methods for monitoring the crop P-status allowing for a more efficient P-fertilization.

Phosphate signalling in mammals

For mammalian organisms Pi is sufficiently abundant in the normal diet and Pi deficiency is unlikely to develop except under conditions of extreme starvation. Still, regulation of Pi levels in plasma is crucial as harmful effects can result from both low and high levels of plasma Pi, including defects in bone formation, soft tissue calcification, defects in development and body weight disorders (Jin et al., 2006; Takeda et al., 2004a; Takeda et al., 2004b). Most of the Pi taken up in the diet is excreted in the urine and Pi homeostasis and plasma Pi concentration primarily depend on the kidney by virtue of its ability to increase or decrease its Pi reabsorptive capacity to accommodate Pi need (Tenenhouse, 2005). The majority of Pi reabsorption is performed by the type IIa Na^+/Pi transporter (Npt2a) expressed in the renal proximal tubule. Npt2a function is largely regulated at the posttranscriptional level via membrane insertion or retrieval/lysosomal degradation of the protein. High serum Pi stimulates parathyroid (PT) cell proliferation and PT hormone (PTH) secretion (Silver, 2001). PTH in turn promotes renal Npt2a degradation via PKA-, PKC- and ERK/MAPK-dependent signalling pathways, thereby encouraging renal Pi wasting and lowering plasma Pi levels (Tenenhouse, 2005). In addition to PTH-mediated regulation of Npt2a function, bone formation comprises another major system affecting Pi homeostasis. Pi is essential with calcium for the mineralization of bone matrix through the formation of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] and evidence suggest that osteoblast cells produce circulating factors, such as the FGF-23 hormone, which regulate renal Pi reabsorption by controlling Npt2a function (Dunstan et al., 2004; Quarles, 2003). Thus, regulation of serum Pi homeostasis is a complex process, controlled by multiple cell types and involving numerous circulating factors.

Although the clinical and physiological consequences of altered Pi homeostasis are well described, it remains unknown how Pi levels are sensed on a cellular level and which molecular mechanisms connect this event to the regulation of hormone production. Further research using micro-array and proteomic analysis may identify new components in mammalian Pi signalling (Conrads et al., 2005). In addition, a role of the Npt2a transporter in phosphate signalling is plausible. Interestingly, in addition to the renal adaptation, rats rapidly develop Pi ingestive behaviour and will seek out a source of Pi when the dietary source is low

(Czarnogorski et al., 2004; Sweeny et al., 1998). Npt2a-like transporters have been characterized in rat brains and they are regulated by both dietary Pi and cerebrospinal fluid (CSF) Pi concentrations (Mulroney et al., 2004). Moreover, the CSF Pi milieu can regulate renal Npt2a function indicating that brain Npt2a transporters may act as Pi sensors that help regulate both brain and whole body Pi homeostasis. A number of Npt2a interacting partners have been identified, which function in regulating the localization and stability of the transporter (Biber et al., 2004; Hernando et al., 2005). Conversely, these interacting partners might also generate a phosphate signal to downstream components upon the Npt2a transport process. Further characterization of Npt2a interacting proteins may provide mechanisms by which a phosphate transporter may interact with downstream signalling cascades.

Yeast as a model system for Pi signalling in higher eukaryotes?

One of the main objectives of studying signalling pathways in yeast is to create a model which may help elucidate similar signalling processes in higher eukaryotes. In the most straightforward situation, the signalling pathway is composed of components which are highly homologues between the organisms. A classical example is the regulation of the cell cycle via CDKs, wherein studies performed in yeast have been of great value for the study of cell cycle regulation in higher eukaryotes (Mendenhall and Hodge, 1998). A more recent example comprises the identification of upstream kinases of the mammalian AMPK kinase, based on the homology of identified upstream kinases of Snf1, the yeast orthologue of mammalian AMPK (Hong et al., 2003; Hong et al., 2005; Woods et al., 2005).

In contrast to the abovementioned examples, little homology has been described between the yeast PHO pathway and Pi signalling components identified in higher eukaryotes. No homologues of the yeast Pho4 transcription factor have been found in plants, implicating other transcription factors and pathways in the response to Pi-deficiency (Franco-Zorrilla et al., 2004). In addition, the mammalian Npt2a does not display any significant similarity with yeast phosphate transporters and Cdk5, the mammalian Pho85 homologue, has not been implicated in regulating Pi homeostasis. Finally, for both plants and mammals no functional Rim15 homologues have been described. These findings make it unlikely that the core of the yeast PHO pathway, i.e.

the Pho81, Pho85-Pho80 and Pho4 proteins, is conserved in Pi signalling pathways operating in plants and mammals and the value of the yeast PHO pathway as a model of Pi signalling may therefore be rather limited.

Still, the study of Pi signalling in yeast may provide important insights into the following topics:

- The nature of the phosphate signal and the mode of detection. Furthermore, as Pho81 is unlikely to directly receive the Pi signal, upstream regulators have to be identified, which may be conserved in higher eukaryotes.
- Possible direct involvement of Pi transporters in the signalling process. In both plants and AM fungi, the high-affinity phosphate transporters show significant homology to the yeast Pho84 transporter, indicating that common regulation mechanisms for this class of Pi-transporters may exist (Maldonado-Mendoza et al., 2001; Rausch and Bucher, 2002; Shin et al., 2004). Consequently, detailed analysis of Pho84 function in yeast may be useful for research performed on plant Pi transporters.
- The connection of Pi signalling with other nutrient-induced signalling pathways. As the knowledge about nutrient-induced signalling pathways increases, it becomes clear that these signalling pathways are integrated into complex networks, wherein information concerning numerous nutrients is integrated in order to produce an optimal response. Pi signalling will be no exception to this phenomenon and studies in yeast may give principal modes of interactions between Pi and other nutrient-induced signalling pathways. As an example, phosphate signalling in both plants and AM fungi has been shown to be regulated by carbon source availability in a manner which is intriguingly similar to the one we described in chapter III (Bucking and Shachar-Hill, 2005; Liu et al., 2005).

Given the fact that CDKs and their CKIs perform many crucial functions in eukaryotic cells, study of the regulatory mechanisms controlling the activity of the PHO pathway can also provide important new insights into the regulation mechanism of a CKI-CDK-cyclin module. Specifically, molecular analysis of the Pho81 CKI may lead to the identification of novel mechanisms involved in the regulation of CDK activity.

Chapter V

Materials and methods

Yeast strains and plasmids

Saccharomyces cerevisiae strains used in this study are listed in Table 1 and plasmids are listed in Table 2. Deletions were made using polymerase chain reaction-based disruption cassettes as described previously (Brachmann et al., 1998). Yeast was transformed with the Gietz method (Gietz et al., 1995). Sporulation was performed by spotting diploid cells on plates containing 1% K-acetate, 0.1% $KHCO_3$, pH 6.0 for 5 to 6 days at 24°C. Tetrads were dissected and separated in a raster using a micromanipulator (Singer Instruments).

Chromosomally *yEGFP3*-tagged *PHO84* was created by transforming a PCR cassette using plasmid pUG23 as template (Fig. V.1). Transformants selected on SD-HIS were verified for correct integration of the cassette at the *PHO84* locus and checked for fluorescence. A similar strategy was employed to generate chromosomally *yEGFP3*-tagged *PHO87* and *PHO90*. Although many transformants with correct integration were obtained, none of them gave a visible fluorescence, most probably because the Pho87 and Pho90 proteins are expressed at low levels (Ghaemmaghami et al., 2003) and are not transcriptionally induced by phosphate starvation (Auesukaree et al., 2003). The primers used for the creation of chromosomally *yEGFP3*-tagged *PHO84* are listed in Table 3.

All experiments were carried out with isogenic wild-type and mutant strains.

Table 1: List of *Saccharomyces cerevisiae* strains used in this study.

All mutant strains have *his3Δ1*, *leu2Δ0*, *ura3Δ0* and *met15Δ0* auxotrophic markers and are Mat**a**, except when indicated otherwise.

Strain	Genotype	Reference
wild-type (BY4741)	Mat**a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Y.K.O. collection
pho4Δ	*pho4::KANMX4*	Y.K.O. collection
pho80Δ	*pho80::HIS3*	This study
pho81Δ	*pho81::KANMX4*	Y.K.O. collection
pho85Δ	*pho85::KANMX4*	Y.K.O. collection
rim15Δ	*rim15::KANMX4*	Y.K.O. collection
sch9Δ	*sch9::HIS3*	This study
pho85Δ Matα	Matα *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 pho85::KANMX4*	Y.K.O. collection

pho81Δ pho85Δ	*pho81::KANMX4 pho85::KANMX4*	This study
pho81Δ pho80Δ	*pho81::KANMX4 pho80::HIS3*	This study
pho85Δ rim15Δ	*pho85::KANMX4 rim15::KANMX4*	This study
pho80Δ rim15Δ	*pho80::HIS3 rim15::KANMX4*	This study
pho85Δ pho4Δ	*pho85::KANMX4 pho4::KANMX4*	This study
pho80Δ pho4Δ	*pho80::HIS3 pho4::KANMX4*	This study
pho84Δ	*pho84::KANMX4*	Y.K.O. collection
pho87Δ	*pho87::KANMX4*	Y.K.O. collection
pho90Δ	*pho90::KANMX4*	Y.K.O. collection
pho91Δ	*pho91::KANMX4*	Y.K.O. collection
pho87Δ pho90Δ pho91Δ	*pho87::KANMX4 pho90::KANMX4 pho91::KANMX4*	This study
pho4Δ pho84Δ	Matα *pho4::KANMX4 pho84::KANMX4*	This study
pho4Δ pho87Δ	*pho4::KANMX4 pho87::KANMX4*	This study
pho4Δ pho90Δ	*pho4::KANMX4 pho90::KANMX4*	This study
pho4Δ pho91Δ	*pho4::KANMX4 pho91::KANMX4*	This study
pho4Δ pho84Δ pho87Δ	*pho4::KANMX4 pho84::KANMX4 pho87::KANMX4*	This study
pho84Δ pho87Δ	*pho84::KANMX4 pho87::KANMX4*	This study
pho84Δ pho89Δ	*pho84::KANMX4 pho89::LEU2*	This study
pho84Δ pho89Δ pho90Δ	*pho84::KANMX4 pho89::LEU2 pho90::KANMX4*	This study
PHO84-GFP	Matα, chromosomal *PHO84-yEGFP3-HIS3*	This study
pho87Δ PHO84-GFP	*pho87::KANMX4* chromosomal *PHO84-yEGFP3-HIS3*	This study

Table 2: List of plasmids used in this study.

Plasmid	**Insert**	**Auxotrophic marker**	**Reference**
EB0730	p*ADH1*pr-Py_2	*URA3*	(Huang et al., 2001)
EB1361	p*ADH1*pr-*PHO81*-Py_2	*URA3*	(Huang et al., 2001)
EB1362	p*ADH1*pr-*PHO81*(aa 400-724)-Py_2	*URA3*	(Huang et al., 2001)
EB1363	p*ADH1*pr-*PHO81*(aa 400-660)-Py_2	*URA3*	(Huang et al., 2001)
EB1364	(p*ADH1*pr-*PHO81*(aa 645-724)-Py_2	*URA3*	(Huang et al., 2001)
EB0347	p*PHO4*pr-*PHO4*-GFP	*URA3*	(Kaffman et al., 1998)
EB0252 (pRS426)	/	*URA3*	(Wykoff and O'Shea, 2001)
EB1368	p*ADH1*pr-*PHO84*	*URA3*	(Wykoff and O'Shea, 2001)
EB1247	p*ADH1*pr-*PHO87*	*URA3*	(Wykoff and O'Shea, 2001)
pUG23	*yEGFP3*	*HIS3*	http://mips.gsf.de/proj/yeast/info/tools/hegemann/gfp.html

Table 3: Primers used for construction of *yEGFP3-PHO84*.

Sequences in italic correspond to the *yEGFP3* starting sequence (4403*) and upstream of the *HIS3* gene (4404*) in the pUG23 plasmid. Underlined sequences are the C-terminus of *PHO84* (without stop codon, 4403*) and the terminator sequence of *PHO84* (4404*). See Fig V.1.

Primer	Sequence
4403*	CCTGCTACGCTAAACTTCAGAAACAAGAATAATGACATTGAATCTTCCAGCCCATCTCAACTT CAACATGAAGCA*ATGTCTAAAGGTGAAGAATTATTC*
4404*	GGGGTTTGATAATAAGTTTATAAAAATGTTTTTGTATTATTTGTTCTAGTTTACAAGTTTTAGT GCATCTTTGAGGC*CTCTTGGCCTCCTCTAGTAC*

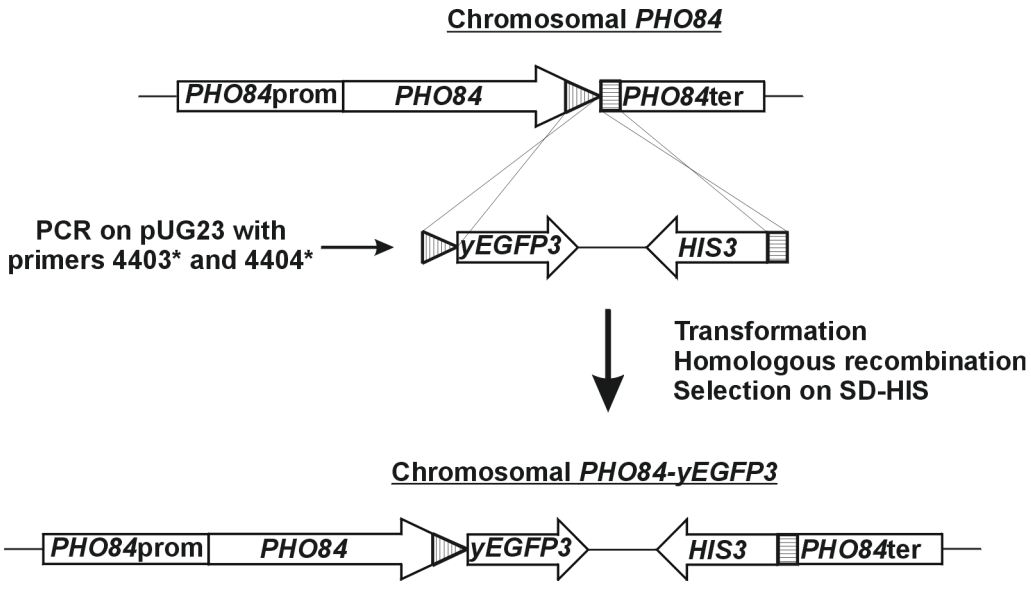


Fig. V.1: Construction of chromosomally tagged *PHO84*. Yeast was transformed with a PCR cassette containing the C-terminal sequence of *PHO84* (without the stop codon, vertical bars), the *yEGFP3* sequence together with the *HIS3* marker (derived from plasmid pUG23) and finally the sequence corresponding to the *PHO84* terminator (horizontal bars). Via homologous recombination a fusion is constructed between the *PHO84* gene and the *yEGFP3* sequence at the chromosomal locus.

Culture and starvation conditions

Yeast cells were grown on rich medium (YP) containing 1% w/v yeast extract and 2% w/v Bactopeptone, supplemented with a carbon source: 2% w/v glucose (YPD) or 3% w/v glycerol (YPGly). Cells containing plasmids were grown on minimal medium

containing 1.7 g/l yeast nitrogen base w/o amino acids supplemented with synthetic drop-out amino acid/nucleotide mixture as required, and with 2% w/v glucose (SD). Solid media contained 1.5 % w/v Bacto-agar in addition. For selection of kanamycin resistant mutants, geneticin was added to a final concentration of 100 µg/ml. For phosphate starvation experiments, yeast cells were grown into mid-exponential phase (OD_{600} = 1.5-2) on either YPD or SD medium. Mid-exponential cells were harvested and transferred to phosphate starvation medium (5.7 g l^{-1} of yeast nitrogen base without phosphate) with 4% glucose and required amino acid/nucleotide supplements. Cells were starved for phosphate for 3 days under continuous shaking, and starvation medium was refreshed daily. For growth monitoring, mid-exponential cells were diluted to an OD_{600} of 0.1 in phosphate starvation medium and grown for 3 days without refreshing. All incubations were carried out at 30°C. For experiments involving the response to phosphate re-addition, the phosphate-starved, glucose-repressed cells were rapidly cooled on ice and harvested by centrifugation. The pellet was washed twice with ice-cold Mes-buffer pH 6.0, resuspended in fresh phosphate starvation medium with 4% glucose and incubated at 30°C with shaking. After 30 min of incubation, 10 mM KH_2PO_4 was added to the culture and samples were taken at the indicated time points.

Determination of trehalose and glycogen levels

Samples were collected by filtration at the indicated time-points, washed once with ice-cold water, weighed ("wet weight") and frozen in liquid nitrogen. The pellets were resuspended in 1 ml of 0.25 M Na_2CO_3 per 40 mg of cells and boiled at 95°C for 60 min. Samples were neutralized and intracellular levels of trehalose were determined using *Humicola* trehalase (Colombo et al., 1998; Neves, 1994). Glycogen levels were determined using *Aspergillus niger* amyloglucosidase (Boehringer Mannheim). Glucose concentrations were measured using the GOD-PAP kit (Dialab). Trehalose and glycogen levels are expressed as % of wet weight.

Sampling, extraction and determination of trehalase activity

Samples of 50 mg cells ml^{-1} were taken at the indicated time-points. Cells were rapidly cooled by addition of ice-cold water, centrifuged and resuspended in 500 µl of

25 mM MES buffer, 50 μM $CaCl_2$, pH 7 for extraction. Crude cell extracts were prepared as described previously (Giots et al., 2003) and dialyzed (BRL microdialysis system) against 25 mM MES buffer pH 7 with 50 μM $CaCl_2$ at 4°C. Trehalase activity in dialyzed cell extracts was determined as described previously (Giots et al., 2003). The specific activity is expressed as nmol glucose liberated min^{-1}mg $protein^{-1}$. Total amount of protein in the samples was determined using a standard method (Lowry et al., 1951).

Phosphate-consumption assay

After addition of 1 mM KH_2PO_4 to Pi-starved cells, samples (e.g. 1 ml) were taken and cells were rapidly pelleted at 4°C. Phosphate levels in the supernatans were determined with the Fiske-Subbarow colorimetric method for the determination of inorganic phosphate (Fiske and Subbarow, 1925).

RNA extraction and Northern blot analysis

Samples of were taken at the indicated time-points and rapidly cooled by addition of ice-cold water. The cells were pelleted and washed once with ice-cold water and stored at -80°C. Isolation of total RNA was performed as described previously (Giots et al., 2003). Probes were labeled with α-^{32}P-dCTP using the High Prime kit (Boehringer Mannheim). Northern blots were made by separation of total RNA on gels containing 1% (w/v) agarose in 50 mM boric acid, 1 mM sodium citrate, 5 mM NaOH pH 7.5 and 1% formaldehyde. Subsequently, RNA was transferred by capillary blotting to a Hybond-N membrane (Amersham) using 10× SCC buffer (1.5 M NaCl, 0.15 M Na-citrate, pH 7.0). The filters were hybridized with the ^{32}P-labelled probes consisting of fragments of the desired coding region. The blots were analyzed using PhosphorImager technology (FUJIX, BAS-1000). Quantification of Northern blot images was performed with TINA 2.0.

GFP-fluorescence microscopy

Cells were used directly without fixation. Cells were viewed using a Zeiss Axioplan 2 fluorescence microscope. Pictures were taken with a Quantix charge-coupled device camera using IP LAB software.

Reproducibility of the results

All experiments were performed at least three times. For trehalose and glycogen levels, mean values for three independent experiments are shown, with error bars representing the corresponding standard deviations (SD). For growth curves, trehalase and Pi-consumption experiments, representative figures are shown. The absolute values are somewhat variable between different experiments, but the relative differences observed between separate experiments and strains are highly reproducible (see (Swinnen et al., 2005)). For Northern blot experiments, representative blots from three independent experiments are shown. Where indicated, quantifications are shown with error bars representing SD calculated from three independent experiments.

References

Abele, U., and Schulz, G. E. (1995). High-resolution structures of adenylate kinase from yeast ligated with inhibitor Ap5A, showing the pathway of phosphoryl transfer. Protein Sci *4*, 1262-1271.

Adams, J., Chen, Z. P., Van Denderen, B. J., Morton, C. J., Parker, M. W., Witters, L. A., Stapleton, D., and Kemp, B. E. (2004). Intrasteric control of AMPK via the gamma1 subunit AMP allosteric regulatory site. Protein Sci *13*, 155-165.

Aerne, B. L., Johnson, A. L., Toyn, J. H., and Johnston, L. H. (1998). Swi5 controls a novel wave of cyclin synthesis in late mitosis. Mol Biol Cell *9*, 945-956.

Almaguer, C., Cheng, W., Nolder, C., and Patton-Vogt, J. (2004). Glycerophosphoinositol, a novel phosphate source whose transport is regulated by multiple factors in Saccharomyces cerevisiae. J Biol Chem *279*, 31937-31942.

Almaguer, C., Mantella, D., Perez, E., and Patton-Vogt, J. (2003). Inositol and phosphate regulate GIT1 transcription and glycerophosphoinositol incorporation in Saccharomyces cerevisiae. Eukaryot Cell *2*, 729-736.

Amezcua, C. A., Harper, S. M., Rutter, J., and Gardner, K. H. (2002). Structure and interactions of PAS kinase N-terminal PAS domain: model for intramolecular kinase regulation. Structure *10*, 1349-1361.

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

App, H., and Holzer, H. (1989). Purification and characterization of neutral trehalase from the yeast ABYS1 mutant. J Biol Chem *264*, 17583-17588.

Arguelles, J. C., Mbonyi, K., Van Aelst, L., Vanhalewyn, M., Jans, A. W., and Thevelein, J. M. (1990). Absence of glucose-induced cAMP signaling in the Saccharomyces cerevisiae mutants cat1 and cat3 which are deficient in derepression of glucose-repressible proteins. Arch Microbiol *154*, 199-205.

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

Auesukaree, C., Homma, T., Kaneko, Y., and Harashima, S. (2003). Transcriptional regulation of phosphate-responsive genes in low-affinity phosphate-transporter-defective mutants in Saccharomyces cerevisiae. Biochem Biophys Res Commun *306*, 843-850.

Auesukaree, C., Homma, T., Tochio, H., Shirakawa, M., Kaneko, Y., and Harashima, S. (2004). Intracellular phosphate serves as a signal for the regulation of the PHO pathway in Saccharomyces cerevisiae. J Biol Chem *279*, 17289-17294.

Auesukaree, C., Tochio, H., Shirakawa, M., Kaneko, Y., and Harashima, S. (2005). Plc1p, Arg82p, and Kcs1p, enzymes involved in inositol pyrophosphate synthesis, are essential for phosphate regulation and polyphosphate accumulation in Saccharomyces cerevisiae. J Biol Chem *280*, 25127-25133.

Baetz, K., Moffat, J., Haynes, J., Chang, M., and Andrews, B. (2001). Transcriptional coregulation by the cell integrity mitogen-activated protein kinase Slt2 and the cell cycle regulator Swi4. Mol Cell Biol *21*, 6515-6528.

Banuelos, M., Gancedo, C., and Gancedo, J. M. (1977). Activation by phosphate of yeast phosphofructokinase. J Biol Chem *252*, 6394-6398.

Barbaric, S., Munsterkotter, M., Goding, C., and Horz, W. (1998). Cooperative Pho2-Pho4 interactions at the PHO5 promoter are critical for binding of Pho4 to UASp1 and for efficient transactivation by Pho4 at UASp2. Mol Cell Biol *18*, 2629-2639.

Barbaric, S., Munsterkotter, M., Svaren, J., and Horz, W. (1996). The homeodomain protein Pho2 and the basic-helix-loop-helix protein Pho4 bind DNA cooperatively at the yeast PHO5 promoter. Nucleic Acids Res *24*, 4479-4486.

Barz, T., Ackermann, K., and Pyerin, W. (2003). Perturbation of protein kinase CK2 uncouples executive part of phosphate maintenance pathway from cyclin-CDK control(1). FEBS Lett *537*, 210-214.

Bateman, A. (1997). The structure of a domain common to archaebacteria and the homocystinuria disease protein. Trends Biochem Sci *22*, 12-13.

Bauer, F., Urdaci, M., Aigle, M., and Crouzet, M. (1993). Alteration of a yeast SH3 protein leads to conditional viability with defects in cytoskeletal and budding patterns. Mol Cell Biol *13*, 5070-5084.

Beauvoit, B., Rigoulet, M., Guerin, B., and Canioni, P. (1989). Polyphosphates as a source of high energy phosphates in yeast mitochondria: A ^{31}P NMR study. FEBS Lett *252*, 17-21.

Beeler, T., Bruce, K., and Dunn, T. (1997). Regulation of cellular Mg2+ by Saccharomyces cerevisiae. Biochim Biophys Acta *1323*, 310-318.

Bell, W., Sun, W., Hohmann, S., Wera, S., Reinders, A., De Virgilio, C., Wiemken, A., and Thevelein, J. M. (1998). Composition and functional analysis of the Saccharomyces cerevisiae trehalose synthase complex. J Biol Chem *273*, 33311-33319.

Berben, G., Legrain, M., Gilliquet, V., and Hilger, F. (1990). The yeast regulatory gene PHO4 encodes a helix-loop-helix motif. Yeast *6*, 451-454.

Berben, G., Legrain, M., and Hilger, F. (1988). Studies on the structure, expression and function of the yeast regulatory gene PHO2. Gene *66*, 307-312.

Bergsma, J. C., Kasri, N. N., Donaton, M. C., De Wever, V., Tisi, R., de Winde, J. H., Martegani, E., Thevelein, J. M., and Wera, S. (2001). PtdIns(4,5)P(2) and phospholipase C-independent Ins(1,4,5)P(3) signals induced by a nitrogen source in nitrogen-starved yeast cells. Biochem J *359*, 517-523.

Berhe, A., Fristedt, U., and Persson, B. L. (1995). Expression and purification of the high-affinity phosphate transporter of Saccharomyces cerevisiae. Eur J Biochem *227*, 566-572.

Best, L., and McLaughlin, J. (2004). Nutrients as regulators of endocrine and neuroendocrine secretion. In Nutrient-Induced Responses in Eukaryotic Cells, J. Winderickx, and P. M. Taylor, eds. (Topics in Current Genetics), pp. 79-112.

Beullens, M., Mbonyi, K., Geerts, L., Gladines, D., Detremerie, K., Jans, A. W., and Thevelein, J. M. (1988). Studies on the mechanism of the glucose-induced cAMP signal in glycolysis and glucose repression mutants of the yeast Saccharomyces cerevisiae. Eur J Biochem *172*, 227-231.

Biber, J., Gisler, S. M., Hernando, N., Wagner, C. A., and Murer, H. (2004). PDZ interactions and proximal tubular phosphate reabsorption. Am J Physiol Renal Physiol *287*, F871-875.

Blazquez, M. A., Lagunas, R., Gancedo, C., and Gancedo, J. M. (1993). Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS Lett *329*, 51-54.

Blomberg, A. (2000). Metabolic surprises in Saccharomyces cerevisiae during adaptation to saline conditions: questions, some answers and a model. FEMS Microbiol Lett *182*, 1-8.

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.

Borst-Pauwels, G. W. (1981). Ion transport in yeast. Biochim Biophys Acta *650*, 88-127.

Bostian, K. A., Lemire, J. M., Cannon, L. E., and Halvorson, H. O. (1980). In vitro synthesis of repressible yeast acid phosphatase: identification of multiple mRNAs and products. Proc Natl Acad Sci U S A *77*, 4504-4508.

Bostian, K. A., Lemire, J. M., and Halvorson, H. O. (1983). Physiological control of repressible acid phosphatase gene transcripts in Saccharomyces cerevisiae. Mol Cell Biol *3*, 839-853.

Bottger, P., and Pedersen, L. (2005). Evolutionary and experimental analyses of inorganic phosphate transporter PiT family reveals two related signature sequences harboring highly conserved aspartic acids critical for sodium-dependent phosphate transport function of human PiT2. Febs J *272*, 3060-3074.

Boustany, L. M., and Cyert, M. S. (2002). Calcineurin-dependent regulation of Crz1p nuclear export requires Msn5p and a conserved calcineurin docking site. Genes Dev *16*, 608-619.

Boy-Marcotte, E., Perrot, M., Bussereau, F., Boucherie, H., and Jacquet, M. (1998). Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in Saccharomyces cerevisiae. J Bacteriol *180*, 1044-1052.

Boy-Marcotte, E., Tadi, D., Perrot, M., Boucherie, H., and Jacquet, M. (1996). High cAMP levels antagonize the reprogramming of gene expression that occurs at the diauxic shift in Saccharomyces cerevisiae. Microbiology *142 (Pt 3)*, 459-467.

Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115-132.

Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S., and Wigler, M. (1987). The S. cerevisiae CDC25 gene product regulates the RAS/adenylate cyclase pathway. Cell *48*, 789-799.

Bucking, H., and Shachar-Hill, Y. (2005). Phosphate uptake, transport and transfer by the arbuscular mycorrhizal fungus Glomus intraradices is stimulated by increased carbohydrate availability. New Phytol *165*, 899-911.

Bun-Ya, M., Harashima, S., and Oshima, Y. (1992). Putative GTP-binding protein, Gtr1, associated with the function of the Pho84 inorganic phosphate transporter in Saccharomyces cerevisiae. Mol Cell Biol *12*, 2958-2966.

Bun-Ya, M., Nishimura, M., Harashima, S., and Oshima, Y. (1991). The PHO84 gene of Saccharomyces cerevisiae encodes an inorganic phosphate transporter. Mol Cell Biol *11*, 3229-3238.

Bun-ya, M., Shikata, K., Nakade, S., Yompakdee, C., Harashima, S., and Oshima, Y. (1996). Two new genes, PHO86 and PHO87, involved in inorganic phosphate uptake in Saccharomyces cerevisiae. Curr Genet *29*, 344-351.

Burglin, T. R. (1988). The yeast regulatory gene PHO2 encodes a homeo box. Cell *53*, 339-340.

Cameroni, E., Hulo, N., Roosen, J., Winderickx, J., and De Virgilio, C. (2004). The novel yeast PAS kinase Rim 15 orchestrates G0-associated antioxidant defense mechanisms. Cell Cycle *3*, 462-468.

Camonis, J. H., and Jacquet, M. (1988). A new RAS mutation that suppresses the CDC25 gene requirement for growth of Saccharomyces cerevisiae. Mol Cell Biol *8*, 2980-2983.

Camus, C., Boy-Marcotte, E., and Jacquet, M. (1994). Two subclasses of guanine exchange factor (GEF) domains revealed by comparison of activities of chimeric genes constructed from CDC25, SDC25 and BUD5 in Saccharomyces cerevisiae. Mol Gen Genet *245*, 167-176.

Cannon, J. F., Pringle, J. R., Fiechter, A., and Khalil, M. (1994). Characterization of glycogen-deficient glc mutants of Saccharomyces cerevisiae. Genetics *136*, 485-503.

Carling, D. (2004). The AMP-activated protein kinase cascade--a unifying system for energy control. Trends Biochem Sci *29*, 18-24.

Carroll, A. S., Bishop, A. C., DeRisi, J. L., Shokat, K. M., and O'Shea, E. K. (2001). Chemical inhibition of the Pho85 cyclin-dependent kinase reveals a role in the environmental stress response. Proc Natl Acad Sci U S A *98*, 12578-12583.

Castro, C. D., Meehan, A. J., Koretsky, A. P., and Domach, M. M. (1995). In situ 31P nuclear magnetic resonance for observation of polyphosphate and catabolite responses of chemostat-cultivated Saccharomyces cerevisiae after alkalinization. Appl Environ Microbiol *61*, 4448-4453.

Castrol, C. D., Koretsky, A. P., and Domach, M. M. (1999). NMR-Observed phosphate trafficking and polyphosphate dynamics in wild-type and vph1-1 mutant Saccharomyces cerevisae in response to stresses. Biotechnol Prog *15*, 65-73.

Celenza, J. L., and Carlson, M. (1989). Mutational analysis of the Saccharomyces cerevisiae SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. Mol Cell Biol *9*, 5034-5044.

Chen, Q., and Haddad, G. G. (2004). Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals. J Exp Biol *207*, 3125-3129.

Cheung, Z. H., and Ip, N. Y. (2004). Cdk5: mediator of neuronal death and survival. Neurosci Lett *361*, 47-51.

Chevtzoff, C., Vallortigara, J., Averet, N., Rigoulet, M., and Devin, A. (2005). The yeast cAMP protein kinase Tpk3p is involved in the regulation of mitochondrial enzymatic content during growth. Biochim Biophys Acta *1706*, 117-125.

Chi, Y., Huddleston, M. J., Zhang, X., Young, R. A., Annan, R. S., Carr, S. A., and Deshaies, R. J. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. Genes Dev *15*, 1078-1092.

Ching, Y. P., Pang, A. S., Lam, W. H., Qi, R. Z., and Wang, J. H. (2002). Identification of a neuronal Cdk5 activator-binding protein as Cdk5 inhibitor. J Biol Chem *277*, 15237-15240.

Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. O., and Herskowitz, I. (1998). The transcriptional program of sporulation in budding yeast. Science *282*, 699-705.

Cohen, A., Perzov, N., Nelson, H., and Nelson, N. (1999). A novel family of yeast chaperons involved in the distribution of V-ATPase and other membrane proteins. J Biol Chem *274*, 26885-26893.

Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J. H., Gorwa, M. F., Colavizza, D., and Thevelein, J. M. (1998). Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast Saccharomyces cerevisiae. Embo J *17*, 3326-3341.

Colombo, S., Ronchetti, D., Thevelein, J. M., Winderickx, J., and Martegani, E. (2004). Activation state of the Ras2 protein and glucose-induced signaling in Saccharomyces cerevisiae. J Biol Chem *279*, 46715-46722.

Colwill, K., Field, D., Moore, L., Friesen, J., and Andrews, B. (1999). In vivo analysis of the domains of yeast Rvs167p suggests Rvs167p function is mediated through multiple protein interactions. Genetics *152*, 881-893.

Conrads, K. A., Yi, M., Simpson, K. A., Lucas, D. A., Camalier, C. E., Yu, L. R., Veenstra, T. D., Stephens, R. M., Conrads, T. P., and Beck, G. R., Jr. (2005). A combined proteome and microarray investigation of inorganic phosphate-induced pre-osteoblast cells. Mol Cell Proteomics *4*, 1284-1296.

Coutinho, C. C., Silva, J. T., and Panek, A. D. (1992). Trehalase activity and its regulation during growth of Saccharomyces cerevisiae. Biochem Int *26*, 521-530.

Crauwels, M., Donaton, M. C., Pernambuco, M. B., Winderickx, J., de Winde, J. H., and Thevelein, J. M. (1997). The Sch9 protein kinase in the yeast Saccharomyces cerevisiae controls cAPK activity and is required for nitrogen activation of the fermentable-growth-medium-induced (FGM) pathway. Microbiology *143 (Pt 8)*, 2627-2637.

Creasy, C. L., Madden, S. L., and Bergman, L. W. (1993). Molecular analysis of the PHO81 gene of Saccharomyces cerevisiae. Nucleic Acids Res *21*, 1975-1982.

Cruz, J. C., and Tsai, L. H. (2004a). Cdk5 deregulation in the pathogenesis of Alzheimer's disease. Trends Mol Med *10*, 452-458.

Cruz, J. C., and Tsai, L. H. (2004b). A Jekyll and Hyde kinase: roles for Cdk5 in brain development and disease. Curr Opin Neurobiol *14*, 390-394.

Czarnogorski, M., Woda, C. B., Schulkin, J., and Mulroney, S. E. (2004). Induction of a phosphate appetite in adult male and female rats. Exp Biol Med (Maywood) *229*, 914-919.

Daniel, T., and Carling, D. (2002). Expression and regulation of the AMP-activated protein kinase-SNF1 (sucrose non-fermenting 1) kinase complexes in yeast and mammalian cells: studies using chimaeric catalytic subunits. Biochem J *365*, 629-638.

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

De Winde, J. H., Crauwels, M., Hohmann, S., Thevelein, J. M., and Winderickx, J. (1996). Differential requirement of the yeast sugar kinases for sugar sensing in establishing the catabolite-repressed state. Eur J Biochem *241*, 633-643.

Dephoure, N., Howson, R. W., Blethrow, J. D., Shokat, K. M., and O'Shea, E. K. (2005). Combining chemical genetics and proteomics to identify protein kinase substrates. Proc Natl Acad Sci U S A *102*, 17940-17945.

DeRisi, J. L., Iyer, V. R., and Brown, P. O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. Science *278*, 680-686.

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.

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

Dhavan, R., and Tsai, L. H. (2001). A decade of CDK5. Nat Rev Mol Cell Biol *2*, 749-759.

Dimmer, K. S., Fritz, S., Fuchs, F., Messerschmitt, M., Weinbach, N., Neupert, W., and Westermann, B. (2002). Genetic basis of mitochondrial function and morphology in Saccharomyces cerevisiae. Mol Biol Cell *13*, 847-853.

Dirick, L., Bohm, T., and Nasmyth, K. (1995). Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of Saccharomyces cerevisiae. Embo J *14*, 4803-4813.

Donaton, M. C., Holsbeeks, I., Lagatie, O., Van Zeebroeck, G., Crauwels, M., Winderickx, J., and Thevelein, J. M. (2003). The Gap1 general amino acid permease acts as an amino acid sensor for activation of protein kinase A targets in the yeast Saccharomyces cerevisiae. Mol Microbiol *50*, 911-929.

Dunn, T., Gable, K., and Beeler, T. (1994). Regulation of cellular Ca2+ by yeast vacuoles. J Biol Chem *269*, 7273-7278.

Dunstan, C. R., Zhou, H., and Seibel, M. J. (2004). Fibroblast growth factor 23: a phosphatonin regulating phosphate homeostasis? Endocrinology *145*, 3084-3086.

Durnez, P., Pernambuco, M. B., Oris, E., Arguelles, J. C., Mergelsberg, H., and Thevelein, J. M. (1994). Activation of trehalase during growth induction by nitrogen sources in the yeast Saccharomyces cerevisiae depends on the free catalytic subunits of cAMP-dependent protein kinase, but not on functional Ras proteins. Yeast *10*, 1049-1064.

El Alami, M., Messenguy, F., Scherens, B., and Dubois, E. (2003). Arg82p is a bifunctional protein whose inositol polyphosphate kinase activity is essential for nitrogen and PHO gene expression but not for Mcm1p chaperoning in yeast. Mol Microbiol *49*, 457-468.

Enjalbert, B., Parrou, J. L., Teste, M. A., and Francois, J. (2004). Combinatorial control by the protein kinases PKA, PHO85 and SNF1 of transcriptional induction of the Saccharomyces cerevisiae GSY2 gene at the diauxic shift. Mol Genet Genomics *271*, 697-708.

Enyenihi, A. H., and Saunders, W. S. (2003). Large-scale functional genomic analysis of sporulation and meiosis in Saccharomyces cerevisiae. Genetics *163*, 47-54.

Espinoza, F. H., Ogas, J., Herskowitz, I., and Morgan, D. O. (1994). Cell cycle control by a complex of the cyclin HCS26 (PCL1) and the kinase PHO85. Science *266*, 1388-1391.

Fascher, K. D., Schmitz, J., and Horz, W. (1990). Role of trans-activating proteins in the generation of active chromatin at the PHO5 promoter in S. cerevisiae. Embo J *9*, 2523-2528.

Feng, Z. H., Wilson, S. E., Peng, Z. Y., Schlender, K. K., Reimann, E. M., and Trumbly, R. J. (1991). The yeast GLC7 gene required for glycogen accumulation encodes a type 1 protein phosphatase. J Biol Chem *266*, 23796-23801.

Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988). Purification of a RAS-responsive adenylyl cyclase complex from Saccharomyces cerevisiae by use of an epitope addition method. Mol Cell Biol *8*, 2159-2165.

Fisher, E., Almaguer, C., Holic, R., Griac, P., and Patton-Vogt, J. (2005). Glycerophosphocholine-dependent growth requires Gde1p (YPL110c) and Git1p in Saccharomyces cerevisiae. J Biol Chem *280*, 36110-36117.

Fiske, C. H., and Subbarow, Y. (1925). The colorimetric determination of phosphorus. J Biol Chem *66*, 375-400.

Flick, J. S., and Thorner, J. (1998). An essential function of a phosphoinositide-specific phospholipase C is relieved by inhibition of a cyclin-dependent protein kinase in the yeast Saccharomyces cerevisiae. Genetics *148*, 33-47.

Floyd, S. R., Porro, E. B., Slepnev, V. I., Ochoa, G. C., Tsai, L. H., and De Camilli, P. (2001). Amphiphysin 1 binds the cyclin-dependent kinase (cdk) 5 regulatory subunit p35 and is phosphorylated by cdk5 and cdc2. J Biol Chem *276*, 8104-8110.

Francois, J., and Hers, H. G. (1988). The control of glycogen metabolism in yeast. 2. A kinetic study of the two forms of glycogen synthase and of glycogen phosphorylase and an investigation of their interconversion in a cell-free extract. Eur J Biochem *174*, 561-567.

Francois, J., Neves, M. J., and Hers, H. G. (1991). The control of trehalose biosynthesis in Saccharomyces cerevisiae: evidence for a catabolite inactivation and repression of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. Yeast *7*, 575-587.

Francois, J., and Parrou, J. L. (2001). Reserve carbohydrates metabolism in the yeast Saccharomyces cerevisiae. FEMS Microbiol Rev *25*, 125-145.

Franco-Zorrilla, J. M., Gonzalez, E., Bustos, R., Linhares, F., Leyva, A., and Paz-Ares, J. (2004). The transcriptional control of plant responses to phosphate limitation. J Exp Bot *55*, 285-293.

Frederick, D. L., and Tatchell, K. (1996). The REG2 gene of Saccharomyces cerevisiae encodes a type 1 protein phosphatase-binding protein that functions with Reg1p and the Snf1 protein kinase to regulate growth. Mol Cell Biol *16*, 2922-2931.

Fristedt, U., Berhe, A., Ensler, K., Norling, and Persson, B. L. (1996). Isolation and characterization of membrane vesicles of Saccharomyces cerevisiae harboring the high-affinity phosphate transporter. Arch Biochem Biophys *330*, 133-141.

Fristedt, U., van Der Rest, M., Poolman, B., Konings, W. N., and Persson, B. L. (1999a). Studies of cytochrome c oxidase-driven H(+)-coupled phosphate transport catalyzed by the Saccharomyces cerevisiae Pho84 permease in coreconstituted vesicles. Biochemistry *38*, 16010-16015.

Fristedt, U., Weinander, R., Martinsson, H. S., and Persson, B. L. (1999b). Characterization of purified and unidirectionally reconstituted Pho84 phosphate permease of Saccharomyces cerevisiae. FEBS Lett *458*, 1-5.

Fu, H. H., and Luan, S. (1998). AtKuP1: a dual-affinity K+ transporter from Arabidopsis. Plant Cell *10*, 63-73.

Gancedo, C., and Flores, C. L. (2004). The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. FEMS Yeast Res *4*, 351-359.

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

Garciadeblas, B., Rubio, F., Quintero, F. J., Banuelos, M. A., Haro, R., and Rodriguez-Navarro, A. (1993). Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in Saccharomyces cerevisiae. Mol Gen Genet *236*, 363-368.

Garreau, H., Hasan, R. N., Renault, G., Estruch, F., Boy-Marcotte, E., and Jacquet, M. (2000). Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in Saccharomyces cerevisiae. Microbiology *146 (Pt 9)*, 2113-2120.

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

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

Geyskens, I., Kumara, S., Donaton, M. C. V., Bergsma, J. C., Thevelein, J., and Wera, S. (2000). Expression of mammalian PKB complements deletion of the yeast protein kinase Sch9. In Molecular Mechanisms in Signal Transduction, J. L. Bos, ed. (IOS Press), pp. 117-126.

Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K., and Weissman, J. S. (2003). Global analysis of protein expression in yeast. Nature *425*, 737-741.

Gietz, R. D., Schiestl, R. H., Willems, A. R., and Woods, R. A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast *11*, 355-360.

Gildor, T., Shemer, R., Atir-Lande, A., and Kornitzer, D. (2005). Coevolution of cyclin Pcl5 and its substrate Gcn4. Eukaryot Cell *4*, 310-318.

Gilles-Gonzalez, M. A., and Gonzalez, G. (2004). Signal transduction by heme-containing PAS-domain proteins. J Appl Physiol *96*, 774-783.

Gillies, R. J., Ugurbil, K., den Hollander, J. A., and Shulman, R. G. (1981). 31P NMR studies of intracellular pH and phosphate metabolism during cell division cycle of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *78*, 2125-2129.

Gilliquet, V., and Berben, G. (1993). Positive and negative regulators of the Saccharomyces cerevisiae 'PHO system' participate in several cell functions. FEMS Microbiol Lett *108*, 333-339.

Giots, F., Donaton, M. C., and Thevelein, J. M. (2003). Inorganic phosphate is sensed by specific phosphate carriers and acts in concert with glucose as a nutrient signal for activation of the protein kinase A pathway in the yeast Saccharomyces cerevisiae. Mol Microbiol *47*, 1163-1181.

Glover, C. V., 3rd (1998). On the physiological role of casein kinase II in Saccharomyces cerevisiae. Prog Nucleic Acid Res Mol Biol *59*, 95-133.

Godon, C., Lagniel, G., Lee, J., Buhler, J. M., Kieffer, S., Perrot, M., Boucherie, H., Toledano, M. B., and Labarre, J. (1998). The H2O2 stimulon in Saccharomyces cerevisiae. J Biol Chem *273*, 22480-22489.

Goodman, J., and Rothstein, A. (1957). The active transport of phosphate into the yeast cell. J Gen Physiol *40*, 915-923.

Gorner, W., Durchschlag, E., Martinez-Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., and Schuller, C. (1998). Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev *12*, 586-597.

Gorner, W., Durchschlag, E., Wolf, J., Brown, E. L., Ammerer, G., Ruis, H., and Schuller, C. (2002). Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. Embo J *21*, 135-144.

Gottlieb, D., Heideman, W., and Saba, J. D. (1999). The DPL1 gene is involved in mediating the response to nutrient deprivation in Saccharomyces cerevisiae. Mol Cell Biol Res Commun *1*, 66-71.

Gray, J. V., Ogas, J. P., Kamada, Y., Stone, M., Levin, D. E., and Herskowitz, I. (1997). A role for the Pkc1 MAP kinase pathway of Saccharomyces cerevisiae in bud emergence and identification of a putative upstream regulator. Embo J *16*, 4924-4937.

Griffioen, G., Anghileri, P., Imre, E., Baroni, M. D., and Ruis, H. (2000). Nutritional control of nucleocytoplasmic localization of cAMP-dependent protein kinase catalytic and regulatory subunits in Saccharomyces cerevisiae. J Biol Chem *275*, 1449-1456.

Griffioen, G., Branduardi, P., Ballarini, A., Anghileri, P., Norbeck, J., Baroni, M. D., and Ruis, H. (2001). Nucleocytoplasmic distribution of budding yeast protein kinase A regulatory subunit Bcy1 requires Zds1 and is regulated by Yak1-dependent phosphorylation of its targeting domain. Mol Cell Biol *21*, 511-523.

Griffioen, G., Laan, R. J., Mager, W. H., and Planta, R. J. (1996). Ribosomal protein gene transcription in Saccharomyces cerevisiae shows a biphasic response to nutritional changes. Microbiology *142 (Pt 8)*, 2279-2287.

Gulli, M. P., Jaquenoud, M., Shimada, Y., Niederhauser, G., Wiget, P., and Peter, M. (2000). Phosphorylation of the Cdc42 exchange factor Cdc24 by the PAK-like kinase Cla4 may regulate polarized growth in yeast. Mol Cell *6*, 1155-1167.

Haguenauer-Tsapis, R., Nagy, M., and Ryter, A. (1986). A deletion that includes the segment coding for the signal peptidase cleavage site delays release of Saccharomyces cerevisiae acid phosphatase from the endoplasmic reticulum. Mol Cell Biol *6*, 723-729.

Hallows, K. R. (2005). Emerging role of AMP-activated protein kinase in coupling membrane transport to cellular metabolism. Curr Opin Nephrol Hypertens *14*, 464-471.

Hammond, J. P., Broadley, M. R., and White, P. J. (2004). Genetic responses to phosphorus deficiency. Ann Bot (Lond) *94*, 323-332.

Hardie, D. G., and Carling, D. (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur J Biochem *246*, 259-273.

Hardie, D. G., Scott, J. W., Pan, D. A., and Hudson, E. R. (2003). Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett *546*, 113-120.

Hardy, T. A., Huang, D., and Roach, P. J. (1994). Interactions between cAMP-dependent and SNF1 protein kinases in the control of glycogen accumulation in Saccharomyces cerevisiae. J Biol Chem *269*, 27907-27913.

Hardy, T. A., and Roach, P. J. (1993). Control of yeast glycogen synthase-2 by COOH-terminal phosphorylation. J Biol Chem *268*, 23799-23805.

Harris, T. E., and Lawrence, J. C., Jr. (2003). TOR signaling. Sci STKE *2003*, re15.

Hartley, A. D., Ward, M. P., and Garrett, S. (1994). The Yak1 protein kinase of Saccharomyces cerevisiae moderates thermotolerance and inhibits growth by an Sch9 protein kinase-independent mechanism. Genetics *136*, 465-474.

Haswell, E. S., and O'Shea, E. K. (1999). An in vitro system recapitulates chromatin remodeling at the PHO5 promoter. Mol Cell Biol *19*, 2817-2827.

Hedbacker, K., Hong, S. P., and Carlson, M. (2004). Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. Mol Cell Biol *24*, 8255-8263.

Henderson, P. J. (1993). The 12-transmembrane helix transporters. Curr Opin Cell Biol *5*, 708-721.

Hengartner, C. J., Myer, V. E., Liao, S. M., Wilson, C. J., Koh, S. S., and Young, R. A. (1998). Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Mol Cell *2*, 43-53.

Hernando, N., Gisler, S. M., Pribanic, S., Deliot, N., Capuano, P., Wagner, C. A., Moe, O. W., Biber, J., and Murer, H. (2005). NaPi-IIa and interacting partners. J Physiol *567*, 21-26.

Herrero, P., Fernandez, R., and Moreno, F. (1989). The hexokinase isoenzyme PII of Saccharomyces cerevisiae ia a protein kinase. J Gen Microbiol *135*, 1209-1216.

Herrero, P., Martinez-Campa, C., and Moreno, F. (1998). The hexokinase 2 protein participates in regulatory DNA-protein complexes necessary for glucose repression of the SUC2 gene in Saccharomyces cerevisiae. FEBS Lett *434*, 71-76.

Herrmann, J. M., Malkus, P., and Schekman, R. (1999). Out of the ER--outfitters, escorts and guides. Trends Cell Biol *9*, 5-7.

Hicke, L. (1999). Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. Trends Cell Biol *9*, 107-112.

Hinnebusch, A. G., and Natarajan, K. (2002). Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. Eukaryot Cell *1*, 22-32.

Hirimburegama, K., Durnez, P., Keleman, J., Oris, E., Vergauwen, R., Mergelsberg, H., and Thevelein, J. M. (1992). Nutrient-induced activation of trehalase in nutrient-starved cells of the yeast Saccharomyces cerevisiae: cAMP is not involved as second messenger. J Gen Microbiol *138 (Pt 10)*, 2035-2043.

Hirst, K., Fisher, F., McAndrew, P. C., and Goding, C. R. (1994). The transcription factor, the Cdk, its cyclin and their regulator: directing the transcriptional response to a nutritional signal. Embo J *13*, 5410-5420.

Hisamoto, N., Frederick, D. L., Sugimoto, K., Tatchell, K., and Matsumoto, K. (1995). The EGP1 gene may be a positive regulator of protein phosphatase type 1 in the growth control of Saccharomyces cerevisiae. Mol Cell Biol *15*, 3767-3776.

Hisamoto, N., Sugimoto, K., and Matsumoto, K. (1994). The Glc7 type 1 protein phosphatase of Saccharomyces cerevisiae is required for cell cycle progression in G2/M. Mol Cell Biol *14*, 3158-3165.

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.

Hohmann, S., Winderickx, J., de Winde, J. H., Valckx, D., Cobbaert, P., Luyten, K., de Meirsman, C., Ramos, J., and Thevelein, J. M. (1999). Novel alleles of yeast hexokinase PII with distinct effects on catalytic activity and catabolite repression of SUC2. Microbiology *145 (Pt 3)*, 703-714.

Holsbeeks, I., Lagatie, O., Van Nuland, A., Van de Velde, S., and Thevelein, J. M. (2004). The eukaryotic plasma membrane as a nutrient-sensing device. Trends Biochem Sci *29*, 556-564.

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

Hong, S. P., Momcilovic, M., and Carlson, M. (2005). Function of mammalian LKB1 and Ca2+/calmodulin-dependent protein kinase kinase alpha as Snf1-activating kinases in yeast. J Biol Chem *280*, 21804-21809.

Hopper, A. K. (1999). Nucleocytoplasmic transport: Inside out regulation. Curr Biol *9*, R803-806.

Horak, J. (2004). Down-regulation of model yeast proteins by ubiquitin-dependent proteolysis. Physiol Res *53 Suppl 1*, S99-102.

Hottiger, T., Schmutz, P., and Wiemken, A. (1987). Heat-induced accumulation and futile cycling of trehalose in Saccharomyces cerevisiae. J Bacteriol *169*, 5518-5522.

Huang, D., Chun, K. T., Goebl, M. G., and Roach, P. J. (1996a). Genetic interactions between REG1/HEX2 and GLC7, the gene encoding the protein phosphatase type 1 catalytic subunit in Saccharomyces cerevisiae. Genetics *143*, 119-127.

Huang, D., Farkas, I., and Roach, P. J. (1996b). Pho85p, a cyclin-dependent protein kinase, and the Snf1p protein kinase act antagonistically to control glycogen accumulation in Saccharomyces cerevisiae. Mol Cell Biol *16*, 4357-4365.

Huang, D., Moffat, J., and Andrews, B. (2002). Dissection of a complex phenotype by functional genomics reveals roles for the yeast cyclin-dependent protein kinase Pho85 in stress adaptation and cell integrity. Mol Cell Biol *22*, 5076-5088.

Huang, D., Moffat, J., Wilson, W. A., Moore, L., Cheng, C., Roach, P. J., and Andrews, B. (1998). Cyclin partners determine Pho85 protein kinase substrate specificity in vitro and in vivo: control of glycogen biosynthesis by Pcl8 and Pcl10. Mol Cell Biol *18*, 3289-3299.

Huang, D., Patrick, G., Moffat, J., Tsai, L. H., and Andrews, B. (1999). Mammalian Cdk5 is a functional homologue of the budding yeast Pho85 cyclin-dependent protein kinase. Proc Natl Acad Sci U S A *96*, 14445-14450.

Huang, D., Wilson, W. A., and Roach, P. J. (1997). Glucose-6-P control of glycogen synthase phosphorylation in yeast. J Biol Chem *272*, 22495-22501.

Huang, S., Jeffery, D. A., Anthony, M. D., and O'Shea, E. K. (2001). Functional analysis of the cyclin-dependent kinase inhibitor Pho81 identifies a novel inhibitory domain. Mol Cell Biol *21*, 6695-6705.

Huang, S., and O'Shea, E. K. (2005). A systematic high-throughput screen of a yeast deletion collection for mutants defective in PHO5 regulation. Genetics *169*, 1859-1871.

Hubbard, E. J., Yang, X. L., and Carlson, M. (1992). Relationship of the cAMP-dependent protein kinase pathway to the SNF1 protein kinase and invertase expression in Saccharomyces cerevisiae. Genetics *130*, 71-80.

Irniger, S., and Braus, G. H. (2003). Controlling transcription by destruction: the regulation of yeast Gcn4p stability. Curr Genet *44*, 8-18.

Iwaki, S., Kihara, A., Sano, T., and Igarashi, Y. (2005). Phosphorylation by Pho85 cyclin-dependent kinase acts as a signal for the down-regulation of the yeast sphingoid long-chain base kinase Lcb4 during the stationary phase. J Biol Chem *280*, 6520-6527.

Jeffery, D. A., Springer, M., King, D. S., and O'Shea, E. K. (2001). Multi-site phosphorylation of Pho4 by the cyclin-CDK Pho80-Pho85 is semi-processive with site preference. J Mol Biol *306*, 997-1010.

Jensen, L. T., Ajua-Alemanji, M., and Culotta, V. C. (2003). The Saccharomyces cerevisiae high affinity phosphate transporter encoded by PHO84 also functions in manganese homeostasis. J Biol Chem *278*, 42036-42040.

Jiang, R., and Carlson, M. (1996). Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. Genes Dev *10*, 3105-3115.

Jiang, R., and Carlson, M. (1997). The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. Mol Cell Biol *17*, 2099-2106.

Jiang, Y., Davis, C., and Broach, J. R. (1998). Efficient transition to growth on fermentable carbon sources in Saccharomyces cerevisiae requires signaling through the Ras pathway. Embo J *17*, 6942-6951.

Jin, H., Hwang, S. K., Yu, K., Anderson, H. K., Lee, Y. S., Lee, K. H., Prats, A. C., Morello, D., Beck, G. R., Jr., and Cho, M. H. (2006). A high inorganic phosphate diet perturbs brain growth, alters Akt-ERK signaling, and results in changes in cap-dependent translation. Toxicol Sci *90*, 221-229.

Jin, M., Fujita, M., Culley, B. M., Apolinario, E., Yamamoto, M., Maundrell, K., and Hoffman, C. S. (1995). sck1, a high copy number suppressor of defects in the cAMP-dependent protein kinase pathway in fission yeast, encodes a protein homologous to the Saccharomyces cerevisiae SCH9 kinase. Genetics *140*, 457-467.

Johnson, D. I. (1999). Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. Microbiol Mol Biol Rev *63*, 54-105.

Johnston, M., and Carlson, M. (1992). Regulation of carbon and phosphate utilization. In The molecular and cellular biology of the yeast Saccharomyces., E. W. Jones, Pringle, J.R. and Broach, J.R., ed. (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press), pp. 193-281.

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

Kaffman, A., Herskowitz, I., Tjian, R., and O'Shea, E. K. (1994). Phosphorylation of the transcription factor PHO4 by a cyclin-CDK complex, PHO80-PHO85. Science *263*, 1153-1156.

Kaffman, A., Rank, N. M., O'Neill, E. M., Huang, L. S., and O'Shea, E. K. (1998a). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature *396*, 482-486.

Kaffman, A., Rank, N. M., and O'Shea, E. K. (1998b). Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. Genes Dev *12*, 2673-2683.

Karandashov, V., and Bucher, M. (2005). Symbiotic phosphate transport in arbuscular mycorrhizas. Trends Plant Sci *10*, 22-29.

Karpichev, I. V., Cornivelli, L., and Small, G. M. (2002). Multiple regulatory roles of a novel Saccharomyces cerevisiae protein, encoded by YOL002c, in lipid and phosphate metabolism. J Biol Chem *277*, 19609-19617.

Kataoka, T., Broek, D., and Wigler, M. (1985). DNA sequence and characterization of the S. cerevisiae gene encoding adenylate cyclase. Cell *43*, 493-505.

Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M., and Johnson, A. D. (1992). Ssn6-Tup1 is a general repressor of transcription in yeast. Cell *68*, 709-719.

Keniry, M. E., Kemp, H. A., Rivers, D. M., and Sprague, G. F., Jr. (2004). The identification of Pcl1-interacting proteins that genetically interact with Cla4 may indicate a link between G1 progression and mitotic exit. Genetics *166*, 1177-1186.

Kim, M. D., Hong, S. P., and Carlson, M. (2005). Role of Tos3, a Snf1 protein kinase kinase, during growth of Saccharomyces cerevisiae on nonfermentable carbon sources. Eukaryot Cell *4*, 861-866.

Klionsky, D. J., Herman, P. K., and Emr, S. D. (1990). The fungal vacuole: composition, function, and biogenesis. Microbiol Rev *54*, 266-292.

Knight, J. P., Daly, T. M., and Bergman, L. W. (2004). Regulation by phosphorylation of Pho81p, a cyclin-dependent kinase inhibitor in Saccharomyces cerevisiae. Curr Genet *46*, 10-19.

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

Konrad, M. (1988). Analysis and in vivo disruption of the gene coding for adenylate kinase (ADK1) in the yeast Saccharomyces cerevisiae. J Biol Chem *263*, 19468-19474.

Kornberg, A., Rao, N. N., and Ault-Riche, D. (1999). Inorganic polyphosphate: a molecule of many functions. Annu Rev Biochem *68*, 89-125.

Kornitzer, D., Raboy, B., Kulka, R. G., and Fink, G. R. (1994). Regulated degradation of the transcription factor Gcn4. Embo J *13*, 6021-6030.

Kota, J., and Ljungdahl, P. O. (2005). Specialized membrane-localized chaperones prevent aggregation of polytopic proteins in the ER. J Cell Biol *168*, 79-88.

Kraakman, L., Lemaire, K., Ma, P., Teunissen, A. W., Donaton, M. C., Van Dijck, P., Winderickx, J., de Winde, J. H., and Thevelein, J. M. (1999a). A Saccharomyces cerevisiae G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. Mol Microbiol *32*, 1002-1012.

Kraakman, L. S., Griffioen, G., Zerp, S., Groeneveld, P., Thevelein, J. M., Mager, W. H., and Planta, R. J. (1993). Growth-related expression of ribosomal protein genes in Saccharomyces cerevisiae. Mol Gen Genet *239*, 196-204.

Kraakman, L. S., Winderickx, J., Thevelein, J. M., and De Winde, J. H. (1999b). Structure-function analysis of yeast hexokinase: structural requirements for triggering cAMP signalling and catabolite repression. Biochem J *343 Pt 1*, 159-168.

Kulaev, I., and Kulakovskaya, T. (2000). Polyphosphate and phosphate pump. Annu Rev Microbiol *54*, 709-734.

Kumble, K. D., and Kornberg, A. (1996). Endopolyphosphatases for long chain inorganic polyphosphate in yeast and mammals. J Biol Chem *271*, 27146-27151.

Kuret, J., Johnson, K. E., Nicolette, C., and Zoller, M. J. (1988). Mutagenesis of the regulatory subunit of yeast cAMP-dependent protein kinase. Isolation of site-directed mutants with altered binding affinity for catalytic subunit. J Biol Chem *263*, 9149-9154.

Lagerstedt, J. O., Reeve, I., Voss, J. C., and Persson, B. L. (2005). Structure and function of the GTP binding protein Gtr1 and its role in phosphate transport in Saccharomyces cerevisiae. Biochemistry *44*, 511-517.

Lagerstedt, J. O., Voss, J. C., Wieslander, A., and Persson, B. L. (2004). Structural modeling of dual-affinity purified Pho84 phosphate transporter. FEBS Lett *578*, 262-268.

Lagerstedt, J. O., Zvyagilskaya, R., Pratt, J. R., Pattison-Granberg, J., Kruckeberg, A. L., Berden, J. A., and Persson, B. L. (2002). Mutagenic and functional analysis of the C-terminus of Saccharomyces cerevisiae Pho84 phosphate transporter. FEBS Lett *526*, 31-37.

Lamb, T. M., Xu, W., Diamond, A., and Mitchell, A. P. (2001). Alkaline response genes of Saccharomyces cerevisiae and their relationship to the RIM101 pathway. J Biol Chem *276*, 1850-1856.

Lanterman, M. M., and Saba, J. D. (1998). Characterization of sphingosine kinase (SK) activity in Saccharomyces cerevisiae and isolation of SK-deficient mutants. Biochem J *332 (Pt 2)*, 525-531.

Lau, W. T., Howson, R. W., Malkus, P., Schekman, R., and O'Shea, E. K. (2000). Pho86p, an endoplasmic reticulum (ER) resident protein in Saccharomyces cerevisiae, is required for ER exit of the high-affinity phosphate transporter Pho84p. Proc Natl Acad Sci U S A *97*, 1107-1112.

Lau, W. W., Schneider, K. R., and O'Shea, E. K. (1998). A genetic study of signaling processes for repression of PHO5 transcription in Saccharomyces cerevisiae. Genetics *150*, 1349-1359.

Lazaro, J. B., Kitzmann, M., Cavadore, J. C., Muller, Y., Clos, J., Fernandez, A., and Lamb, N. J. (1996). cdk5 expression and association with p35nck5a in early stages of rat cerebellum neurogenesis; tyrosine dephosphorylation and activation in post-mitotic neurons. Neurosci Lett *218*, 21-24.

Lecoq, K., Belloc, I., Desgranges, C., and Daignan-Fornier, B. (2001). Role of adenosine kinase in Saccharomyces cerevisiae: identification of the ADO1 gene and study of the mutant phenotypes. Yeast *18*, 335-342.

Lee, J., Colwill, K., Aneliunas, V., Tennyson, C., Moore, L., Ho, Y., and Andrews, B. (1998). Interaction of yeast Rvs167 and Pho85 cyclin-dependent kinase complexes may link the cell cycle to the actin cytoskeleton. Curr Biol *8*, 1310-1321.

Lee, M., O'Regan, S., Moreau, J. L., Johnson, A. L., Johnston, L. H., and Goding, C. R. (2000). Regulation of the Pcl7-Pho85 cyclin-cdk complex by Pho81. Mol Microbiol *38*, 411-422.

Lemaire, K., Van de Velde, S., Van Dijck, P., and Thevelein, J. M. (2004). Glucose and sucrose act as agonist and mannose as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast Saccharomyces cerevisiae. Mol Cell *16*, 293-299.

Lenburg, M. E., and O'Shea, E. K. (1996). Signaling phosphate starvation. Trends Biochem Sci *21*, 383-387.

Lenburg, M. E., and O'Shea, E. K. (2001). Genetic evidence for a morphogenetic function of the Saccharomyces cerevisiae Pho85 cyclin-dependent kinase. Genetics *157*, 39-51.

Lenssen, E., James, N., Pedruzzi, I., Dubouloz, F., Cameroni, E., Bisig, R., Maillet, L., Werner, M., Roosen, J., Petrovic, K.*, et al.* (2005). The Ccr4-Not complex independently controls both Msn2-dependent transcriptional activation--via a newly identified Glc7/Bud14 type I protein phosphatase module--and TFIID promoter distribution. Mol Cell Biol *25*, 488-498.

Lenssen, E., Oberholzer, U., Labarre, J., De Virgilio, C., and Collart, M. A. (2002). Saccharomyces cerevisiae Ccr4-not complex contributes to the control of Msn2p-dependent transcription by the Ras/cAMP pathway. Mol Microbiol *43*, 1023-1037.

Lesage, P., Yang, X., and Carlson, M. (1996). Yeast SNF1 protein kinase interacts with SIP4, a C6 zinc cluster transcriptional activator: a new role for SNF1 in the glucose response. Mol Cell Biol *16*, 1921-1928.

Lichko, L., Kulakovskaya, T., and Kulaev, I. (1998). Membrane-bound and soluble polyphosphatases of mitochondria of Saccharomyces cerevisiae: identification and comparative characterization. Biochim Biophys Acta *1372*, 153-162.

Lichko, L. P., Andreeva, N. A., Kulakovskaya, T. V., and Kulaev, I. S. (2003). Exopolyphosphatases of the yeast Saccharomyces cerevisiae. FEMS Yeast Res *3*, 233-238.

Lillie, S. H., and Pringle, J. R. (1980). Reserve carbohydrate metabolism in Saccharomyces cerevisiae: responses to nutrient limitation. J Bacteriol *143*, 1384-1394.

Lin, K., Hwang, P. K., and Fletterick, R. J. (1995). Mechanism of regulation in yeast glycogen phosphorylase. J Biol Chem *270*, 26833-26839.

Lin, K., Rath, V. L., Dai, S. C., Fletterick, R. J., and Hwang, P. K. (1996). A protein phosphorylation switch at the conserved allosteric site in GP. Science *273*, 1539-1542.

Liu, C., Yang, Z., Yang, J., Xia, Z., and Ao, S. (2000). Regulation of the yeast transcriptional factor PHO2 activity by phosphorylation. J Biol Chem *275*, 31972-31978.

Liu, J., Samac, D. A., Bucciarelli, B., Allan, D. L., and Vance, C. P. (2005). Signaling of phosphorus deficiency-induced gene expression in white lupin requires sugar and phloem transport. Plant J *41*, 257-268.

Liu, K. H., and Tsay, Y. F. (2003). Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. Embo J *22*, 1005-1013.

Lorenz, M. C., Pan, X., Harashima, T., Cardenas, M. E., Xue, Y., Hirsch, J. P., and Heitman, J. (2000). The G protein-coupled receptor gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in Saccharomyces cerevisiae. Genetics *154*, 609-622.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J Biol Chem *193*, 265-275.

Ludin, K., Jiang, R., and Carlson, M. (1998). Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *95*, 6245-6250.

Ma, P., Wera, S., Van Dijck, P., and Thevelein, J. M. (1999). The PDE1-encoded low-affinity phosphodiesterase in the yeast Saccharomyces cerevisiae has a specific function in controlling agonist-induced cAMP signaling. Mol Biol Cell *10*, 91-104.

MacKelvie, S. H., Andrews, P. D., and Stark, M. J. (1995). The Saccharomyces cerevisiae gene SDS22 encodes a potential regulator of the mitotic function of yeast type 1 protein phosphatase. Mol Cell Biol *15*, 3777-3785.

Madden, K., Sheu, Y. J., Baetz, K., Andrews, B., and Snyder, M. (1997). SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. Science *275*, 1781-1784.

Magasanik, B., and Kaiser, C. A. (2002). Nitrogen regulation in Saccharomyces cerevisiae. Gene *290*, 1-18.

Magbanua, J. P., Fujisawa, K., Ogawa, N., and Oshima, Y. (1997a). The homeodomain protein Pho2p binds at an A/T-rich segment flanking the binding site of the basic-helix-loop-helix protein Pho4p in the yeast PHO promoters. Yeast *13*, 1299-1308.

Magbanua, J. P., Ogawa, N., Harashima, S., and Oshima, Y. (1997b). The transcriptional activators of the PHO regulon, Pho4p and Pho2p, interact directly with each other and with components of the basal transcription machinery in Saccharomyces cerevisiae. J Biochem (Tokyo) *121*, 1182-1189.

Maj, M., Singh, B., and Gupta, R. S. (2000). The influence of inorganic phosphate on the activity of adenosine kinase. Biochim Biophys Acta *1476*, 33-42.

Maj, M. C., Singh, B., and Gupta, R. S. (2002). Pentavalent ions dependency is a conserved property of adenosine kinase from diverse sources: identification of a novel motif implicated in phosphate and magnesium ion binding and substrate inhibition. Biochemistry *41*, 4059-4069.

Maldonado-Mendoza, I. E., Dewbre, G. R., and Harrison, M. J. (2001). A phosphate transporter gene from the extra-radical mycelium of an arbuscular mycorrhizal fungus Glomus intraradices is regulated in response to phosphate in the environment. Mol Plant Microbe Interact *14*, 1140-1148.

Malik, S., and Roeder, R. G. (2000). Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. Trends Biochem Sci *25*, 277-283.

Manning, G., Plowman, G. D., Hunter, T., and Sudarsanam, S. (2002). Evolution of protein kinase signaling from yeast to man. Trends Biochem Sci *27*, 514-520.

Martinez, P., and Persson, B. L. (1998). Identification, cloning and characterization of a derepressible Na+-coupled phosphate transporter in Saccharomyces cerevisiae. Mol Gen Genet *258*, 628-638.

Martinez, P., Zvyagilskaya, R., Allard, P., and Persson, B. L. (1998). Physiological regulation of the derepressible phosphate transporter in Saccharomyces cerevisiae. J Bacteriol *180*, 2253-2256.

Matsumoto, K., Uno, I., and Ishikawa, T. (1984). Identification of the structural gene and nonsense alleles for adenylate cyclase in Saccharomyces cerevisiae. J Bacteriol *157*, 277-282.

Matsumoto, K., Uno, I., and Ishikawa, T. (1985). Genetic analysis of the role of cAMP in yeast. Yeast *1*, 15-24.

Mayordomo, I., and Sanz, P. (2001). Hexokinase PII: structural analysis and glucose signalling in the yeast Saccharomyces cerevisiae. Yeast *18*, 923-930.

Mbonyi, K., Beullens, M., Detremerie, K., Geerts, L., and Thevelein, J. M. (1988). Requirement of one functional RAS gene and inability of an oncogenic ras variant to mediate the glucose-induced cyclic AMP signal in the yeast Saccharomyces cerevisiae. Mol Cell Biol *8*, 3051-3057.

Mbonyi, K., van Aelst, L., Arguelles, J. C., Jans, A. W., and Thevelein, J. M. (1990). Glucose-induced hyperaccumulation of cyclic AMP and defective glucose repression in yeast strains with reduced activity of cyclic AMP-dependent protein kinase. Mol Cell Biol *10*, 4518-4523.

McBride, H. J., Sil, A., Measday, V., Yu, Y., Moffat, J., Maxon, M. E., Herskowitz, I., Andrews, B., and Stillman, D. J. (2001). The protein kinase Pho85 is required for asymmetric accumulation of the Ash1 protein in Saccharomyces cerevisiae. Mol Microbiol *42*, 345-353.

McCartney, R. R., Rubenstein, E. M., and Schmidt, M. C. (2005). Snf1 kinase complexes with different beta subunits display stress-dependent preferences for the three Snf1-activating kinases. Curr Genet *47*, 335-344.

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

McDonald, A. E., Niere, J. O., and Plaxton, W. C. (2001). Phosphite disrupts the acclimation of Saccharomyces cerevisiae to phosphate starvation. Can J Microbiol *47*, 969-978.

Measday, V., McBride, H., Moffat, J., Stillman, D., and Andrews, B. (2000). Interactions between Pho85 cyclin-dependent kinase complexes and the Swi5 transcription factor in budding yeast. Mol Microbiol *35*, 825-834.

Measday, V., Moore, L., Ogas, J., Tyers, M., and Andrews, B. (1994). The PCL2 (ORFD)-PHO85 cyclin-dependent kinase complex: a cell cycle regulator in yeast. Science *266*, 1391-1395.

Measday, V., Moore, L., Retnakaran, R., Lee, J., Donoviel, M., Neiman, A. M., and Andrews, B. (1997). A family of cyclin-like proteins that interact with the Pho85 cyclin-dependent kinase. Mol Cell Biol *17*, 1212-1223.

Meimoun, A., Holtzman, T., Weissman, Z., McBride, H. J., Stillman, D. J., Fink, G. R., and Kornitzer, D. (2000). Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex. Mol Biol Cell *11*, 915-927.

Mendenhall, M. D., and Hodge, A. E. (1998). Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev *62*, 1191-1243.

Mitchelhill, K. I., Stapleton, D., Gao, G., House, C., Michell, B., Katsis, F., Witters, L. A., and Kemp, B. E. (1994). Mammalian AMP-activated protein kinase shares structural and functional homology with the catalytic domain of yeast Snf1 protein kinase. J Biol Chem *269*, 2361-2364.

Miyakawa, Y. (2000). Identification of a Candida albicans homologue of the PHO85 gene, a negative regulator of the PHO system in Saccharomyces cerevisiae. Yeast *16*, 1045-1051.

Moffat, J., and Andrews, B. (2004). Late-G1 cyclin-CDK activity is essential for control of cell morphogenesis in budding yeast. Nat Cell Biol *6*, 59-66.

Moskvina, E., Schuller, C., Maurer, C. T., Mager, W. H., and Ruis, H. (1998). A search in the genome of Saccharomyces cerevisiae for genes regulated via stress response elements. Yeast *14*, 1041-1050.

Mouillon, J. M., and Persson, B. L. (2005). Inhibition of the protein kinase A alters the degradation of the high-affinity phosphate transporter Pho84 in Saccharomyces cerevisiae. Curr Genet *48*, 226-234.

Mouillon, J. M., and Persson, B. L. (2006). New aspects on phosphate sensing and signalling in Saccharomyces cerevisiae. FEMS Yeast Res *6*, 171-176.

Mulroney, S. E., Woda, C. B., Halaihel, N., Louie, B., McDonnell, K., Schulkin, J., Haramati, A., and Levi, M. (2004). Central control of renal sodium-phosphate (NaPi-2) transporters. Am J Physiol Renal Physiol *286*, F647-652.

Nagiec, M. M., Skrzypek, M., Nagiec, E. E., Lester, R. L., and Dickson, R. C. (1998). The LCB4 (YOR171c) and LCB5 (YLR260w) genes of Saccharomyces encode sphingoid long chain base kinases. J Biol Chem *273*, 19437-19442.

Nakafuku, M., Obara, T., Kaibuchi, K., Miyajima, I., Miyajima, A., Itoh, H., Nakamura, S., Arai, K., Matsumoto, K., and Kaziro, Y. (1988). Isolation of a second yeast Saccharomyces cerevisiae gene (GPA2) coding for guanine nucleotide-binding regulatory protein: studies on its structure and possible functions. Proc Natl Acad Sci U S A *85*, 1374-1378.

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

Neef, D. W., and Kladde, M. P. (2003). Polyphosphate loss promotes SNF/SWI- and Gcn5-dependent mitotic induction of PHO5. Mol Cell Biol *23*, 3788-3797.

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.

Nelissen, B., Mordant, P., Jonniaux, J. L., De Wachter, R., and Goffeau, A. (1995). Phylogenetic classification of the major superfamily of membrane transport facilitators, as deduced from yeast genome sequencing. FEBS Lett *377*, 232-236.

Neves, M. J., Terenzi, H.F., Leone, F.A., and Jorge, J.A. (1994). Quantification of trehalose in biological samples with a conidial trehalase from the thermophilic fungus Humicola grisea var Thermoidea. World J Microbiol Biotechnol *10*, 17-19.

Nigavekar, S. S., Tan, Y. S., and Cannon, J. F. (2002). Glc8 is a glucose-repressible activator of Glc7 protein phosphatase-1. Arch Biochem Biophys *404*, 71-79.

Nikawa, J., Cameron, S., Toda, T., Ferguson, K. M., and Wigler, M. (1987). Rigorous feedback control of cAMP levels in Saccharomyces cerevisiae. Genes Dev *1*, 931-937.

Nishizawa, M., Kanaya, Y., and Toh, E. A. (1999a). Mouse cyclin-dependent kinase (Cdk) 5 is a functional homologue of a yeast Cdk, pho85 kinase. J Biol Chem *274*, 33859-33862.

Nishizawa, M., Katou, Y., Shirahige, K., and Toh-e, A. (2004). Yeast Pho85 kinase is required for proper gene expression during the diauxic shift. Yeast *21*, 903-918.

Nishizawa, M., Kawasumi, M., Fujino, M., and Toh-e, A. (1998). Phosphorylation of sic1, a cyclin-dependent kinase (Cdk) inhibitor, by Cdk including Pho85 kinase is required for its prompt degradation. Mol Biol Cell *9*, 2393-2405.

Nishizawa, M., Suzuki, K., Fujino, M., Oguchi, T., and Toh-e, A. (1999b). The Pho85 kinase, a member of the yeast cyclin-dependent kinase (Cdk) family, has a regulation mechanism different from Cdks functioning throughout the cell cycle. Genes Cells *4*, 627-642.

Nishizawa, M., Tanabe, M., Yabuki, N., Kitada, K., and Toh, E. A. (2001). Pho85 kinase, a yeast cyclin-dependent kinase, regulates the expression of UGP1 encoding UDP-glucose pyrophosphorylase. Yeast *18*, 239-249.

Norbeck, J., Pahlman, A. K., Akhtar, N., Blomberg, A., and Adler, L. (1996). Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from Saccharomyces cerevisiae. Identification of the corresponding GPP1 and GPP2 genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. J Biol Chem *271*, 13875-13881.

Nourani, A., Utley, R. T., Allard, S., and Cote, J. (2004). Recruitment of the NuA4 complex poises the PHO5 promoter for chromatin remodeling and activation. Embo J *23*, 2597-2607.

Nwaka, S., and Holzer, H. (1998). Molecular biology of trehalose and the trehalases in the yeast Saccharomyces cerevisiae. Prog Nucleic Acid Res Mol Biol *58*, 197-237.

Nwaka, S., Mechler, B., Destruelle, M., and Holzer, H. (1995). Phenotypic features of trehalase mutants in Saccharomyces cerevisiae. FEBS Lett *360*, 286-290.

Nwaka, S., Mechler, B., and Holzer, H. (1996). Deletion of the ATH1 gene in Saccharomyces cerevisiae prevents growth on trehalose. FEBS Lett *386*, 235-238.

Ogas, J., Andrews, B. J., and Herskowitz, I. (1991). Transcriptional activation of CLN1, CLN2, and a putative new G1 cyclin (HCS26) by SWI4, a positive regulator of G1-specific transcription. Cell *66*, 1015-1026.

Ogawa, N., DeRisi, J., and Brown, P. O. (2000). New components of a system for phosphate accumulation and polyphosphate metabolism in Saccharomyces cerevisiae revealed by genomic expression analysis. Mol Biol Cell *11*, 4309-4321.

Ogawa, N., Noguchi, K., Sawai, H., Yamashita, Y., Yompakdee, C., and Oshima, Y. (1995). Functional domains of Pho81p, an inhibitor of Pho85p protein kinase, in the transduction pathway of Pi signals in Saccharomyces cerevisiae. Mol Cell Biol *15*, 997-1004.

Ogawa, N., Noguchi, K., Yamashita, Y., Yasuhara, T., Hayashi, N., Yoshida, K., and Oshima, Y. (1993). Promoter analysis of the PHO81 gene encoding a 134 kDa protein bearing ankyrin repeats in the phosphatase regulon of Saccharomyces cerevisiae. Mol Gen Genet *238*, 444-454.

Ogawa, N., and Oshima, Y. (1990). Functional domains of a positive regulatory protein, PHO4, for transcriptional control of the phosphatase regulon in Saccharomyces cerevisiae. Mol Cell Biol *10*, 2224-2236.

O'Neill, E. M., Kaffman, A., Jolly, E. R., and O'Shea, E. K. (1996). Regulation of PHO4 nuclear localization by the PHO80-PHO85 cyclin-CDK complex. Science *271*, 209-212.

Ortiz, C. H., Maia, J. C., Tenan, M. N., Braz-Padrao, G. R., Mattoon, J. R., and Panek, A. D. (1983). Regulation of yeast trehalase by a monocyclic, cyclic AMP-dependent phosphorylation-dephosphorylation cascade system. J Bacteriol *153*, 644-651.

Oshima, Y. (1997). The phosphatase system in Saccharomyces cerevisiae. Genes Genet Syst *72*, 323-334.

Pan, X., and Heitman, J. (1999). Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in Saccharomyces cerevisiae. Mol Cell Biol *19*, 4874-4887.

Panek, A. C., Araujo, P. S., Poppe, S. C., and Panek, A. D. (1990). On the determination of trehalose-6-phosphate synthase in Saccharomyces. Biochem Int *21*, 695-704.

Panek, A. C., de Araujo, P. S., Moura Neto, V., and Panek, A. D. (1987). Regulation of the trehalose-6-phosphate synthase complex in Saccharomyces. I. Interconversion of forms by phosphorylation. Curr Genet *11*, 459-465.

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

Papamichos-Chronakis, M., Gligoris, T., and Tzamarias, D. (2004). The Snf1 kinase controls glucose repression in yeast by modulating interactions between the Mig1 repressor and the Cyc8-Tup1 co-repressor. EMBO Rep *5*, 368-372.

Pardons, K. (2005) Dissecting novel regulatory mechanisms for glucose-induced cAMP signalling in *Saccharomyces cerevisiae*., PhD thesis, Laboratory of Functional Biology, Katholieke Universiteit Leuven.

Park, J. I., Grant, C. M., and Dawes, I. W. (2005). The high-affinity cAMP phosphodiesterase of Saccharomyces cerevisiae is the major determinant of cAMP levels in stationary phase: involvement of different branches of the Ras-cyclic AMP pathway in stress responses. Biochem Biophys Res Commun *327*, 311-319.

Parrou, J. L., Enjalbert, B., Plourde, L., Bauche, A., Gonzalez, B., and Francois, J. (1999). Dynamic responses of reserve carbohydrate metabolism under carbon and nitrogen limitations in Saccharomyces cerevisiae. Yeast *15*, 191-203.

Parrou, J. L., Teste, M. A., and Francois, J. (1997). Effects of various types of stress on the metabolism of reserve carbohydrates in Saccharomyces cerevisiae: genetic evidence for a stress-induced recycling of glycogen and trehalose. Microbiology *143 (Pt 6)*, 1891-1900.

Pattison-Granberg, J., and Persson, B. L. (2000). Regulation of cation-coupled high-affinity phosphate uptake in the yeast Saccharomyces cerevisiae. J Bacteriol *182*, 5017-5019.

Patton-Vogt, J. L., and Henry, S. A. (1998). GIT1, a gene encoding a novel transporter for glycerophosphoinositol in Saccharomyces cerevisiae. Genetics *149*, 1707-1715.

Paulsen, I. T., Sliwinski, M. K., Nelissen, B., Goffeau, A., and Saier, M. H., Jr. (1998). Unified inventory of established and putative transporters encoded within the complete genome of Saccharomyces cerevisiae. FEBS Lett *430*, 116-125.

Pederson, B. A., Cheng, C., Wilson, W. A., and Roach, P. J. (2000). Regulation of glycogen synthase. Identification of residues involved in regulation by the allosteric ligand glucose-6-P and by phosphorylation. J Biol Chem *275*, 27753-27761.

Pederson, B. A., Wilson, W. A., and Roach, P. J. (2004). Glycogen synthase sensitivity to glucose-6-P is important for controlling glycogen accumulation in Saccharomyces cerevisiae. J Biol Chem *279*, 13764-13768.

Pedruzzi, I., Burckert, N., Egger, P., and De Virgilio, C. (2000). Saccharomyces cerevisiae Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1. Embo J *19*, 2569-2579.

Pedruzzi, I., Dubouloz, F., Cameroni, E., Wanke, V., Roosen, J., Winderickx, J., and De Virgilio, C. (2003). TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into G0. Mol Cell *12*, 1607-1613.

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.

Pernambuco, M. B., Winderickx, J., Crauwels, M., Griffioen, G., Mager, W. H., and Thevelein, J. M. (1996). Glucose-triggered signalling in Saccharomyces cerevisiae: different requirements for sugar phosphorylation between cells grown on glucose and those grown on non-fermentable carbon sources. Microbiology *142 (Pt 7)*, 1775-1782.

Persson, B. L., Berhe, A., Fristedt, U., Martinez, P., Pattison, J., Petersson, J., and Weinander, R. (1998). Phosphate permeases of Saccharomyces cerevisiae. Biochim Biophys Acta *1365*, 23-30.

Persson, B. L., Lagerstedt, J. O., Pratt, J. R., Pattison-Granberg, J., Lundh, K., Shokrollahzadeh, S., and Lundh, F. (2003). Regulation of phosphate acquisition in Saccharomyces cerevisiae. Curr Genet *43*, 225-244.

Persson, B. L., Petersson, J., Fristedt, U., Weinander, R., Berhe, A., and Pattison, J. (1999). Phosphate permeases of Saccharomyces cerevisiae: structure, function and regulation. Biochim Biophys Acta *1422*, 255-272.

Pestov, N. A., Kulakovskaya, T. V., and Kulaev, I. S. (2005). Effects of inactivation of the PPN1 gene on exopolyphosphatases, inorganic polyphosphates and function of mitochondria in the yeast Saccharomyces cerevisiae. FEMS Yeast Res *5*, 823-828.

Petersson, J., Pattison, J., Kruckeberg, A. L., Berden, J. A., and Persson, B. L. (1999). Intracellular localization of an active green fluorescent protein-tagged Pho84 phosphate permease in Saccharomyces cerevisiae. FEBS Lett *462*, 37-42.

Pick, U., Bental, M., Chitlaru, E., and Weiss, M. (1990). Polyphosphate-hydrolysis--a protective mechanism against alkaline stress? FEBS Lett *274*, 15-18.

Pinson, B., Merle, M., Franconi, J. M., and Daignan-Fornier, B. (2004). Low affinity orthophosphate carriers regulate PHO gene expression independently of internal orthophosphate concentration in Saccharomyces cerevisiae. J Biol Chem *279*, 35273-35280.

Pisoni, R. L., and Lindley, E. R. (1992). Incorporation of [32P]orthophosphate into long chains of inorganic polyphosphate within lysosomes of human fibroblasts. J Biol Chem *267*, 3626-3631.

Plankert, U., Purwin, C., and Holzer, H. (1991). Yeast fructose-2,6-bisphosphate 6-phosphatase is encoded by PHO8, the gene for nonspecific repressible alkaline phosphatase. Eur J Biochem *196*, 191-196.

Plaxton, W. C. (2004). Plant Response to Stress: Biochemical Adaptations to Phosphate Deficiency. In Encyclopedia of Plant and Crop Science (Marcel Dekker, Inc.), pp. 976-980.

Plourde-Owobi, L., Durner, S., Parrou, J. L., Wieczorke, R., Goma, G., and Francois, J. (1999). AGT1, encoding an alpha-glucoside transporter involved in uptake and intracellular accumulation of trehalose in Saccharomyces cerevisiae. J Bacteriol *181*, 3830-3832.

Poon, R. Y., Lew, J., and Hunter, T. (1997). Identification of functional domains in the neuronal Cdk5 activator protein. J Biol Chem *272*, 5703-5708.

Popova Iu, G., Padkina, M. V., and Sambuk, E. V. (2000). [Effect of mutations in PHO85 and PHO4 genes on utilization of proline in Saccharomyces cerevisiae yeasts]. Genetika *36*, 1622-1628.

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

Pratt, J. R., Mouillon, J. M., Lagerstedt, J. O., Pattison-Granberg, J., Lundh, K. I., and Persson, B. L. (2004). Effects of methylphosphonate, a phosphate analogue, on the expression and degradation of the high-affinity phosphate transporter Pho84, in Saccharomyces cerevisiae. Biochemistry *43*, 14444-14453.

Pries, R., Bomeke, K., Irniger, S., Grundmann, O., and Braus, G. H. (2002). Amino acid-dependent Gcn4p stability regulation occurs exclusively in the yeast nucleus. Eukaryot Cell *1*, 663-672.

Proud, C. G., Hundal, H. S., and Taylor, P. M. (2004). Nutrient sensing in animal cells and integration of nutrient and endocrine signalling pathways. In Nutrient-Induced Responses in Eukaryotic Cells, J. Winderickx, and P. M. Taylor, eds. (Topics in Current Genetics), pp. 25-64.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R*., et al.* (2005). Global analysis of protein phosphorylation in yeast. Nature *438*, 679-684.

Quarles, L. D. (2003). Evidence for a bone-kidney axis regulating phosphate homeostasis. J Clin Invest *112*, 642-646.

Randez-Gil, F., Bojunga, N., Proft, M., and Entian, K. D. (1997). Glucose derepression of gluconeogenic enzymes in Saccharomyces cerevisiae correlates with phosphorylation of the gene activator Cat8p. Mol Cell Biol *17*, 2502-2510.

Randez-Gil, F., Herrero, P., Sanz, P., Prieto, J. A., and Moreno, F. (1998). Hexokinase PII has a double cytosolic-nuclear localisation in Saccharomyces cerevisiae. FEBS Lett *425*, 475-478.

Rausch, C., and Bucher, M. (2002). Molecular mechanisms of phosphate transport in plants. Planta *216*, 23-37.

Reinders, A., Burckert, N., Boller, T., Wiemken, A., and De Virgilio, C. (1998). Saccharomyces cerevisiae cAMP-dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase. Genes Dev *12*, 2943-2955.

Requena, N. (2005). Measuring quality of service: phosphate 'a la carte' by arbuscular mycorrhizal fungi. New Phytol *168*, 268-271.

Robertson, L. S., Causton, H. C., Young, R. A., and Fink, G. R. (2000). The yeast A kinases differentially regulate iron uptake and respiratory function. Proc Natl Acad Sci U S A *97*, 5984-5988.

Robertson, L. S., and Fink, G. R. (1998). The three yeast A kinases have specific signaling functions in pseudohyphal growth. Proc Natl Acad Sci U S A *95*, 13783-13787.

Robinson, K. A., and Lopes, J. M. (2000). SURVEY AND SUMMARY: Saccharomyces cerevisiae basic helix-loop-helix proteins regulate diverse biological processes. Nucleic Acids Res *28*, 1499-1505.

Rolland, F., De Winde, J. H., Lemaire, K., Boles, E., Thevelein, J. M., and Winderickx, J. (2000). Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. Mol Microbiol *38*, 348-358.

Rolland, F., Wanke, V., Cauwenberg, L., Ma, P., Boles, E., Vanoni, M., de Winde, J. H., Thevelein, J. M., and Winderickx, J. (2001). The role of hexose transport and phosphorylation in cAMP signalling in the yeast Saccharomyces cerevisiae. FEMS Yeast Res *1*, 33-45.

Ronne, H. (1995). Glucose repression in fungi. Trends Genet *11*, 12-17.

Roomans, G. M., Blasco, F., and Borst-Pauwels, G. W. (1977). Cotransport of phosphate and sodium by yeast. Biochim Biophys Acta *467*, 65-71.

Roosen, J., Engelen, K., Marchal, K., Mathys, J., Griffioen, G., Cameroni, E., Thevelein, J. M., De Virgilio, C., De Moor, B., and Winderickx, J. (2005). PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability. Mol Microbiol *55*, 862-880.

Roosen, J., Oesterhelt, C., Pardons, K., Swinnen, E., and Winderickx, J. (2004). Integration of nutrient signalling pathways in the yeast *Saccharomyces cerevisiae*. In Nutrient-Induced Responses in Eukaryotic Cells, J. Winderickx, and P. M. Taylor, eds. (Topics in Current Cenetics), pp. 277-318.

Rosales, J. L., Nodwell, M. J., Johnston, R. N., and Lee, K. Y. (2000). Cdk5/p25(nck5a) interaction with synaptic proteins in bovine brain. J Cell Biochem *78*, 151-159.

Rotin, D., Staub, O., and Haguenauer-Tsapis, R. (2000). Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. J Membr Biol *176*, 1-17.

Rudoni, S., Colombo, S., Coccetti, P., and Martegani, E. (2001). Role of guanine nucleotides in the regulation of the Ras/cAMP pathway in Saccharomyces cerevisiae. Biochim Biophys Acta *1538*, 181-189.

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

Rutter, J., Michnoff, C. H., Harper, S. M., Gardner, K. H., and McKnight, S. L. (2001). PAS kinase: an evolutionarily conserved PAS domain-regulated serine/threonine kinase. Proc Natl Acad Sci U S A *98*, 8991-8996.

Rutter, J., Probst, B. L., and McKnight, S. L. (2002). Coordinate regulation of sugar flux and translation by PAS kinase. Cell *111*, 17-28.

Saito, K., Ohtomo, R., Kuga-Uetake, Y., Aono, T., and Saito, M. (2005). Direct Labeling of Polyphosphate at the Ultrastructural Level in Saccharomyces cerevisiae by Using the Affinity of the Polyphosphate Binding Domain of Escherichia coli Exopolyphosphatase. Appl Environ Microbiol *71*, 5692-5701.

Salaun, C., Rodrigues, P., and Heard, J. M. (2001). Transmembrane topology of PiT-2, a phosphate transporter-retrovirus receptor. J Virol *75*, 5584-5592.

Saldanha, A. J., Brauer, M. J., and Botstein, D. (2004). Nutritional homeostasis in batch and steady-state culture of yeast. Mol Biol Cell *15*, 4089-4104.

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

Sass, P., Field, J., Nikawa, J., Toda, T., and Wigler, M. (1986). Cloning and characterization of the high-affinity cAMP phosphodiesterase of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *83*, 9303-9307.

Schmelzle, T., Beck, T., Martin, D. E., and Hall, M. N. (2004). Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. Mol Cell Biol *24*, 338-351.

Schmelzle, T., and Hall, M. N. (2000). TOR, a central controller of cell growth. Cell *103*, 253-262.

Schmidt, M. C., and McCartney, R. R. (2000). beta-subunits of Snf1 kinase are required for kinase function and substrate definition. Embo J *19*, 4936-4943.

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

Schneider, K. R., Smith, R. L., and O'Shea, E. K. (1994). Phosphate-regulated inactivation of the kinase PHO80-PHO85 by the CDK inhibitor PHO81. Science *266*, 122-126.

Schomerus, C., and Kuntzel, H. (1992). CDC25-dependent induction of inositol 1,4,5-trisphosphate and diacylglycerol in Saccharomyces cerevisiae by nitrogen. FEBS Lett *307*, 249-252.

Schuddemat, J., de Boo, R., van Leeuwen, C. C., van den Broek, P. J., and van Steveninck, J. (1989). Polyphosphate synthesis in yeast. Biochim Biophys Acta *1010*, 191-198.

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

Serrano, R., Ruiz, A., Bernal, D., Chambers, J. R., and Arino, J. (2002). The transcriptional response to alkaline pH in Saccharomyces cerevisiae: evidence for calcium-mediated signalling. Mol Microbiol *46*, 1319-1333.

Sethuraman, A., Rao, N. N., and Kornberg, A. (2001). The endopolyphosphatase gene: essential in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *98*, 8542-8547.

Shao, D., Creasy, C. L., and Bergman, L. W. (1998). A cysteine residue in helixII of the bHLH domain is essential for homodimerization of the yeast transcription factor Pho4p. Nucleic Acids Res *26*, 710-714.

Shemer, R., Meimoun, A., Holtzman, T., and Kornitzer, D. (2002). Regulation of the transcription factor Gcn4 by Pho85 cyclin PCL5. Mol Cell Biol *22*, 5395-5404.

Shi, X., and Kornberg, A. (2005). Endopolyphosphatase in Saccharomyces cerevisiae undergoes post-translational activations to produce short-chain polyphosphates. FEBS Lett *579*, 2014-2018.

Shin, H., Shin, H. S., Dewbre, G. R., and Harrison, M. J. (2004). Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J *39*, 629-642.

Shirahama, K., Yazaki, Y., Sakano, K., Wada, Y., and Ohsumi, Y. (1996). Vacuolar function in the phosphate homeostasis of the yeast Saccharomyces cerevisiae. Plant Cell Physiol *37*, 1090-1093.

Sillje, H. H., Paalman, J. W., ter Schure, E. G., Olsthoorn, S. Q., Verkleij, A. J., Boonstra, J., and Verrips, C. T. (1999). Function of trehalose and glycogen in cell cycle progression and cell viability in Saccharomyces cerevisiae. J Bacteriol *181*, 396-400.

Sillje, H. H., ter Schure, E. G., Rommens, A. J., Huls, P. G., Woldringh, C. L., Verkleij, A. J., Boonstra, J., and Verrips, C. T. (1997). Effects of different carbon fluxes on G1 phase duration, cyclin expression, and reserve carbohydrate metabolism in Saccharomyces cerevisiae. J Bacteriol *179*, 6560-6565.

Silver, J. (2001). Cycling with the parathyroid. J Clin Invest *107*, 1079-1080.

Silver, J., and Dranitzki-Elhalel, M. (2003). Sensing phosphate across the kingdoms. Curr Opin Nephrol Hypertens *12*, 357-361.

Simon, I., Barnett, J., Hannett, N., Harbison, C. T., Rinaldi, N. J., Volkert, T. L., Wyrick, J. J., Zeitlinger, J., Gifford, D. K., Jaakkola, T. S., and Young, R. A. (2001). Serial regulation of transcriptional regulators in the yeast cell cycle. Cell *106*, 697-708.

Singer, M. A., and Lindquist, S. (1998). Thermotolerance in Saccharomyces cerevisiae: the Yin and Yang of trehalose. Trends Biotechnol *16*, 460-468.

Singh, K. K., Rasmussen, A. K., and Rasmussen, L. J. (2004). Genome-wide analysis of signal transducers and regulators of mitochondrial dysfunction in Saccharomyces cerevisiae. Ann N Y Acad Sci *1011*, 284-298.

Smith, A., Ward, M. P., and Garrett, S. (1998). Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. Embo J *17*, 3556-3564.

Smith, D. S., and Tsai, L. H. (2002). Cdk5 behind the wheel: a role in trafficking and transport? Trends Cell Biol *12*, 28-36.

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

Sopko, R., Huang, D., Preston, N., Chua, G., Papp, B., Kafadar, K., Snyder, M., Oliver, S. G., Cyert, M., Hughes, T. R., *et al.* (2006). Mapping Pathways and Phenotypes by Systematic Gene Overexpression. Mol Cell *21*, 319-330.

Souza, A. C., De Mesquita, J. F., Panek, A. D., Silva, J. T., and Paschoalin, V. M. (2002). Evidence for a modulation of neutral trehalase activity by Ca2+ and cAMP signaling pathways in Saccharomyces cerevisiae. Braz J Med Biol Res *35*, 11-16.

Spain, B. H., Koo, D., Ramakrishnan, M., Dzudzor, B., and Colicelli, J. (1995). Truncated forms of a novel yeast protein suppress the lethality of a G protein alpha subunit deficiency by interacting with the beta subunit. J Biol Chem *270*, 25435-25444.

Springer, M., Wykoff, D. D., Miller, N., and O'Shea, E. K. (2003). Partially phosphorylated Pho4 activates transcription of a subset of phosphate-responsive genes. PLoS Biol *1*, 261-270.

Steger, D. J., Haswell, E. S., Miller, A. L., Wente, S. R., and O'Shea, E. K. (2003). Regulation of chromatin remodeling by inositol polyphosphates. Science *299*, 114-116.

Sugajska, E., Swiatek, W., Zabrocki, P., Geyskens, I., Thevelein, J. M., Zolnierowicz, S., and Wera, S. (2001). Multiple effects of protein phosphatase 2A on nutrient-induced signalling in the yeast Saccharomyces cerevisiae. Mol Microbiol *40*, 1020-1026.

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

Sweeny, J. M., Seibert, H. E., Woda, C., Schulkin, J., Haramati, A., and Mulroney, S. E. (1998). Evidence for induction of a phosphate appetite in juvenile rats. Am J Physiol *275*, R1358-1365.

Swinnen, E., Rosseels, J., and Winderickx, J. (2005). The minimum domain of Pho81 is not sufficient to control the Pho85-Rim15 effector branch involved in phosphate starvation-induced stress responses. Curr Genet *48*, 18-33.

Takeda, E., Taketani, Y., Sawada, N., Sato, T., and Yamamoto, H. (2004a). The regulation and function of phosphate in the human body. Biofactors *21*, 345-355.

Takeda, E., Yamamoto, H., Nashiki, K., Sato, T., Arai, H., and Taketani, Y. (2004b). Inorganic phosphate homeostasis and the role of dietary phosphorus. J Cell Mol Med *8*, 191-200.

Tamai, Y., Toh-e, A., and Oshima, Y. (1985). Regulation of inorganic phosphate transport systems in Saccharomyces cerevisiae. J Bacteriol *164*, 964-968.

Tan, Y. S., Morcos, P. A., and Cannon, J. F. (2003). Pho85 phosphorylates the Glc7 protein phosphatase regulator Glc8 in vivo. J Biol Chem *278*, 147-153.

Tanaka, K., Lin, B. K., Wood, D. R., and Tamanoi, F. (1991). IRA2, an upstream negative regulator of RAS in yeast, is a RAS GTPase-activating protein. Proc Natl Acad Sci U S A *88*, 468-472.

Tanaka, K., Matsumoto, K., and Toh, E. A. (1989). IRA1, an inhibitory regulator of the RAS-cyclic AMP pathway in Saccharomyces cerevisiae. Mol Cell Biol *9*, 757-768.

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

Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K., and Toh-e, A. (1990b). IRA2, a second gene of Saccharomyces cerevisiae that encodes a protein with a domain homologous to mammalian ras GTPase-activating protein. Mol Cell Biol *10*, 4303-4313.

Taylor, B. L., and Zhulin, I. B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. Microbiol Mol Biol Rev *63*, 479-506.

Tenenhouse, H. S. (2005). Regulation of phosphorus homeostasis by the type iia na/phosphate cotransporter. Annu Rev Nutr *25*, 197-214.

Tennyson, C. N., Lee, J., and Andrews, B. J. (1998). A role for the Pcl9-Pho85 cyclin-cdk complex at the M/G1 boundary in Saccharomyces cerevisiae. Mol Microbiol *28*, 69-79.

Teusink, B., Walsh, M. C., van Dam, K., and Westerhoff, H. V. (1998). The danger of metabolic pathways with turbo design. Trends Biochem Sci *23*, 162-169.

Thevelein, J. M. (1991). Fermentable sugars and intracellular acidification as specific activators of the RAS-adenylate cyclase signalling pathway in yeast: the relationship to nutrient-induced cell cycle control. Mol Microbiol *5*, 1301-1307.

Thevelein, J. M. (1992). The RAS-adenylate cyclase pathway and cell cycle control in Saccharomyces cerevisiae. Antonie Van Leeuwenhoek *62*, 109-130.

Thevelein, J. M. (1994). Signal transduction in yeast. Yeast *10*, 1753-1790.

Thevelein, J. M., Cauwenberg, L., Colombo, S., De Winde, J. H., Donation, M., Dumortier, F., Kraakman, L., Lemaire, K., Ma, P., Nauwelaers, D*., et al.* (2000). Nutrient-induced signal transduction through the protein kinase A pathway and its role in the control of metabolism, stress resistance, and growth in yeast. Enzyme Microb Technol *26*, 819-825.

Thevelein, J. M., and de Winde, J. H. (1999). Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast Saccharomyces cerevisiae. Mol Microbiol *33*, 904-918.

Thevelein, J. M., and Hohmann, S. (1995). Trehalose synthase: guard to the gate of glycolysis in yeast? Trends Biochem Sci *20*, 3-10.

Thomas, M. R., and O'Shea, E. K. (2005). An intracellular phosphate buffer filters transient fluctuations in extracellular phosphate levels. Proc Natl Acad Sci U S A *102*, 9565-9570.

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

Ticconi, C. A., and Abel, S. (2004). Short on phosphate: plant surveillance and countermeasures. Trends Plant Sci *9*, 548-555.

Timblin, B. K., and Bergman, L. W. (1997). Elevated expression of stress response genes resulting from deletion of the PHO85 gene. Mol Microbiol *26*, 981-990.

Timblin, B. K., Tatchell, K., and Bergman, L. W. (1996). Deletion of the gene encoding the cyclin-dependent protein kinase Pho85 alters glycogen metabolism in Saccharomyces cerevisiae. Genetics *143*, 57-66.

Toda, T., Cameron, S., Sass, P., and Wigler, M. (1988). SCH9, a gene of Saccharomyces cerevisiae that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. Genes Dev *2*, 517-527.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D., McMullen, B., Hurwitz, M., Krebs, E. G., and Wigler, M. (1987a). Cloning and characterization of BCY1, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in Saccharomyces cerevisiae. Mol Cell Biol *7*, 1371-1377.

Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987b). Three different genes in S. cerevisiae encode the catalytic subunits of the cAMP-dependent protein kinase. Cell *50*, 277-287.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985). In yeast, RAS proteins are controlling elements of adenylate cyclase. Cell *40*, 27-36.

Tomizawa, K., Sunada, S., Lu, Y. F., Oda, Y., Kinuta, M., Ohshima, T., Saito, T., Wei, F. Y., Matsushita, M., Li, S. T*., et al.* (2003). Cophosphorylation of amphiphysin I and dynamin I by Cdk5 regulates clathrin-mediated endocytosis of synaptic vesicles. J Cell Biol *163*, 813-824.

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

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

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

Tu, J., Song, W., and Carlson, M. (1996). Protein phosphatase type 1 interacts with proteins required for meiosis and other cellular processes in Saccharomyces cerevisiae. Mol Cell Biol *16*, 4199-4206.

Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P*., et al.* (2000). A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature *403*, 623-627.

Unnikrishnan, I., Miller, S., Meinke, M., and LaPorte, D. C. (2003). Multiple positive and negative elements involved in the regulation of expression of GSY1 in Saccharomyces cerevisiae. J Biol Chem *278*, 26450-26457.

Uno, I., Matsumoto, K., Adachi, K., and Ishikawa, T. (1983). Genetic and biochemical evidence that trehalase is a substrate of cAMP-dependent protein kinase in yeast. J Biol Chem *258*, 10867-10872.

Urech, K., Durr, M., Boller, T., Wiemken, A., and Schwencke, J. (1978). Localization of polyphosphate in vacuoles of Saccharomyces cerevisiae. Arch Microbiol *116*, 275-278.

Vagabov, V. M., Trilisenko, L. V., and Kulaev, I. S. (2000). Dependence of inorganic polyphosphate chain length on the orthophosphate content in the culture medium of the yeast Saccharomyces cerevisiae. Biochemistry (Mosc) *65*, 349-354.

Vagabov, V. M., Trilisenko, L. V., Shchipanova, I. N., Sibel'dina, L. A., and Kulaev, I. S. (1998). [Change in inorganic polyphosphate chain length depending on the stage of Saccharomyces cerevisiae growth]. Mikrobiologiia *67*, 188-193.

Vallier, L. G., and Carlson, M. (1994). Synergistic release from glucose repression by mig1 and ssn mutations in Saccharomyces cerevisiae. Genetics *137*, 49-54.

Van Belle, D., and Andre, B. (2001). A genomic view of yeast membrane transporters. Curr Opin Cell Biol *13*, 389-398.

Van Nuland, A., Vandormael, P., Donaton, M., Alenquer, M., Lourenco, A., Quintino, E., Versele, M., and Thevelein, J. M. (2006). Ammonium permease-based sensing mechanism for rapid ammonium activation of the protein kinase A pathway in yeast. Mol Microbiol *59*, 1485-1505.

Vandebroek, T., Vanhelmont, T., Terwel, D., Borghgraef, P., Lemaire, K., Snauwaert, J., Wera, S., Van Leuven, F., and Winderickx, J. (2005). Identification and isolation of a hyperphosphorylated, conformationally changed intermediate of human protein tau expressed in yeast. Biochemistry *44*, 11466-11475.

Versaw, W. K., and Metzenberg, R. L. (1995). Repressible cation-phosphate symporters in Neurospora crassa. Proc Natl Acad Sci U S A *92*, 3884-3887.

Versele, M., de Winde, J. H., and Thevelein, J. M. (1999). A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. Embo J *18*, 5577-5591.

Vincent, O., and Carlson, M. (1999). Gal83 mediates the interaction of the Snf1 kinase complex with the transcription activator Sip4. Embo J *18*, 6672-6681.

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

Wang, Z., Wilson, W. A., Fujino, M. A., and Roach, P. J. (2001a). Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase, and the cyclin-dependent kinase Pho85p. Mol Cell Biol *21*, 5742-5752.

Wang, Z., Wilson, W. A., Fujino, M. A., and Roach, P. J. (2001b). The yeast cyclins Pc16p and Pc17p are involved in the control of glycogen storage by the cyclin-dependent protein kinase Pho85p. FEBS Lett *506*, 277-280.

Wanke, V., Pedruzzi, I., Cameroni, E., Dubouloz, F., and De Virgilio, C. (2005). Regulation of G(0) entry by the Pho80-Pho85 cyclin-CDK complex. Embo J *24*, 4271-4278.

Waters, N. C., Knight, J. P., Creasy, C. L., and Bergman, L. W. (2004). The yeast Pho80-Pho85 cyclin-CDK complex has multiple substrates. Curr Genet *46*, 1-9.

Wek, R. C., Cannon, J. F., Dever, T. E., and Hinnebusch, A. G. (1992). Truncated protein phosphatase GLC7 restores translational activation of GCN4 expression in yeast mutants defective for the eIF-2 alpha kinase GCN2. Mol Cell Biol *12*, 5700-5710.

Wera, S., Bergsma, J. C., and Thevelein, J. M. (2001). Phosphoinositides in yeast: genetically tractable signalling. FEMS Yeast Res *1*, 9-13.

Wera, S., De Schrijver, E., Geyskens, I., Nwaka, S., and Thevelein, J. M. (1999). Opposite roles of trehalase activity in heat-shock recovery and heat-shock survival in Saccharomyces cerevisiae. Biochem J *343 Pt 3*, 621-626.

Werner, A., and Kinne, R. K. (2001). Evolution of the Na-P(i) cotransport systems. Am J Physiol Regul Integr Comp Physiol *280*, R301-312.

Werner, T. P., Amrhein, N., and Freimoser, F. M. (2005). Novel method for the quantification of inorganic polyphosphate (iPoP) in Saccharomyces cerevisiae shows dependence of iPoP content on the growth phase. Arch Microbiol *184*, 129-136.

Werner-Washburne, M., Braun, E. L., Crawford, M. E., and Peck, V. M. (1996). Stationary phase in Saccharomyces cerevisiae. Mol Microbiol *19*, 1159-1166.

Werner-Washburne, M., Brown, D., and Braun, E. (1991). Bcy1, the regulatory subunit of cAMP-dependent protein kinase in yeast, is differentially modified in response to the physiological status of the cell. J Biol Chem *266*, 19704-19709.

Wilson, R. B., and Tatchell, K. (1988). SRA5 encodes the low-Km cyclic AMP phosphodiesterase of Saccharomyces cerevisiae. Mol Cell Biol *8*, 505-510.

Wilson, W. A., Hawley, S. A., and Hardie, D. G. (1996). Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. Curr Biol *6*, 1426-1434.

Wilson, W. A., Mahrenholz, A. M., and Roach, P. J. (1999). Substrate targeting of the yeast cyclin-dependent kinase Pho85p by the cyclin Pcl10p. Mol Cell Biol *19*, 7020-7030.

Wilson, W. A., and Roach, P. J. (2002). Nutrient-regulated protein kinases in budding yeast. Cell *111*, 155-158.

Wilson, W. A., Wang, Z., and Roach, P. J. (2002). Analysis of respiratory mutants reveals new aspects of the control of glycogen accumulation by the cyclin-dependent protein kinase Pho85p. FEBS Lett *515*, 104-108.

Wilson, W. A., Wang, Z., and Roach, P. J. (2005). Regulation of yeast glycogen phosphorylase by the cyclin-dependent protein kinase Pho85p. Biochem Biophys Res Commun *329*, 161-167.

Winderickx, J., de Winde, J. H., Crauwels, M., Hino, A., Hohmann, S., Van Dijck, P., and Thevelein, J. M. (1996). Regulation of genes encoding subunits of the trehalose synthase complex in Saccharomyces cerevisiae: novel variations of STRE-mediated transcription control? Mol Gen Genet *252*, 470-482.

Winderickx, J., Holsbeeks, I., Lagatie, O., Giots, F., Thevelein, J. M., and De Winde, H. (2003). From feast to famine; adaptation to nutrient availability in yeast. In Yeast Stress Responses, S. Hohmann, and P. W. H. Mager, eds. (Topics in Current Genetics), pp. 305-386.

Wingender-Drissen, R., and Becker, J. U. (1983). Regulation of yeast phosphorylase by phosphorylase kinase and cAMP-dependent protein kinase. FEBS Lett *163*, 33-36.

Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., *et al.* (1999). Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science *285*, 901-906.

Woods, A., Dickerson, K., Heath, R., Hong, S. P., Momcilovic, M., Johnstone, S. R., Carlson, M., and Carling, D. (2005). C(Ca2+)/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab *2*, 21-33.

Woods, A., Munday, M. R., Scott, J., Yang, X., Carlson, M., and Carling, D. (1994). Yeast SNF1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase in vivo. J Biol Chem *269*, 19509-19515.

Wu, J., Zhang, N., Hayes, A., Panoutsopoulou, K., and Oliver, S. G. (2004). Global analysis of nutrient control of gene expression in Saccharomyces cerevisiae during growth and starvation. Proc Natl Acad Sci U S A *101*, 3148-3153.

Wurst, H., and Kornberg, A. (1994). A soluble exopolyphosphatase of Saccharomyces cerevisiae. Purification and characterization. J Biol Chem *269*, 10996-11001.

Wurst, H., Shiba, T., and Kornberg, A. (1995). The gene for a major exopolyphosphatase of Saccharomyces cerevisiae. J Bacteriol *177*, 898-906.

Wykoff, D. D., and O'Shea, E. K. (2001). Phosphate transport and sensing in Saccharomyces cerevisiae. Genetics *159*, 1491-1499.

Xue, Y., Batlle, M., and Hirsch, J. P. (1998). GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Galpha subunit and functions in a Ras-independent pathway. Embo J *17*, 1996-2007.

Yang, X., Hubbard, E. J., and Carlson, M. (1992). A protein kinase substrate identified by the two-hybrid system. Science *257*, 680-682.

Yang, X., Jiang, R., and Carlson, M. (1994). A family of proteins containing a conserved domain that mediates interaction with the yeast SNF1 protein kinase complex. Embo J *13*, 5878-5886.

Yompakdee, C., Bun-ya, M., Shikata, K., Ogawa, N., Harashima, S., and Oshima, Y. (1996a). A putative new membrane protein, Pho86p, in the inorganic phosphate uptake system of Saccharomyces cerevisiae. Gene *171*, 41-47.

Yompakdee, C., Ogawa, N., Harashima, S., and Oshima, Y. (1996b). A putative membrane protein, Pho88p, involved in inorganic phosphate transport in Saccharomyces cerevisiae. Mol Gen Genet *251*, 580-590.

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

Zabrocki, P., Pellens, K., Vanhelmont, T., Vandebroek, T., Griffioen, G., Wera, S., Van Leuven, F., and Winderickx, J. (2005). Characterization of alpha-synuclein aggregation and synergistic toxicity with protein tau in yeast. Febs J *272*, 1386-1400.

Zahringer, H., Burgert, M., Holzer, H., and Nwaka, S. (1997). Neutral trehalase Nth1p of Saccharomyces cerevisiae encoded by the NTH1 gene is a multiple stress responsive protein. FEBS Lett *412*, 615-620.

Zahringer, H., Holzer, H., and Nwaka, S. (1998). Stability of neutral trehalase during heat stress in Saccharomyces cerevisiae is dependent on the activity of the catalytic subunits of cAMP-dependent protein kinase, Tpk1 and Tpk2. Eur J Biochem *255*, 544-551.

Zahringer, H., Thevelein, J. M., and Nwaka, S. (2000). Induction of neutral trehalase Nth1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: variations of PKA effect during stress and growth. Mol Microbiol *35*, 397-406.

Zhang, S., Guha, S., and Volkert, F. C. (1995). The Saccharomyces SHP1 gene, which encodes a regulator of phosphoprotein phosphatase 1 with differential effects on glycogen metabolism, meiotic differentiation, and mitotic cell cycle progression. Mol Cell Biol *15*, 2037-2050.

Zorzano, A., Palacin, M., and Guma, A. (2005). Mechanisms regulating GLUT4 glucose transporter expression and glucose transport in skeletal muscle. Acta Physiol Scand *183*, 43-58.

List of publications

Roosen, J., Oesterhelt, C., Pardons, K., Swinnen, E. and Winderickx, J. (2004). **Integration of nutrient signalling pathways in the yeast *Saccharomyces cerevisiae*.** In: Nutrient-Induced Responses in Eukaryotic Cells, J. Winderickx, and P.M. Taylor, eds. Topics in Current Genetics, pp. 277-318

Swinnen, E., Rosseels, J. and Winderickx, J. (2005). **The minimum domain of Pho81 is not sufficient to control the Pho85-Rim15 effector branch involved in phosphate starvation-induced stress responses.** Curr Genet 48, 18-33

Swinnen, E., Wanke, V., Roosen, J., Smets, B., Dubouloz, F., Pedruzzi, I., Cameroni, E., De Virgilio, C. and Winderickx, J. (2006) **Rim15 and the crossroads of nutrient signalling pathways in *Saccharomyces cerevisiae*.** Cell Div (accepted for publication)

Swinnen, E., Rosseels, J., Thevelein, J. and Winderickx, J. **The Pho4 transcription factor controls the activity of the Pho87 low-affinity phosphate transporter.** (manuscript in preparation)