Nutrient availability regulates cell cycle through a Pho85 CDK-dependent control of Cln3 cyclin stability

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Als meus pares,

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INTRODUCTION

From the beginning of our existence, human beings have been distinguished by our desire to understand and influence the environment, seeking how to explain and manipulate it through science, philosophy and art. Probably, one of the highest challenges we face is to get to know ourselves and to take advantage of this knowledge to improve our own health. This natural curiosity has led to the development of advanced tools and skills on Biomedical Sciences field, such as the use of a huge variety of organisms to understand how human cells work: from mammal organisms to unicellular eukaryotes, such as yeasts.

Saccharomyces cerevisiae is a unicellular eukaryote organism that belongs to the Fungi kingdom and to the Ascomycota phylum. It is generally known as “yeast”, term from an Indo-European root shared by Greek *zein* that means 'to boil' (or in Catalan “llevat” that comes from Latin “levare”, to raise) because of its properties on bread, wine and beer production.

Over the last centuries, yeast has been used as a model organism in several fields of biomedical science research. Among the advantages as a laboratory tool, yeast is easy to culture, it is easy to manipulate genetically and was the first organism to be sequenced. Moreover, despite of thousand millions of years of evolutionary divergence, more than one third of the yeast genes are conserved in humans (they are homologues), and in many cases do similar functions (they are orthologues). Most of the conserved genes in both species are those involved in regulating cell division. The fast growth rate and the high conserved regulation in upper eukaryotes make yeast one of the best model organism to elucidate how cell cycle works.

1. REGULATION OF CELL CYCLE IN *Saccharomyces cerevisiae*

Yeast cells multiply by two different reproductive strategies: sexual and asexual. Upon sustained absence of nutrients, the favorite reproductive strategy is the sexual one, which entails Meiosis. Meiosis is the process by which the cell switch from a diploid number of chromosomes set to a haploid one. However, upon optimum nutrient conditions, *Saccharomyces cerevisiae* divides rapidly by budding, an asymmetric process of asexual reproduction, known as haploid mitosis: one single cell give raise to a protuberance that grows until it becomes another unicellular organism. The whole budding process takes about two hours.

Between every cell division, all essential components of the cell are needed to be duplicated. The most important component is the genetic material (DNA molecules organized in distinct pieces called chromosomes), which must be accurately replicated

and carefully segregated to the two daughter cells. In eukaryotic cells, chromosome duplication is accomplished by initiating replication forks at many origins of replication on each chromosome (Donaldson et al., 1999; Stillman, 2001; Raghuraman et al, 2001; Iyer et al., 2001; Heun et al., 2001; Wyrick et al., 2001). Spindle Pole Body (SPB) duplication is also required. SPB is the sole site of microtubule organization in the budding yeast. SPBs are embedded in the nuclear envelope throughout the yeast life cycle and are able to nucleate both nuclear and cytoplasmic microtubules. Importantly, SPBs are not synthesized de novo. Therefore, every time a cell divides, SPB must be duplicated, as well as its genome, to ensure that both the mother and daughter cells contain one copy of all 16 chromosomes and one SPB (reviewed in Jaspersen and Winey, 2004)

The processes of DNA replication, SPB duplication and sister chromatid separation occur in temporally different phases of the eukaryotic cell cycle. DNA replication and SPB duplication occurs at S-phase (DNA Synthesis), whereas sister chromatid separation takes place in M-phase (Mitosis). S and M phases are separated by two gaps, known as G_1, where cell grows, and G_2, where the cell perpares for next division. This cell growth at G1 phase is essential for cell cycle progression as yeast cells must reach a critical size before carry on the next division. The key point that controls cell size in the cell cycle is called “START”. START is the transition that initiates processes like budding, spindle pole body duplication and, later, DNA synthesis in S phase. Once cells have passed START, they are irreversibly committed to replicate their DNA and progress through the cell cycle. START thus coordinates cell cycle with cell growth. Nutrient starvation, as well as induction of mating, blocks passage through START. There are additional checkpoints that arrest cells during the cell cycle to prevent DNA damage or cell death due to different defective events. These control points are situated at the G_1-S and G_2-M boundaries and can be considered as internal regulatory systems whose inhibits cell cycle if prerequisites for progression are not met (Hartwell et al., 1974). This events are represented at Figure #1 of Introduction (Fig. I1).

Progression through cell cycle can be monitored by light microscopy in *S. cerevisiae* through the emergence and evolution of a daughter protuberance, known as “bud”, that indicates which events are taking place. Yeast buds are initiated when mother cells attain a critical cell size at a time almost coinciding with the onset of DNA synthesis at G_1/S transition. This is followed by localized weakening of the cell wall and this, together with tension exerted by turgor pressure, allows extrusion of cytoplasm into an area bounded by new cell wall material. All this latter events are accompanied by bud enlargement from S phase to M phase.

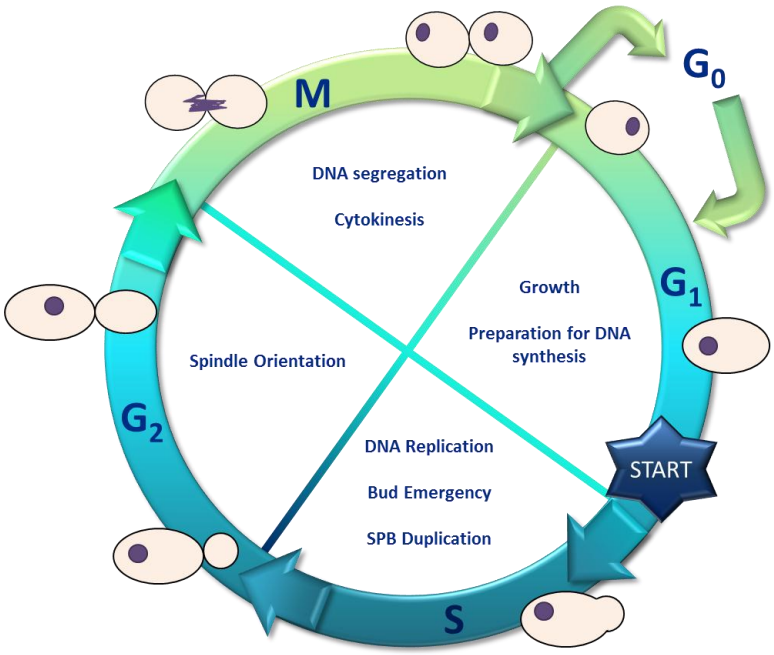

Figure I1. *Saccharomyces cerevisiae* cell cycle. The four cell cycle phases are represented: G_1, when cell grows; S, where DNA is replicated, bud emerges and Spindle Pole Body (SPB) is duplicated; G_2, when Spindle is oriented; and M, or Mitosis, where the DNA is segregated and cytokinesis takes place. Between G_1 and S phase, there is an event known as "START" in which, once all the requirements to enter S phase are reached, cell is destined irreversibly to enter the mitotic cell cycle. Morphology cell changes due to bud emergency are also represented.

Finally, the G_0 phase or resting phase is a period in the cell cycle in which the cell remains in a quiescent state mainly due to a dramatic absence of growth factors (reviewed in Gray et al., 2004).

It has been established, by biochemical and genetic experiments, that the main regulators of the cell cycle are Cyclin-Dependent protein Kinases (CDKs) that act together with their associated regulators called cyclins and CKIs (Cyclin-dependent Kinase Inhibitors).

There are five CDKs in *S. cerevisiae*: Kin28, Ssn3, Ctk1 (which has as main function to phosphorylate the C-terminus region of the RNApolimerase II), Cdc28 and Pho85. Cdc28 and Pho85 play the most important roles on regulation of cell cycle. Activation of Cdc28 and Pho85 depends on a large family of cyclin subunits, whereas the other CDKs seem to be activated by a single dedicated cyclin (Andrews and Measday, 1998).

1.1. Cell Cycle Regulators: CDKs, Cyclins and Inhibitors

CDKs are kinases, a type of enzyme that transfers phosphate groups from high-energy donor molecules (such as ATP) to specific substrates. The process of phosphate transference is called phosphorylation. CDKs have regulatory functions in the cell cycle that have been evolutionarily conserved in high eukaryotes. In fact, yeast cells can normally proliferate when a single CDK gene is substituted by its human homologue (Lee and Nurse, 1987).

CDKs bind to a regulatory protein called "cyclin", that changes the conformation of the CDK in T-loop and PSTAIRE sequences, allowing the ATP binding and activating the complex. This cyclin-CDK complex phosphorylates their substrates on serines and threonines, when they appear in the following amino acid consensus sequence: [S/T*]P, where S/T* is the phosphorylated serine or threonine and P is a proline. Sometimes, the phosphorylation by a CAK (CDK-activating kinase) is necessary.

Cell cycle process is carefully regulated because, though CDKs levels remain constant during the whole cycle, cyclins are temporally regulated by transcription, translation, posttranslational modification, inhibition and proteolysis.

Cyclins were first discovered as proteins that appear and disappear in synchrony during cell cycle. They were early defined by their ability to bind and activate a CDK and are often recognized by the presence of a conserved domain, the *cyclin box*, which is a region required for binding and activation of CDKs (Evans et al., 1983; Kobayashi et al., 1992; Mendenhall and Hodge, 1998).

1.1.1. The cyclin dependent kinase Cdc28

Cdc28 CDK (or Cdk1 in mammals) is encoded by the essential gene *CDC28*. It was originally described as a *ts* mutation that blocked cell cycle at Start and was rapidly recognized as the main coordinator of budding yeast cell cycle in normal conditions (Hartwell et al., 1973).

Cdc28 activity and specificity are timely and tightly controlled due to the binding of different cyclins, phase-specific phosphorylations and inhibitors.

1.1.1.1. Cdc28 cyclins

Cdc28 cyclins have been historically classified into two broad groups: G_1 cyclins that are named Cln1, Cln2 and Cln3 and regulate events during the interval between cytokinesis and DNA replication; and B-type cyclins, named Clb1, Clb2, Clb3, Clb4, Clb5 and Clb6, that are needed for replication, G_2 progression and passage through Mitosis (Mendenhall and Hodge, 1998). All cyclins, but Cln3, experience waves of production and destruction in normal conditions.

G_1 cyclins split into two functional groups: on the one hand, Cln3, which levels are rather low and steady along cell cycle, is mainly regulated by relocation under high nutrient conditions and is the primary initiator of events at Start (Tyers et al., 1993; Verges et al., 2007). One of the main roles of Cln3 is to activate the transcription of the other two Clns. On the other hand, among Start-specific events that lead to the transcription of the highly redundant Cln1,2 cyclins, bud emerges, spindle pole body (SPB) duplicates, the S-phase inhibitor Sic1 is degraded, and a whole transcriptional regulon that encompasses the subsequent S-phase cyclins and Cln1,2 themselves is triggered (Breeden, 1996; Cross and Tinkelenberg, 1991; Koch et al., 1993; Nash et al., 2001; Nashmyth and Dirick, 1991; Spellman et al., 1998).

B-type cyclins form a family of six proteins commonly subdivided into three pairs, based on their homology and transcriptional pattern. The first pair being transcribed along cell cycle is Clb5,6, which are produced at Start and their primary role is to initiate S-phase in a timely fashion way (Schwob and Nasmyth, 1993) and to inhibit Clns. Clb5,6-Cdc28 complexes phosphorylate components of the pre-replication complexes to fire replication origins (Dahmann et al., 1995; Masumoto et al., 2002; Tanaka et al., 2007). The second pair of cyclins is formed by Clb3,4. They appear in mid S-phase and contribute in DNA replication and drive spindle assembly (Richardson et al., 1992). The last wave of B-type cyclins appears before anaphase, it generates two highly homologous cyclins, Clb1,2, that promote bud growth, chromosome separation and inhibit G_1-specific events (Amon et al., 1993; Fitch et al., 1992; Lew and Reed, 1993). All this events are summarized in Fig. I2.

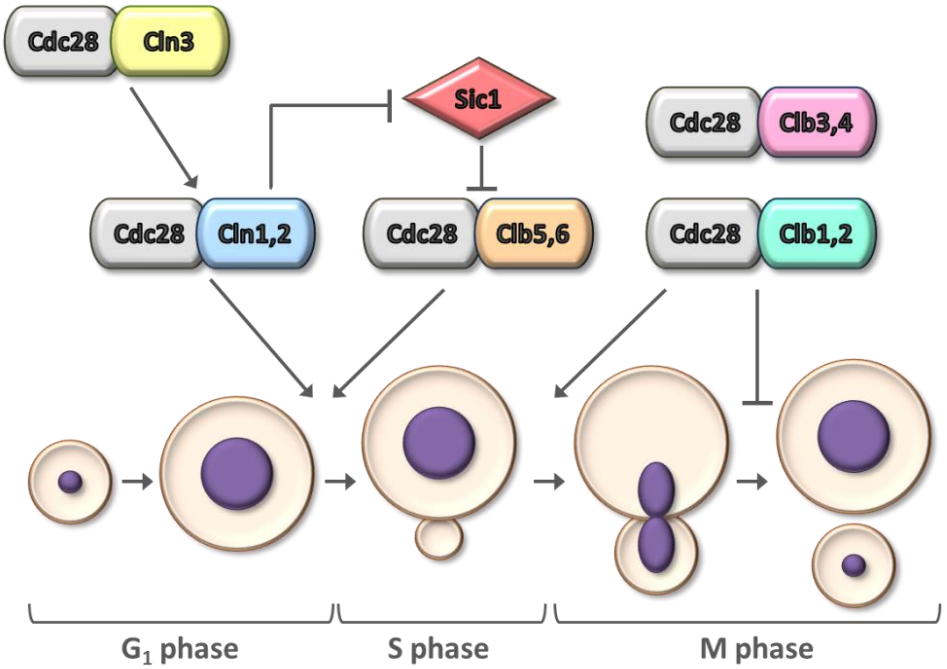

Figure I2. Cdc28 regulates cell cycle progression. Cdc28 CDK and its cyclins are represented through the cell cycle: Cln3 is the G_1 cyclin that initiates START events and leads to the transcription of Cln1 and Cln2 cyclins, which are involved in budding and in the inhibition of Sic1 that prevents the activity of Clb5 and Clb6. Once these B-type cyclins are active in late G_1, DNA synthesis is initiated and finish with the appearance of Clb3 and Clb4 cyclins. Finally, Clb1 and Clb2 lead some mitotic events and inhibit G_1-specific events.

1.1.1.2. Cdc28 activators and inhibitors

Besides binding to cyclins, Cdc28 is also regulated by phosphorylation and cycle specific inhibitors. A required step in its activation is the phosphorylation of a conserved threonine residue within the activation domain, the T-loop (Espinoza et al., 1995). In budding yeast, the activating phosphorylation of Cdc28 at Thr169 by a single CDK-activating Kinase (CAK), named Cak1, is essential for cell cycle progression (Thuret et al., 1996). A second reversible phosphorylation on tyrosine 19 of Cdc28, results in the inhibition of its CDK activity. It is mediated by a kinase expressed in S-phase and G_2, named Swe1 (Booher et al., 1993; Lim et al., 1996). Swe1 specifically recognizes Clb1,2-Cdc28 molecules. Therefore phosphorylation on Cdc28 by Swe1, selectively restricts Clb1,2-Cdc28 activity. Swe1 has a less effect on Clb3,4-Cdc28, and Clb5,6-Cdc28 show to be insensitive to this phosphorylation (Hu and Aparicio, 2005). Hence, Swe1 primarily restricts Clb1,2 activity and is the major mechanism by which cells delay entry into mitosis until critical cell size has been reached (Kellogg, 2003; Rupes,

2002) or bud morphogenesis has been successfully completed (Cid et al., 2002; Lew, 2003). Swe1 phosphorylation is opposed at mitosis by the Mih1 phosphatase (Russel et al., 1989). Swe1 degradation depends on both Clb-Cdc28 activity and Hsl1, a conserved upstream kinase, in association with the adaptor protein Hsl7, which together target Swe1 for degradation once the bud site is properly assembled (Asano et al., 2005; McMillan et al., 1999).

Yet the presence of some proteins can also inhibit some Cdc28 complexes. Sic1 is a potent Clb-Cdc28 specific inhibitor that excludes substrates from the active site of Clb-bound Cdc28. Sic1 plays a role in the timing and robustness of regulation of DNA replication by holding under inhibition Clb5,6-Cdc28 complexes and setting a threshold for Clb-Cdc28 activation (Cross et al., 2007). The levels of Sic1 are maximal at early G_1, but at late G_1 a rise in Cln1,2-Cdc28 activity results in the phosphorylation of Sic1, which targets it for ubiquitination and degradation. Adequate progression into S phase requires degradation of Sic1 or an overcoming activity of Cyclin B-associated CDK activity (Schwob et al., 1994; Verma et al., 1997). Sic1 has also an important role in down-regulating Clb2 to allow spindle degradation and exit from mitosis.

Moreover, there are certain inhibitors that play an important role in specific situations like mating. Far1 is a Cln2-Cdc28 specific inhibitor that acts upon activation of the pheromone pathway. The activation of Far1 represses Start-specific transcription, causing in Cln-Cdc28 activity total depletion and resulting in a G_1 arrest in preparation for mating (Peter et al., 1993; Peter and Herskowitz, 1994).

1.1.2. The cyclin dependent kinase Pho85

Pho85 is a multifunctional CDK in *S. cerevisiae* that has emerged as an important model for the role of CDKs in cell cycle control and other processes. This CDK is targeted to its substrates by 10 different cyclins or Pcls (Pho85 cyclins) and only some of them exhibit cell cycle-periodic expression (Measday et al., 1997). Three of these Pcls have specific roles in G_1 such as regulation of G_1-specific gene expression and control of polarized cell growth. Many known substrates of the G_1 specific-Pho85 are also phosphorylated by the homologous CDK Cln-Cdc28, suggesting parallel or overlapping roles. Most of the remaining Pcls have different roles in signaling: Pho85 is generally active when environmental conditions are satisfactory, phosphorylating proteins involved in transcription and other regulatory events to keep the stress response and inappropriate activities turned off. Therefore, Pho85 seems to regulate a variety of cellular processes (Huang et al., 2007).

Pho85 regulates G_1 progression, cell polarity and the actin cytoskeleton, gene expression, phosphate and glycogen metabolism, and the signaling of changes in the

environment (Carrol and O'Shea, 2002). Despite its many roles, Pho85 is not essential for viability.

Pho85 is a functional homologue of Cdk5, the mammalian CDK involved in development of the central nervous system and neurite outgrowth. Overexpression of *CDK5* in yeast cells complements many defects associated with *pho85Δ*. On the other hand, Pho85 expressed in mammalian cells associates with Cdk5 activators to form functional kinase complexes (Huang et al., 1999; Nishizawa et al., 1999). This suggests that functionally conserved pathways could exist in yeast and mammalian cells concerning Pho85 and some of its substrates.

1.1.2.1. Pho85 cyclins

Pcls have been divided into two subfamilies based on sequence similarities within the cyclin-box region.

The Pho80 subfamily, consisting of Pho80, Pcl6, Pcl7, Pcl8 and Pcl10, has important roles in regulating metabolism and sensing environmental changes (Carrol and O'Shea, 2002). For instance, the phosphorylation of Pho4 transcription factor or Rim15 that regulates the entry to G_0 depends on the Pho80 subfamily (except for Pcl7 with a peak in mid- to late S suggesting a role in this phase).

The Pcl1,2 subfamily includes Pcl1, Pcl2, Pcl9, Clg1 and Pcl5 (Measday et al., 1997). Pcl5 seems to have a role in nutrient sensing (Bömeke et al., 2006). The other cyclins have functions related to the timing of expression of their genes: Pcl9 has a role in early G_1 inhibiting the Whi5 repressor; and Pcl1 and Pcl2 have redundant roles in cell cycle regulation in late G_1 phase. Clg1 function remains unknown. Some of these functions are summarized in Table #1 of Introduction (Tab. I1).

Gel filtration of yeast cell lysates has demonstrated that most of the Pho85 protein is present as a monomer, although a portion is present in high-molecular-weight fractions, for instance associated with cyclins and cyclin-associated substrates. Overproduction of one cyclin leads to the sequestration of most of the Pho85 with that cyclin. This suggests a model for Pho85 function where the Pcls are limiting and Pho85 is in excess, with a significant pool of free monomer (Wilson et al., 1999).

Pho85 is directed to specific substrates via interactions with substrate-associated Pcls. Pcl10-Pho85 specifically phosphorylates Gsy2, but not Pho4, whereas Pho80-Pho85 specifically phosphorylates Pho4 but not Gsy2 (Huang et al., 1998).

1.1.2.2. Pho85 activators and inhibitors

Contrary to Cdc28, Pho85 do not need to be activated by a CAK, but it is modulated by some allosteric changes of some interacting proteins.

The PHO81 gene is known to encode an inhibitor of the Pho85-Pho80. This protein contains six repeats of an ankyrin-like sequence in its terminal region, with significant similarities to p16INK4, which inhibits the human cyclinD-CDK4 kinase complex (Ogawa et al., 1995). Inhibitory activity for Pho80-Pho85 requires myo-D-inositol heptakisphosphate (IP7) generated by Vip1 protein during phosphates starvation, which changes the allosteric conformation of Pho81 (see more detailed explanation on chapter 2.2.1).

Not only Pho80, but also Pcl7, interacts with the phosphate-regulated inhibitor Pho81 and is inhibited in response to phosphate starvation (Lee et al., 2000).

Substrate	Biological role	Cyclin	Role of phosphorylation
Whi5[a]	G1-specific transcriptional repressor	Pcl9	Negatively regulates Whi5
Swi5	Transcriptional activator	?	Negatively regulates Swi5
Ash1	Transcriptional repressor	?	Destabilizes Ash1
Rvs167	Amphiphysin homologue required for endocytosis	Pcl1, Pcl2	May negatively regulate protein–protein interactions
Bni4	Bud-neck adapter protein	Pcl1, Pcl2	Positively regulates bud-neck localization
Rga2	GAP for Cdc42	Pcl1, Pcl2	Cell morphogenesis
Cdc24	GTP–GDP exchange factor for Cdc42	Pcl1, Pcl2	Cell morphogenesis
Sic1	Cdc28–Clb inhibitor	?	Targets Sic1 for degradation
Clb6	S-phase cyclin	?	Targets Clb6 for degradation
Lcb4	Long-chain base kinase	Pcl1,2	Targets Lcb4 for degradation
Shs1	Septin	Pcl1,2	May enhance its association with Gin4
Gcn4	Transcription factor involved in amino-acid biosynthesis	Pcl5	Targets Gcn4 for degradation
Glc8	Regulatory subunit for Glc7 phosphatase	Pcl6, Pcl7	Required for activity
Gsy2	Glycogen synthase	Pcl8,10	Inactivates glycogen synthase activity
Crz1	Transcription factor involved in response to calcium stress	Pho80	Negatively regulates nuclear localization
Pho4	Transcription factor involved in phosphate and vacuole regulation	Pho80	Negatively regulates nuclear localization
Rim15	Protein kinase regulating entry into G0	Pho80	Sequesters Rim15 in the cytoplasm via its association with 14-3-3 proteins
Pho80	Pho85 cyclin	Pho80?	Required for active kinase
Pho81	Pho85 CDK inhibitor	Pho80?	Positively regulates binding to Pho80–Pho85

Table I1. Cyclins target Pho85 CDK to different substrates. Depending on the complex formed by Pho85 CDK and a specific cyclin, the phosphorylated substrate will be different. Confirmed substrates are showed in boldface type. (Adapted from Huang et al., 2007).

1.1.2.3. Pho85 functions on polarized growth

Two of the G_1-specific Pho85 cyclins have an essential role that is genetically redundant with Cln1,2-Cdc28 in late G_1 phase (Measday et al., 1994; Moffat and Andrews, 2004). A *cln1Δ cln2Δ pcl1Δ pcl2Δ* strain is lethal because fails to extend a discernible bud. Pcl1,2-Pho85 and Cln1,2-Cdc28 redundantly phosphorylate Cdc24 and some other proteins that associate with Cdc42. Moreover, a number of Cdc24-associated proteins including the GTPase activating protein (GAP) Rga2, and the cell polarity regulators, Boi1 and Boi2, are likely among the key targets of G_1 Pho85 cyclins (McCusker et al., 2007).

Some other Pcl1,2-Pho85 targets have been identified: Rvs167 (the amphiphysin homologue), an actin cytoskeleton protein required for endocytosis (Lee et al., 1998; Friesen et al., 2003) and Lcb4, the major enzyme that produces long-chain base phosphates (LCBPs), precursors of sphingolipids that are important growth regulators in mammalian cells (Hannun et al., 2001).

Polarized growth functions as well as cell cycle regulation by Pho85, are summed up in Fig. I3.

1.1.2.4. Deletion of PHO85

Consistent with diverse roles for Pho85 in the regulation of cellular events, deletion of *PHO85* results in a broad spectrum of defects. A *pho85Δ* strain exhibits:

- Slow growth, with a G_1 delay on rich medium and poor growth on non-fermentable sources (Timblin et al., 1996).
- Defects associated with polarity: irregular budding, sensitivity to high concentrations of NaCl, depolarized actin and defects in endocytosis (Lee et al., 1998).
- Cell wall integrity defects as suggested by its sensitivity to tunicamycin, zymoliase and calcofluor (Huang et al., 2002).
- Sensitivity to many types of stress, including high osmolarity, high concentrations of CaCl2, 4-nitroquinolin oxide (4-NQO), cycloheximide, geneticin and hygromycin B, probably due to defects in vacuole function (Huang et al., 2002).
- Constitutive expression of phosphate-starvation-genes (Uesono et al., 1987).
- Hyperaccumulation of glycogen (Timblin et al., 1996).

Loss of a particular Pho85 cyclin can mimic as well some of the *pho85Δ* cells:

- *pho80Δ* cells show a constitutive expression of phosphate-starvation-dependent genes, grow slowly, are unable to grow on non-fermentable carbon sources, and exhibit a broad range of stress sensitivities (Uesono et al., 1987; Huang et al., 2002).
- *pcl8Δ* and *pcl10Δ* show high levels of glycogen accumulation (Huang et al., 1998).
- Deletion of Pcl1,2 subfamily have a retarded cell cycle, with defects on polarity, morphology and cell wall (Moffat et al., 2000).

Pho85 could have a relevant role in sensing nutrients to allow a proper cell cycle progression, since it is known that Pho85 is involved in the phosphate signaling pathway. In particular, the Pho80-Pho85 complex regulates the expression of phosphate-starvation genes. Whether the presence/absence of phosphates could regulate the cell cycle through Pho85 is one of the main objectives of this thesis. Hence, this phosphate-signaling pathway will be explained on a more detailed way in chapter 2.2.1, in the nutrient signaling part of this work.

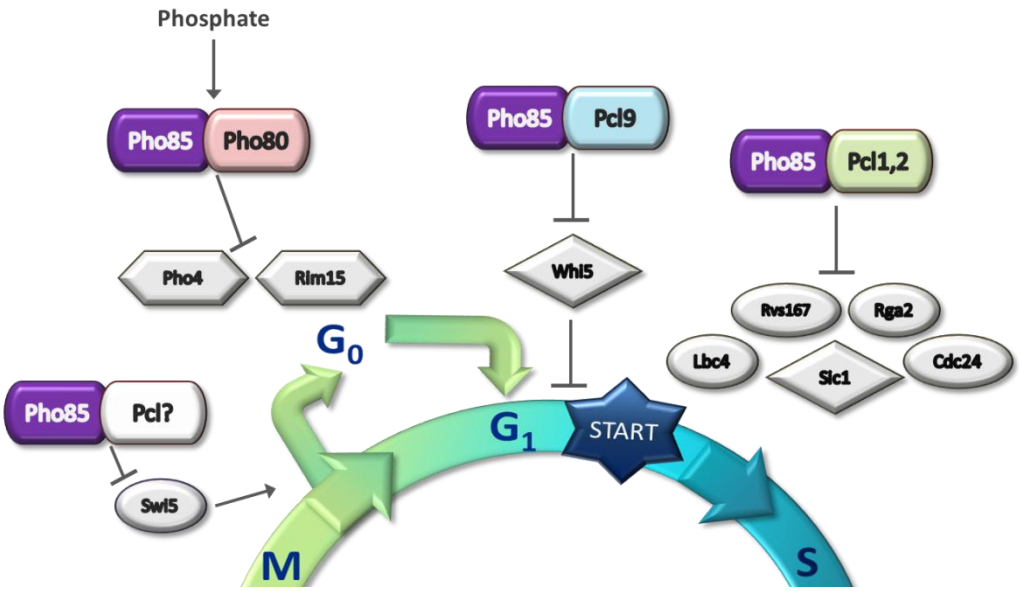

Figure I3. Pho85 regulates cell cycle progression. Pho85, in combination with several different cyclins, phosphorylates multiple substrates to regulate the G_1 and G_0 phases of the cell cycle. In presence of phosphate, Pho85 binds to Pho80 to inhibit the G_0 entry through Pho4 and Rim15, binds to Pcl9 to trigger START by inhibiting Whi5 and binds to Pcl1,2 to inhibit Sic1 and to lead some morphogenesis functions in late G_1. Binding to unknown cyclins promote phosphorylation to M phase proteins. T-bars indicate negative regulation by phosphorylation; arrows indicate positive regulation.

1.2. Cell Cycle Transcriptional Regulation

In *S. cerevisiae*, a fraction of genes (more than 10%) are transcribed with cell cycle periodicity (Spellman et al., 1998). These genes can be organized into clusters exhibiting similar patterns of periodic transcription, which are achieved via both repressive and activating mechanisms, and are ruled by CDKs and a network of transcription factors that has an oscillatory property by itself (Orlando et al., 2008; Wittenberg and Reed, 2005). Roughly, these clusters are the G_1, the S-phase, the Clb2 and the M-G_1 clusters.

1.2.1. The mid-G_1 to late-G_1 cluster

The G_1-specific gene cluster is triggered at Start. It is targeted by two heterodimeric transcription factors that share the same transactivating protein (Swi6) and two different DNA binding proteins (Swi4 and Mbp1). The first complex Swi6-Swi4 is the Swi4 cell cycle box Binding Factor (SBF) and the second complex Swi6-Mbp1 is the Mlu cell cycle box Binding Factor (MBF). SBF typically targets genes involved in cell morphogenesis and the cyclins Cln1,2, whereas MBF target genes are those necessary for DNA replication and the cyclins Clb5,6 (Breeden, 1996; Koch et al., 1993; Nasmyth and Dirick, 1991).

Under physiological conditions, Cln3-Cdc28 is the primary activator of these transcription factors (Tyers et al., 1993). Upon Cln3 activation, the CDK phosphorylates and inactivates the G_1 specific inhibitor Whi5, analogous to the retinoblastoma (Rb) in mammals, exiting the nucleus (Costanzo et al., 2004) and releasing both SBF and MBF dependent transcription. Recently, it has been suggested that a common substrate, redundantly phosphorylated by Cdc28 and Pho85, may regulate the timing of G_1 transcription: a kinase inhibitor for both Cdc28 and Pho85 simultaneous inactivation, GW400426, prevents the expression of specific transcripts in late G_1, including *CLN2* and *PCL1*, but not inhibition of either kinase alone. During the carrying out of this thesis, a work of Brenda Andrew's lab was published: Pho85 was indeed regulating Whi5 through phosphorylation (Huang et al., 2009). How these results affected to the current work is explained in the Results chapter.

When cells reach START, a burst of Cln1,2-Cdc28 activity further activates SBF and MBF through a positive feedback loop that involves phosphorylation of Whi5 (Cross and Tinkelenberg, 1991; Dirick et al., 1995; Skotheim et al., 2008).

1.2.2. The S to G_2 cluster

During S-phase, the histone cluster seems to be upon SBF/MBF control but its timing of expression is shifted by two co-repressors Hir1 and Hir2 (Sherwood et al., 1993). It has been recently described that Hcm1, a transcription factor from the Forkhead family, is responsible for the correct timing of transcription of a number of genes involved in chromosome segregation. Hcm1 belongs to this S-phase cluster (Pramila et al., 2006).

The Clb2 cluster encloses 35 genes that are transcribed from the end of S-phase until nuclear division. Several genes important for progression through mitosis are included in this cluster; the mitotic cyclins *CLB1,2*; *CDC5*, the yeast polo-like kinase homolog; *CDC20*, a mitotic specificity factor for the APC protein-ubiquitin ligase; and *SWI5* and *ACE2*, transcription factors required for late M/early G_1-specific gene expression (Ghiara et al., 1991; Spellman et al., 1998). The promoters of these genes are permanently bound by the transcription factors Fkh1, Fhk2 and Mcm1 and the linker protein to cell cycle regulation is the co-activator Ndd1, expressed during S-phase and turned off during mitosis (Koranda et al., 2000; Loy et al., 1999). Ndd1 activation is mediated by CDK-dependent phosphorylation, being the primary activator Clb2 itself. Therefore the regulation of this cluster accounts with a positive loop, similarly to the G_1 cluster (Reynolds et al., 2003).

Pho85 seems to have a role in S phase since its cyclin gene *PCL7* is cell-cycle regulated with a peak in mid-to late S phase (Lee et al., 2000). This may be related to its role in activating Glc8 for a proper spindle assembly. Glc8 is the yeast orthologous of mammalian phosphatase inhibitor-2, which modulates the regulator that controls kinetochore-microtubule interactions (Nigavekar et al., 2002; Sassoon et al., 1999). Glc8 function depends on the phosphorylation of the Thr-118, which is phosphorylated by Pho85 associated with cyclins Pcl7 and Pcl6, and possibly Pcl8 and Pcl10 (Tan et al., 2003). Consistent with a role for Pho85 in spindle function, *PHO85* has synthetic lethal interactions with the spindle assembly checkpoint genes *MAD1* and *BUB3*, and is required for benomyl (a drug that binds and destabilizes microtubules) resistance (Daniel et al., 2006).

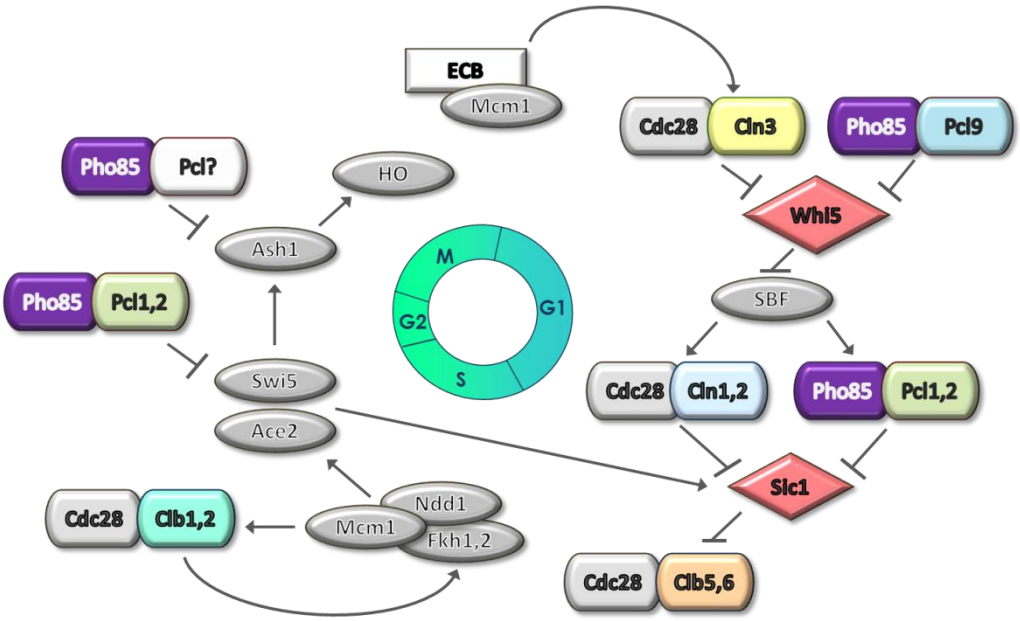

Figure I4. Both Pho85 and Cdc28 have redundant and opposite roles on cell cycle regulation. G_1 progression depends on the Whi5 inhibitor nuclear exclusion by Cdc28-Cln3 and Pho85-PCl9 that leads to the transcription of Cln1,2, which, bound to Cdc28 and together with Pho85- Pcl1 complex, induce Sic1 degradation, and consequently Clb5,6 activity. S and M phases transcription is regulated by Cdc28-Clb1,2 and Pho85-Pcl1,2 complexes.

1.2.3. The M to early-G_1 cluster

The M and early G_1 cluster is formed by genes required for G_1 functions, like the MCM complex (component of the prereplication complex), transcription factors required for G_1 gene expression and proteins involved in the yeast mating response, which occurs during G_1 (Cho et al., 1998; Spellman et al., 1998). A large number of these genes requires Mcm1 and the repressors Yox1 and Yhp1, which bind to the "early cell cycle box" (ECB) for their correct timing of expression (Mclnerny et al., 1997; Pramila et al., 2002). Other genes, like *SIC1, PCL2, PCL9, ASH1* and *HO* depend on Ace2 and Swi5 for their expression. Ace2 depends on Fkh1 and Fkh2 that are regulated by Clb2-Cdc28, whereas Swi5 depends on the complex Pcl2-Pho85 that negatively regulates Swi5 (Nasmyth and Shore, 1987; Zhu et al., 2000; Measday et al., 2000). Moreover, the expression of *ASH1* that depends on Swi5 is regulated again by Pho85. Pho85 destabilizes the protein Ash1 that acts as a transcription factor of *HO*, which encodes an endonuclease that generates homothallic switching and must be repressed for cell cycle progression (McBride et al., 2000). A third group of genes is formed by those normally induced by mating pheromone and depends on the transcription factor Ste12 and two co-repressors, Dig1 and Dig2, that are targeted by Fus3 upon

pheromone sensing (Breitkreutz et al., 2003; Kusari et al., 2004; Oehlen et al., 1996; Spellman et al., 1998).

To sum up, transcription is globally controlled by both Pho85 and Cdc28 CDKs, having either redundant or opposite roles on cell cycle regulation, as represented on Figure I4.

1.3. Cell Cycle Proteolytic Regulation

Besides transcriptional control, cell cycle regulators are also timely regulated by ubiquitin-proteasome-mediated proteolysis to promote cell cycle irreversibly (Hershko and Ciechanover, 1998). Ubiquitin targets proteins for degradation when it is covalently bound to Lysine residues on the target for degradation. Other ubiquitins are bound to the first one by its K48 residue to form a chain. This ubiquitin chain is the degradation signal recognized by the proteasome, a big complex of proteases highly conserved during evolution. The process of ubiquitination includes three enzymes. Firstly, an "Ubiquitin-activating enzyme" (E1) activates ubiquitin by an ATP-dependent way. Secondly, an "Ubiquitin-conjugating enzyme" (E2) takes the ubiquitin to the target protein. And finally, an "Ubiquitin-ligase enzyme" (E3) gives substrate specificity and is bound to the E2 and the target protein. The unique E1 in *Saccharomyces cerevisiae* is named Uba1, whereas there are 13 E2, although is Cdc34 the E2 that always acts at Start. Some other like Ubc4, Rad6, Rub1... operate at M. E3 can be classified depending on common domains called HECT, the first and best known, RING and U-box (both described below, chapter 1.3.3).

Almost all the proteins that regulate cell cycle have a short half-life since their stability is mostly regulated through phosphorylations and ubiquitinations by complexes that have different E2 and E3 according to the different cell cycle phases. The more relevant complexes of ubiquitin ligases (E3) with HECT domains in *S. cerevisiae* are: Skp1/Cullin/F-box (SCF) that covers the constitutive phosphorylation and START dependent-proteolysis, and the Anaphase Promoting Complex (APC), also called the cyclosome which is turned off on S phase and switched on during anaphase to promote completion of mitosis (Jorgensen and Tyers, 1999; Zachariae and Nasmyth, 1999; Desalle and Pagano, 2001).

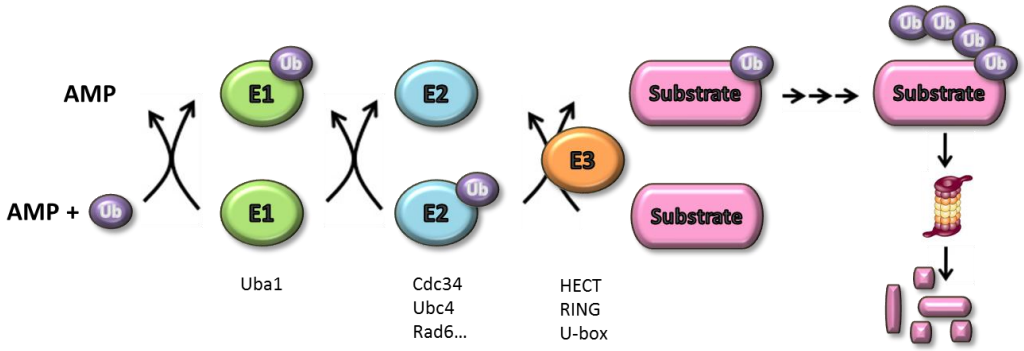

Figure I5. Ubiquitination system in *S. cerevisiae*. Ubiquitin is activated by an E1 ubiquitin-activating enzyme in a process requiring ATP. Then, ubiquitin from E1 is transferred to the active site cysteine of a ubiquitin-conjugating enzyme E2 via a trans(thio)esterification reaction. The final step of the ubiquitination cascade creates an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin. In general, this step requires the activity of one of the hundreds of E3 ubiquitin-protein ligases. E3 enzymes function as the substrate recognition modules of the system and are capable to interact with both E2 and substrate.

1.3.1. START proteolysis

The SCF system is built of an F-box binding protein (Skp1), a scaffold protein (Cdc53 or Cullin), and an interchangeable F-box protein, which is responsible for substrate recognition. Skp1 is homolog of the binding protein to Cyclin F and the Cyclin A-Cdk2 complex in mammals. Cdc53 is an essential gene that needs a conformation change by Rub1 to be activated. The F-box proteins hold a sequence found in Cdc4, Grr1, Met30, Skp2 and Ctf13 in yeast and in Cyclin F and Skp2 in humans (Willems et al., 1996). It catalyzes ubiquitination of phosphorylation-targeted cell-cycle-regulating proteins, including the G_1 cyclins, CDK inhibitors (Sic1 and Far1), and proteins involved in DNA replication, like Cdc6 and Clb6 (Barral et al., 1995; Henchoz et al., 1997; Schwob et al., 1994; Tyers and Jorgensen, 2000; Willems et al., 1996; Jackson et al, 2006).

SCF is active throughout the cell cycle and the degradation of its substrates is controlled at the level of phosphorylation, which is in many cases mediated by the CDK activity (Lanker et al., 1996). A classic example of an SCF-regulated event of cell cycle is the Sic1 and Far1 degradation at the G_1/S transition. It is triggered by a Cln1,2-Cdc28-dependent phosphorylation (Nash et al., 2001). Phospho-Sic1 and phospho-Far1 are both bound by the F-box protein Cdc4 and are ubiquitinated by the E2 enzyme Cdc34. Once Sic1 is degraded, Clb5,6-Cdc28 activity is released from the Sic1-dependent inhibition, and is able to initiate DNA replication (Schwob et al., 1994). Furthermore, Sic1 destruction in particular depends on a more complex process in which, to reach its

full phosphorylation *in vivo*, Pho85 is required (Nishizawa et al., 1998). It has been recently discovered that both Cln2-Cdc28 and Clb5-Cdc28 complexes act in processive multiphosphorylation cascades leading to the phosphorylation of a small number of specific phosphodegrons: after some Sic1 phosphorylations by Cln complex, the Clb5-Cdc28 complex is now able to phosphorylate other Sic1 sites that are required for its real destruction, generating a positive feedback to reach the high level of free Clb5 not in a progressively way but at the same time to enter properly into S phase (Kõivomägi et al., 2011). See Figure #6 of introduction (Fig. I6).

The phosphorylation of Sic1 by Pho85 is essential during DNA damage response: when DNA damage occurs in G_1, Cln-Cdc28 is downregulated, leading to a delay in the cell cycle (Sidorova and Breeden, 1997). Pho85 is required for a proper return to cell cycle progression after activation of the G_1 DNA damage checkpoint. Pho85 is active at this time to antagonize the cell cycle arrest and restart the cell cycle possibly by phosphorylating and inhibiting Swi5 and by phosphorylating and promoting the degradation of Sic1 to reactivate Cdc28 (Wysocki et al., 2006). About Far1 regulation, exposure to pheromone induces arrest in G_1 by enhancing the association of the CKI Far1 with Cln-Cdc28 complexes to inhibit their catalytic activity (Peter and Herskowitz, 1994). In contrast, *pho85Δ* cells are hypersensitive to α-factor (Measday et al., 1997; Lee et al., 1998), indicating that Pcl-Pho85 kinase activity is important after mating process. Once Far1 is degraded cells become insensitive to the arrest imposed by mating pheromone (Henchoz et al., 1997).

Again, it is observed that Pho85 and Cdc28 play redundant roles on cell cycle regulation. But this time, despite having similar functions, both CDKs are essential under different conditions.

Phosphorylation of PEST sequences is an important targeting signal for Clns to be degraded. The PEST sequence is a peptide sequence which is rich in proline (P), glutamic acid (E), serine (S), and threonine (T). This sequence is associated with proteins that have a short intracellular half-life, such as Cln1,2,3 or Sic1, with a half-life of 3 to 10 minutes. The autophosphorylation of Cln-Cdc28 complexes on the PEST domain of these cyclins tethers them to Grr1, a different F-box protein, for ubiquitination and degradation. Thus, active Cln1,2-Cdc28 complexes are intrinsically labile and highly responsive to changes in rates of transcription brought on by different environmental conditions (Schneider et al., 1998). However, Cln3 is not degraded by a Grr1 complex, as it is in *Candida albicans* (Li et al., 2006). Therefore, this is a special case of a cyclin degraded by a complex still uncovered. Another cyclin that has to be targeted to degrade by phosphorylation is Clb6. Cdc28 and Pho85 phosphorylate Clb6 in S phase in a redundant manner as a signal for degradation (Jackson et al., 2006), leading to a negative feedback that regulates this cyclin levels, which are highly

increased before S phase entry. These positive and negative loops are represented in Figure I6.

The F-box protein Met30 mediates another cell-cycle role for the SCF. It targets kinase Swe1 for degradation in the G_2/M phase transition and, thereby, restricts the window in which Swe1 can inhibit Cdc28 (Kaiser et al., 1998).

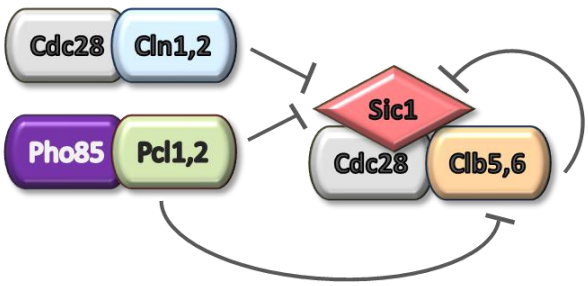

Figure I6. S phase entry regulation by Pho85 and Cdc28. Clb5,6 activity is regulated on the one hand by Sic1, which inhibits the complex until it is targeted for degradation by Cdc28-Cln1,2, Pho85-Pcl1 and the same Clb5,6-Cdc28 complex, and on the other hand directly by Cdc28 and Pho85 that targets the complex for degradation once its function is accomplished.

1.3.2. Anaphase proteolysis

The APC core complex is necessary for progression through anaphase, exit from mitosis and maintenance of G_1 phase (Zacharie and Nasmyth, 1999). It is turned off on S phase and switched on during anaphase. It leads Clb2,3,5 to have a short half-life of about 2 minutes at G_1 phase and to increase their stability to 15 minutes or more at S and G_2 phases.

The regulation of the APC is achieved by association with two conserved accessory factors, Cdc20 and Cdh1, which serve as substrate-specific subunits for the APC complex. Cdc20 activates the APC at the transition from metaphase to anaphase. Its abundance is cell-cycle-regulated, accumulating in S-phase, peaking in mitosis, due to transcriptional up-regulation by Clb-Cdc28 and dropping in G_1 due to protein degradation by Cdh1-APC (Pesin and Orr-Weaver, 2008; Prinz et al., 1998). Its activity requires the phosphorylation of APC core subunits by the polo-like kinase (Cdc5) and Cdc20 by Clb-Cdc28 (Nigg, 1998; Rudner and Murray, 2000). Clb3, Clb5 and the anaphase inhibitor Pds1 (Securin) are the only essential substrates of Cdc20-APC (Thornton and Toczyski, 2003). Anaphase is initiated upon Clb2-Cdc28-mediated activation of Cdc20-APC. Then active Cdc20-APC eliminates the anaphase inhibitor

Pds1, which holds Esp1 inactive. Esp1 cleaves the cohesin protein Scc1 to allow sister chromatid segregation (Uhlmann et al., 1999).

Complementarily to Cdc20, Cdh1 mediates APC activation from the end of anaphase until late G_1. It has other targets from M throughout G_1, including Clb1,2, factors that regulate spindle function, sister chromatid cohesins, and even Cdc20 (Zachariae and Nasmyth, 1999). In contrast to Cdc20, Cdh1 is expressed throughout the cell cycle and is held inactive by Clb-Cdc28-dependent phosphorylation until the end of mitosis, when it is dephosphorylated by Cdc14, upon activation of the Mitotic Exit Network (MEN) (Jaspersen et al., 1999; Zachariae and Nasmyth, 1999).

1.3.3. Other proteolytic systems

As it has been said before, E3 enzymes possess different domains: The HECT (Homologous to the E6-AP Carboxyl Terminus) domain, where a covalent E3-ubiquitin intermediate is formed before ubiquitin is transferred to the substrate protein, and the RING (Really Interesting New Gene) domain (or the closely related U-box domain), which transfer ubiquitin directly from E2.

A RING domain is a protein structural domain of zinc finger type that contains a $Cys_3HisCys_4$ amino acid motif, which binds two zinc cations. Many RING finger domains simultaneously bind ubiquitination enzymes and their substrates and hence function as ligases (Lorick et al., 1999).

Dma1 is, for instance, one well known E3 ubiquitin-ligase enzyme, containing a RING domain and a fork-head associated (FHA) phosphopeptide-binding domain. Dma1, in association with the E2 Ubc4, is involved in nutritional control of the cell cycle, proper spindle positioning, and likely regulating septin ring deposition at the bud neck.

1.4. Cln3 cyclin regulation

START G_1 phase is firstly promoted by Cln3 cyclin that triggers activation of transcription of the genes that depend on SBF (Swi6-Swi4) and MBF (Mbp1-Swi6) transcription factors. Cln3 binds to Cdc28 to promote the formation of the RNA polymerase II holoenzyme at the TATA boxes of target genes (Cosma et al., 2001) and the phosphorylation of the inhibitor Whi5, forcing its nuclear exclusion (Costanzo et al., 2004; de Bruin et al., 2004). This process leads to *CLN1* and *CLN2* gene expression. Although Cln1 and Cln2 have the potential to activate their own transcription in absence of Cln3, since they can phosphorylate Whi5 (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991), this positive feedback activation does not contribute strongly to the timing of initial *CLN1,2* induction and cells have significantly increased doubling time (Dirick et al., 1995; Stuart and Wittenberg, 1995; Bean et al., 2006).

Cyclins as Cln3 are regulated by cell cycle-specific transcription, activation, localization and degradation. As a result of this redundant regulation, defects in a single regulatory step rarely disrupt the cell cycle.

Cln3 has a very short half-life that allows its steady-state abundance to be rapidly and sensitively regulated by changes in growth rate, protein synthesis, and cell size. Thus, the instability of Cln3 may underlie the ability of cells to adapt rapidly to fluctuating growth conditions and to avoid undergoing cell division under adverse conditions.

1.4.1. *CLN3* transcriptional regulation

Changes in *CLN3* expression alter G_1 duration: increased expression of *CLN3* shortens G_1, whereas loss of *CLN3* extends G_1 (Cross, 1988; Nash et al., 1988).

CLN3 transcription is maintained throughout the cell cycle but displays some cell cycle periodicity that depends on early cell cycle boxes (ECB) sites upstream of the *CLN3* promoter (Mai et al., 2002; McInerny et al., 1997; McKay et al., 2001). The ECB element includes a 16-bp palindrome where Mcm1 is bound to protect from other transcription factors that promote the expression of *CLN3* (Mai et al., 2002).

Yox1 is another binding factor upstream the sequence of *CLN3*. This is one of the proteins that repress ECB activity and is required to turn off the targets of Swi4-Swi6 until late M phase, as a negative feedback loop that determines the length of the pulse of ECB activity (Pramila et al., 2002).

CLN3 expression is in turn regulated by nutrients through several distinct pathways. Indeed, it is thought that glucose induces *CLN3* transcription and promotes rapid G_1 progression (Hubler et al., 1993). *CLN3* mRNA levels are induced by glucose and decreased in the presence of non-fermentable carbon sources. This is not regulated by Ras/cAMP or Tor pathways (as it is during translation regulation; see 1.4.2), but involves a set of repeated sequences upstream of the *CLN3* coding region with the sequence AAGAAAAA, that drives glucose-dependent transcription of reporter genes and binds DNA sequence specific proteins like Azf1, a nuclear zinc finger (Newcomb et al., 2002). Azf1 acts as a glucose-dependent transcription factor that interacts with the *CLN3* A_2GA_5 repeats to play a positive role in the regulation of *CLN3* mRNA expression by glucose. Mcm1 binding to ECB is known to be affected by changes in the carbon source as well (Mai et al., 2002).

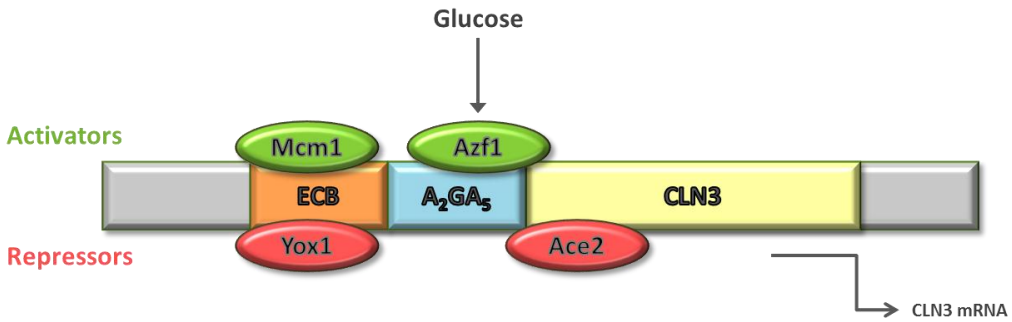

Figure I7. *CLN3* transcription is regulated by different transcription factors and inhibitors. *CLN3* transcription is positively regulated by Mcm1 and Azf1, which in turn is stimulated by glucose, and negatively modulated by Ace2 in daughter cells and Yox1.

However, there are some situations where G_1 has not a rapid progression but a delay instead. This is the case of daughter cells, with a G_1 delay that has long been thought to be due to a requirement for attaining a certain critical cell size before passing the commitment point START. But in the case of these daughter cells there is one more important mechanism for this delay: the presence of Ace2 in daughter cells inhibits *CLN3* expression, extending G_1 in daughter cells (Laabs et al., 2003).

Thus, *CLN3* is an integrator for signals that regulate the rate of G_1 progression in yeast.

1.4.2. *CLN3* translational regulation

Although *CLN3* mRNA displays almost constant expression levels throughout the cell cycle, the Cln3 protein is extremely unstable (Tyers et al., 1993; Cross and Blake, 1993; Yaglom et al., 1995). This instability indicates that the main regulation of Cln3 occurs at a posttranscriptional level.

A short upstream ORF makes *CLN3* translation extremely sensitive to decreases in the abundance of translational initiation complexes as well as to the rate of overall protein synthesis, which is highly affected when cells are small. A 50% decrease in protein synthesis produces a 10-fold fall in Cln3 protein levels. This mechanism probably accounts for regulation of Cln3 translation by the Tor or the protein Kinase A pathways (Barbet et al., 1996; Gallego et al., 1997; Polymenis and Schmidt, 1997). The Tor, the phosphatidylinositol 3-kinase and the RAS/GPA2/cyclin AMP (cAMP) pathways are thought to regulate Cln3 translation through effects on protein synthesis rates.

This pathway may involve the Tor1- and Tor2-stimulated association of Sit4 with Tap42, since this association is also inhibited by rapamycin, an effect not seen in strains carrying alleles of *TOR1* or *TOR2* that confer rapamycin resistance (Di Como et al., 1996). In addition to this pathway, nutritional deprivation or interruption of the Tor-dependent signal transduction pathway induces the degradation of eIF4G, a factor required for initiation of translation of capped mRNAs (Berset et al., 1998). These results suggest that nutritional signals favouring growth are mediated by the Tor1 and Tor2 proteins. Tor1 and Tor2 somehow inhibit the degradation of eIF4G and increase the association of Tap42 with Sit4 that, presumably, promotes the translation of *CLN3* (see Fig. I8).

However, other researchers have argued against the importance of this pathway as a specific mechanism controlling the nutritional response. They claim that translational rates are dramatically reduced by rapamycin treatment whereas down-regulation of the Cln3 translation rate occurs when overall translation rates are still quite high. Aldea and colleagues found that Cln3 protein levels decrease sharply in the first two hours following nitrogen starvation. This decrease is mainly due to an eightfold decrease in the rate of *CLN3* mRNA translation. During the same time interval, general protein synthesis decreases only twofold, indicating that *CLN3* translation is particularly sensitive to the loss of nitrogen. It has been demonstrated that Cln3 is down-regulated mainly at the translation level by nitrogen deprivation, whereas the faster turnover observed under these condition would help to decrease the Cln3 levels more rapidly (Gallego et al., 1997).

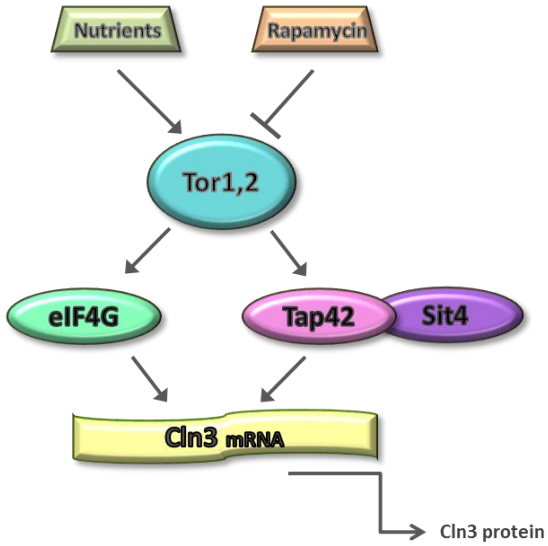

Figure I8. *CLN3* mRNA translation is affected by general protein synthesis mechanisms. *CLN3* mRNA translation is positively regulated by TORC pathway in presence of nutrients, by stabilizing eIF4G and promoting association of Tap42 and Sit4.

Another well characterized way to modulate *CLN3* translation, discovered by the same group, is by Whi3. Whi3 is a RNA binding protein that contains a C-terminal RNA recognition motif (RRM) that binds *CLN3* mRNA and sequesters it in cytoplasmic foci to locally restrict synthesis of this G_1 cyclin (Garí et al., 2001) and to avoid entry to G_1 (Vergés et al., 2007). See Fig. I9.

1.4.3. Cln3 half-life regulation

Proteolysis is an important regulating point for the life of this cyclin. Cln3 contains an ubiquitin-dependent degradation signal composed by PEST sequences (residues 437 to 536). *CLN3* mutant in the C-terminal one-third of *CLN3*, which contains this region, makes Cln3 hyperstable (Tyers et al., 1992; Cross et al., 1993). This mutant, generally known as Cln3-1, does not contain the PEST sequence, indicating that Cln3 is regulated by ubiquitin-dependent proteolysis.

However, Cln3 seems not to follow the usual way of ubiquitin-degradation pathway via the Grr1 F-box protein. Therefore, the E2 that directs Cln3 degradation remains still unknown.

It has been suggested that this ubiquitin-dependent proteolysis needs multiple phosphorylations by Cdc28 at the PEST sequences of Cln3 to ensure its degradation, which is mediated by the ubiquitin-conjugating enzyme Cdc34 (Gallego et al., 1997; Tyers et al., 1992; Yaglom et al., 1995; Cross et al., 1993).

In addition to Cln3 stabilization on *cdc34ts* mutants, a Cln3-β-galactosidase fusion is also stabilized in ubc4Δubc5Δ double mutants but is not stabilized by mutations in other five ubiquitin-conjugating enzyme mutants (Yaglom et al., 1995). Ubc4 and Ubc5 are E2 that share responsibility for degrading a variety of short-lived and abnormal proteins (Seufert and Jentsch, 1990).

Furthermore, a role for Ydj1 has been recently found in the regulation of Cln3. Cln3 contains a chaperone-regulatory J_i domain that keeps the protein retained at the Endoplasmic Reticulum. Ydj1 would promote the release of Cln3 and its nuclear accumulation in late G_1. Moreover Ydj1 could act as a transmitter of growth capacity as synthesis of this chaperone is affected by the protein synthesis rate (Vergés et al., 2007).

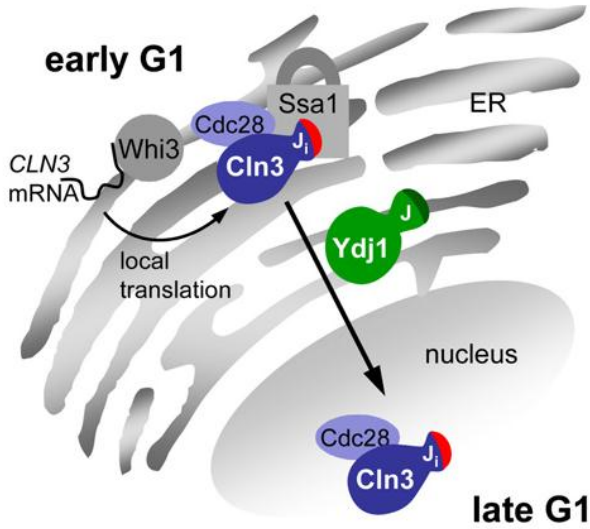

Figure I9. Cln3 translation and half-life. G_1 cyclin Cln3 retention at the ER is modulated during G_1 by the J_i domain and the Ydj1 chaperone. By inhibiting the Hsp70 conformational cycle, the J_i domain would lock Ssa1,2 chaperones into a tightly associated ER complex with Cdc28 in early G_1, thus preventing unscheduled nuclear import of Cln3. In late G_1, once a relative surplus of Ydj1 (and most likely other folding activities) is achieved, ATPase activation by Ydj1 would unlock the Ssa1,2 complex and trigger ER release and nuclear accumulation of Cln3 to initiate cell cycle entry (Vergés et al., 2007).

2. NUTRIENT SIGNALING IN *Saccharomyces cerevisiae*

Normal cell cycle progression can be perturbed by a huge variety of inputs as DNA damage, osmostress, high temperature, pheromones, etc. Nowadays, all these inputs are very well described by several groups. However, nutrient environmental sensing and how it affects cell cycle progression still remains not completely understood.

The constantly fluctuating nutrient content on the environment is a determinant key of cell cycle progression and growth, stress resistance and metabolism. The nutrient-induced signaling network enables yeast both to optimally profit from rich nutrient conditions by stimulating cell proliferation and to survive periods of nutrient scarceness by inducing the entry into a quiescent, resting phase, called stationary phase G_0 (Pedruzzi et al., 2003; Zaman et al., 2008). However, it is not always reproduced in "general lab growth conditions".

S. cerevisiae cells use a wide variety of substances as carbon source, but some of them are preferred over others. Glucose is the favorite one and yeast grows exponentially if its concentration is high. When glucose becomes limiting, cells go through the diauxic shift, in which metabolism shifts from fermentation to respiration to allow usage of ethanol and acetate, which have been accumulated during the fermentative growth phase. Finally, when these late carbon sources have been exhausted, cells enter the stationary phase, called G_0. Cells in G_0 present a number of distinguishing characteristics, including enhanced resistance to heat and high osmolarity, substantially reduced translation, a specific transcriptional profile, increased levels of storage carbohydrates and a thickened cell wall (Fabrizio and Longo, 2003; Gray et al., 2004; Coller et al., 2006).

Importantly, when nitrogen, another essential nutrient, becomes limiting before glucose, yeast stops cell growth at a specific point of the cell cycle. If the lack of nitrogen is kept for long time, cells finally enter to G_0 as well (Weinberger et al., 2007; Wanke et al., 2005).

Finally, phosphate source is the latest nutrient discovered that is able to do some kind of regulation, but it remains not completely understood (Gray et al, 2004; Klosinska et al., 2010).

There are several signaling pathways that regulate cell cycle progression depending on the nutrients concentration. Most of them are well known kinases, but some others still remain uncovered. Among the most important nutrients required by yeast it is known that carbon, nitrogen and amino acids sources are sensed by well

described signalling pathways. However, very few data has been reported about how phosphate signalling works.

2.1. Carbon source signaling pathways

Yeast cells preferably ferment glucose and other rapidly fermentable sugars to ethanol and acetate although respiration would be energetically more favorable. This is because the ethanol produced during fermentation inhibits growth of other micro-organisms.

2.1.1. The Snf1 pathway

In the presence of glucose, the serine/threonine kinase Snf1 is inactivated, resulting in the transcriptional repression of genes that are not needed during fermentative growth on glucose, for instance genes encoding enzymes involved in gluconeogenesis, the Krebs cycle, respiration or the uptake and metabolism of alternative carbon sources (Ronne et al., 1995; Gancedo, 1998; Hedbacker and Carlson, 2008).

The Sfn1 kinase belongs to the well-conserved Snf1/AMP-activated protein kinase family (AMPK) and remains phosphorylated and activated while levels of glucose are low (Celenza et al., 1989; Yang et al., 1994; Jiang and Carlson, 1997; McCarteney et al., 2005; Sutherland et al., 2003).

Upon high levels of glucose, dephosphorylation of Snf1 by the protein phosphatase complex Gcl7-Reg1 leads to its inactivation. The catalytic activity of the Glc7-Reg1 phosphatase itself is not controlled by glucose, but rather Snf1 itself that exposes its activation loop to act as a substrate for Gcl7-Reg1 depending on glucose availability (Rubenstein et al., 2008).

The most important Snf1 target is the transcriptional repressor Mig1. In the presence of glucose, Mig1 is localized to the nucleus inhibiting the expression of many glucose-repressed genes by binding to their promoter in association with the co-repressor complex Cyc8/Ssn6-Tup1. Glucose exhaustion induces phosphorylation of Mig1 by the activated Snf1 complex, which triggers the nuclear exclusion of Mig1 (Treitel and Carlson, 1995; Papamichos-Chronakis et al., 2004; De Vit et al., 1997).

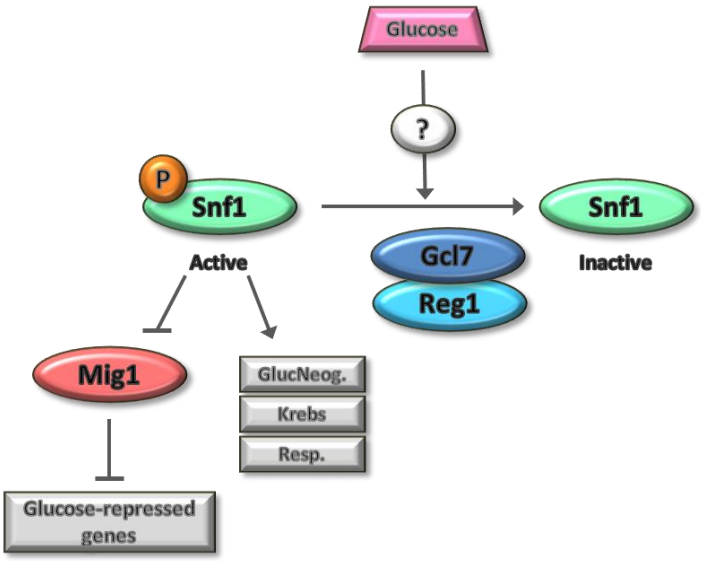

Figure I10. Carbon source signaling by Snf1. High levels of glucose induces inhibitory phosphorylation of Snf1 through Gcl7-Reg1 complex, leading Mig1 nuclear exclusion, which represses multiple non-necessary genes during fermentation. The sensor of glucose concentration still remains uncovered.

Though this pathway is partially well described, how the glucose signal is exactly transduced to the Snf1 complex or to its regulators still remains unclear.

2.1.2. The cAMP-PKA pathway

In response to high levels of glucose after a previous depletion of this carbon source, the cAMP-PKA pathway induces the synthesis of cAMP to stimulate the activity of the cAMP-dependent protein kinase PKA. PKA targets several proteins that help cells to adapt for fermentative growth as upregulation of glycolysis, stimulation of cell growth, cell cycle progression, downregulation of stress resistance and gluconeogenesis, and mobilization of the reserve carbohydrate glycogen and the stress protectant trehalose (Thevelein et al., 2000; Santangelo, 2006; Tamaki, 2007; Gancedo, 2008).

PKA is a hetero-tetramer composed by the catalytic subunits Tpk1, Tpk2 and Tpk3 that dissociate from the regulatory subunit Bcy1 in presence of cAMP to lead to their activation. Both intracellular and extracellular glucose sensing positively affect this pathway. How this pathway exactly works is represented in Fig. I11.

However, how the signals of both glucose sensing systems are exactly integrated to control adenylate cyclase activity remains unsolved.

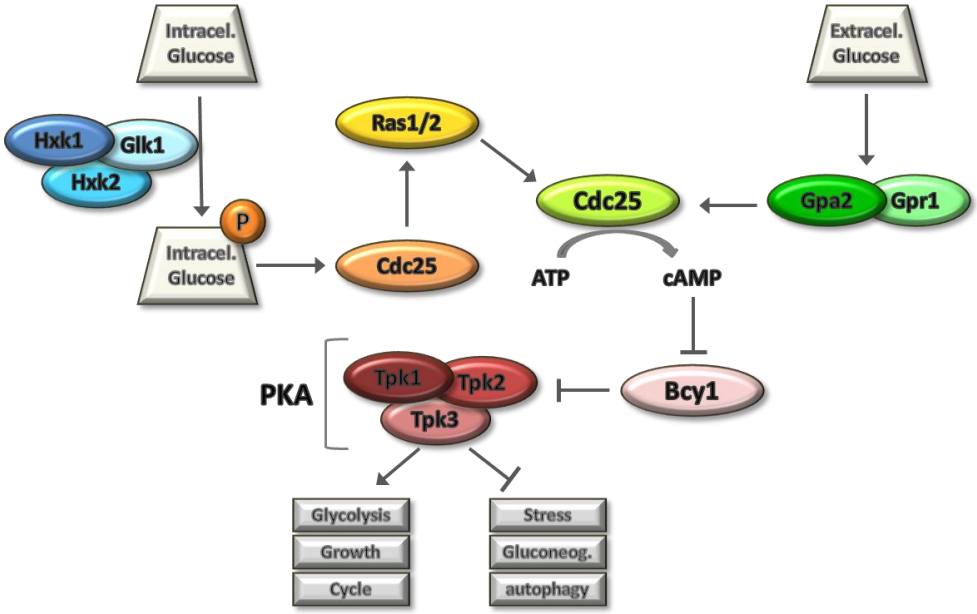

Figure I11. Carbon source signaling by cAMP-PKA. Three glucose phosphorylating enzymes (Hxk1, Hxk2 and Glk1) phosphorylates intracellular glucose leading to the activation of cAMP-PKA pathway via the Ras proteins (Ras1 and Ras2), which belong to the group of small G proteins. Active Ras proteins (when they are bound to GTP) stimulate the activity of Cdc35 (known as the adenylate cyclase Cyr1), which catalyzes the synthesis of cAMP from ATP (Field et al., 1988; Camonis and Jacquet, 1988). Extracellular glucose detection occurs through a G protein-coupled receptor (GPCR) system, composed of Gpr1 and Gpa2. Addition of glucose activates Gpr1 which in turn stimulates the exchange of GDP to GTP on Gpa2 that leads to the activation of the cAMP-PKA pathway (Kraakman et al, 1999; Colombo et al., 1998; Rolland et al, 2000; Versele et al., 1999). The Gpr1-Gpa2 complex displays low affinity for glucose and intracellular sensing of glucose is a prerequisite for the activation of this system; these requirements ensure that the cAMP-PKA pathway is only fully activated when glucose levels are high enough to switch easily from respiration to fermentation (Rolland et al., 2000; Lemaire et al., 2004).

Several of the known PKA targets affect gene transcript levels, either directly or indirectly. Two of those are Msn2 and Msn4 that are phosphorylated by PKA in presence of glucose to inhibit their nuclear import and avoid the transcription of the stress response element-controlled genes (STRE). The STRE genes protect against diverse types of stress such as heat, oxidative and osmotic stress, carbohydrate metabolism, and growth regulation (Estruch and Carlson, 1993; Martinez-Pastor et al., 1996; Gasch et al., 2000). Msn2,4 are also negatively regulated by the Ccr4-Not

complex and by the protein kinases Yak1 and Rim15 (both under negative control of PKA) (Lenssen et al., 2005; Moriya et al., 2001). Rim15 is essential for the accumulation of the carbohydrate reserve glycogen and the stress protectant trehalose, the induction of several stress response genes, the induction of thermo-tolerance and a proper G_1-arrest upon nutrient starvation (Reinders et al., 1998; Cameroni et al., 2004; Zhang et al., 2009).

Finally, PKA is a known inhibitor of autophagy, a degradative process that recycles non-essential proteins and organelles during periods of nutrient starvation, through some Atg proteins.

2.2. Nitrogen source signaling pathways

The target of Rapamycin (TOR), a highly conserved Ser/Thr protein kinase, is the central component of a major regulatory signaling network that controls cell growth in diverse eukaryotic organisms, ranging from yeast to human cells. The TOR proteins were first identified in yeast as the targets of the antifungal and immunosuppresive agent rapamycin (Heitman et al., 1991).

Yeast cells contain two TOR homologues, Tor1 and Tor2, which are found in the TOR multiprotein complexes TORC1 and TORC2. However, TORC1 is the only one specifically inhibited by rapamycin. The addition of rapamycin induces dramatic phenotypic changes such as cell cycle arrest and entry into G_0, general downregulation of protein synthesis, accumulation of the reserve carbohydrate glycogen and the stress protectant trehalose, upregulation of stress response genes, autophagy and alterations in nitrogen and carbon metabolism. TORC1 signaling controls the temporal aspects of cell growth in response to the amount of available nitrogen and carbon source. TORC2, which is insensitive to rapamycin, is thought to regulate the spatial aspects of growth, such as the control of actin polarization (De Virgilio and Loewith, 2006).

The multiprotein TORC1 involves, apart from Tor1, other proteins such as Tor2, Lst8, Kog1 and Tco89, that might play a role to bind the TOR complexes to their substrates, receive upstream signals and/or determine the localization of the complexes (Loewith et al., 2002; Fadri et al., 2005).

It has been found that TOR complexes associate with membranes ranging from the plasma to the vacuolar and internal membranes of the lysosomes and endosomes,

being TORC1 mainly found at the vacuolar membrane, the main reservoir of nutrients in the yeast cells (Wedaman et al., 2003).

2.2.1. TORC1 signaling via Tap42-Sit4-PP2AC

Several TORC1-mediated processes are via the PP2A and the PP2A-related protein phosphatases. The PP2A holoenzyme contains proteins such as Pph21, Pph22, Tpd3, and Cdc55/Rts1. On the other hand, the PP2A-related phosphatase is mainly found as a complex between the catalytic subunit, Sit4, and one of the four regulatory subunits, Sap4, Sap155, Sap185 or Sap190 (Luke et al., 1996). TORC1 phosphorylates Tap42, which competes for binding the catalytic subunit of the phosphatases. This competition leads to the exclusion of other subunits to redirect the substrate specificity of the holoenzymes.

Some processes where this signaling pathway is crucial are: nitrogen, amino acids, protein synthesis, stress response and autophagy signaling. Some of them are represented on Figure I12.

Nitrogen signaling by TOR via PP2A and Sit4 is under the control of GATA family zinc-finger transcription factors, which are involved in the transcription of genes required for the usage of poor nitrogen sources in the lack of rich nitrogen sources as glutamine.

TORC1 also negatively controls the general amino acid control (GAAC) pathway (Natarajan et al., 2001). Moreover, TORC1 also appears to control the turnover of several amino acid permeases through regulation of the Tap42-Sit4 phosphatase complex. This regulation sorts the permeases depending on the amount of nitrogen sources in the medium. Under rich nutrient conditions, Tat2 is targeted to the plasma membrane, whereas the nitrogen-responsive permeases, such as Gap1, are sorted to the vacuole for degradation (Beck et al., 1999; Roberg et al., 1997).

Besides, TORC1 has a major regulatory role in protein synthesis as it promotes expression of genes needed for translation machinery such as translation factors, rRNA and Ribosomal Proteins (RP) modifying and processing enzymes, tRNA synthetases and subunits of RNA Polimerases I and III (Jorgensen et al., 2004).

TORC1 also exerts a major impact on the transcription of stress response genes. Here, TORC1 has a dual control: on the one hand, it prevents nuclear translocation of the protein kinase Rim15, thereby ensuring that this kinase is maintained inactive through PKA-mediated phosphorylation (Urban et al., 2007). On

the other hand, TORC1 inhibits the transcription of stress-responsive genes via Tap42-PP2A to promote the phosphorylation and cytoplasmic retention of Msn2.

TORC1 is also a known negative regulator of autophagy by phosphorylating the Atg13 protein via Tap42-PP2A (Kamada et al., 2000; Yorimitsu et al., 2009).

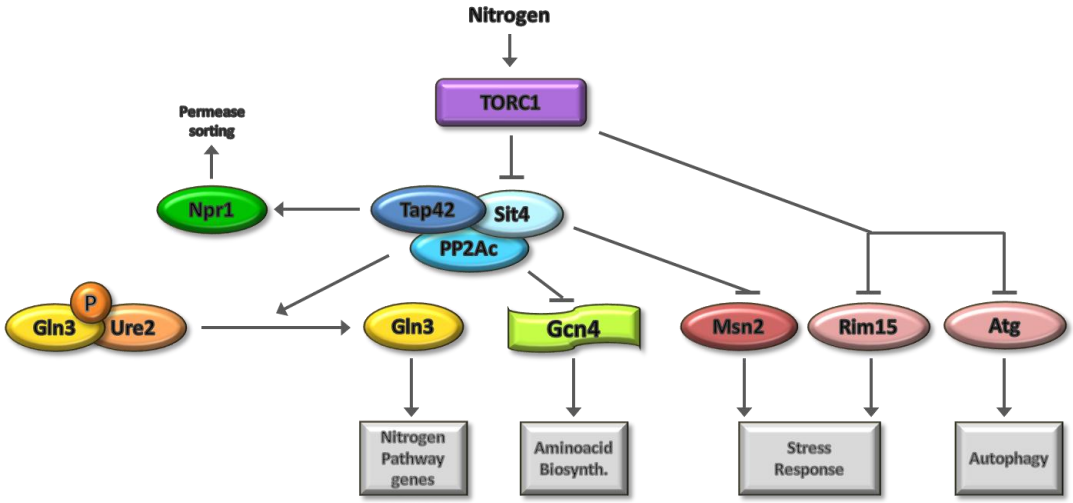

Figure I12. Nitrogen signaling by TORC1 via Tap42-Sit4-PP2AC. *Nitrogen signaling:* In presence of rich nitrogen sources TORC1 phosphorylates Gln3, triggering to its sequestration in the cytoplasm through its binding with the cytoplasmic anchor protein Ure2. Rapamycin treatment or nitrogen starvation abrogates the TORC1 association and releases the Tap42-PP2A complex into the cytosol for its slowly dissociation. Then Gln3 is not phosphorylated anymore, dissociates from Ure2 and enters into the nucleus, where Gln3 can exert its transcriptional activator function (Beck et al., 1999). *Amino acid signaling:* TORC1 inhibits *GCN4* mRNA translation, a transcription factor important for activating transcription of genes needed for amino acid biosynthesis in response to amino acid starvation (Natarajan et al., 2001). Moreover, Npr1 is a protein kinase that stabilizes the general amino acid permease Gap1 at the plasma membrane. Phosphorylation of Npr1 by TORC1 activity inhibits the protein triggering to the degradation of Gap1 and to the stabilization at the plasma membrane of Tat2, the high-affinity tryptophan permease. *Stress response signaling:* TORC1 inhibits Rim15 and Msn2 entry to nucleus, the last one via Tap42-PP2A. *Autophagy:* TORC1 inhibits Atg proteins.

Finally, as others nutrient signaling pathways described before, understanding of how nutrients are sensed and how this information is transduced to TORC1 is still one of the major challenges in the TORC1 field.

2.2.2. TORC1 Signaling via Sch9

During the lag phase, when cells prepare themselves for fermentative growth, cAMP-PKA pathway is transiently activated. Then, Sch9 is essential for the proper regulation of several essential processes in glucose-repressed cells, such as the activation of trehalase, the repression of stress response genes and the induction of ribosomal protein genes (Crauwels et al., 1997). Both kinases have an additive effect in some processes, such as the expression of proteins required for nucleotide metabolism. But they also have opposite effects in other situations, such as the expression of proteins involved in detoxification or proteolysis (Roosen et al., 2005).

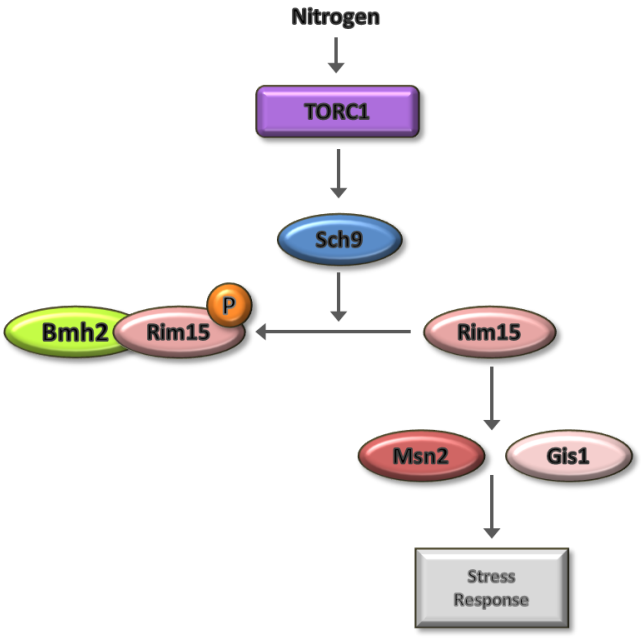

Figure I13. Nitrogen signaling by TORC1 via Sch9. Rim15 controls the expression of stress-responsive genes through the transcriptional activators Gis1 and Msn2/4 that, respectively, bind to the PDS and STRE elements (Roosen et al., 2005). Sch9 phosphorylates Rim15 to stimulate its binding to Bmh2, which guarantee optimal sequestration of Rim15 in the cytoplasm (Wanke et al., 2008). As expected, the regulation of Rim15 localization by Sch9 is also found to be dependent on TORC1 signaling as rapamycin treatment causes dephosphorylation of Bmh2-binding site and enforces nuclear accumulation of Rim15 (Urban et al., 2007).

Sch9 is directly phosphorylated by TORC1 on at least five residues in the C-terminal half and these phosphorylation events are critical for its catalytic activity. It allows TORC1 to prevent the induction of typical G_0 traits as well as to stimulate

ribosomal biogenesis and translation initiation (Urban et al., 2007). Moreover, Sch9 appears to be phosphorylated by other undefined kinases that remain unknown.

Like TORC1, Sch9 is predominantly localized at the vacuolar membrane, but a fraction is localized as well in the nucleus, recruited to the chromatin of osmostress-responsive genes (Pascual-Ahuir and Proft, 2007).

One major role of Sch9 is to regulate translation in function of nutrient availability and growth potential. Sch9 controls the expression of RP genes and the Ribi regulon, by interfering with the transcriptional processes conducted by the RNA polymerases I, II and III.

Several studies implicate Sch9 (together with Pho85-Pho80, see below) in the regulation of the cellular responses to stress. Its role in the regulation of the protein kinase Rim15 is the best characterized (represented in Fig. I13).

Autophagy is induced in yeast cells upon the simultaneous inactivation of Sch9 and PKA, via the Atg1 kinase, acting in parallel to the TORC1 pathways to triggers autophagy (Yorimitsu et al., 2007).

2.3. Phosphate source signaling pathways

Inorganic phosphate is an essential nutrient for all organisms required for biosynthesis of nucleotides, phospholipids and metabolites. This makes phosphate an important messenger to signal a growth limiting metabolic state and reduced developmental capacities of the cell. Similar to glucose or nitrogen starvation conditions, depletion of phosphorous sources forces yeast cells to enter the quiescent G_0-state (Swinnen et al., 2006). Pho85 is one of the most important kinases involved in nutrient signaling of phosphate concentration.

2.3.1. Pho85 in phosphate sensing

Nutrient sensing by Pho85 is mainly performed together with Pho80, which is the major cyclin involved in phosphate metabolism and regulation of proper entry into G_0 under phosphate starving conditions.

The Pho85-Pho80 kinase complex plays a central role in the PHO pathway, which allows cells to properly respond to changes in extra- and/or intracellular phosphate levels. Pho85-Pho80 complex controls the localization of the Pho4 transcription factor, which is essential for induction of the PHO genes, leading to optimized phosphate acquisition (Kaffman et al., 1994).

Under limiting conditions, the low-phosphate signal activates the PHO pathway by triggering activation of the CDK-inhibitor (CKI) Pho81. In turn, the active Pho81 maintains the Pho85-Pho80 complex in its inactive form and prevents that the kinase complex hyperphosphorylates Pho4. Under these conditions, Pho4 can interact with its nuclear import factor Pse1 and is translocated to the nucleus, where it activates the transcription of the PHO genes (O'Neill et al., 1996)

When phosphate is abundant, Pho81 is inactivated and as a result the Pho85-Pho80 kinase complex becomes active. Then, Pho4 is phosphorylated, and interacts with the export factor Msn5 to be excluded from the nucleus. It leads to a Pho4 retention at the cytoplasm and the repression of the PHO genes (Kaffman et al., 1998).

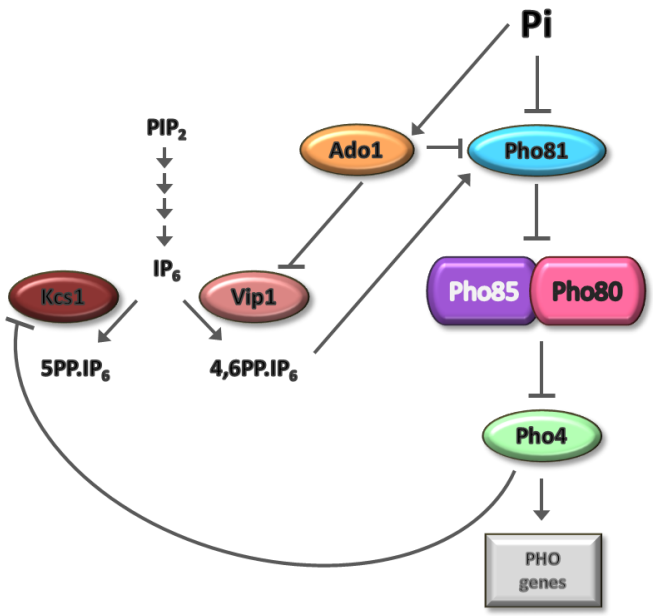

Figure I14. Phosphate signaling by Pho85-Pho80 complex. Activation of the PHO pathway in response to P_i-limiting conditions requires inhibition of Pho85-Pho80 by Pho81 and subsequent transcription of the PHO genes via interaction of Pho4. Inositol phosphate metabolism induces Pho81 activity through the Vip1 product, which in turn is inhibited by Ado1 in high phosphate conditions.

Since Pho81 always interacts with the Pho80 cyclin, but only inhibits the Pho85-Pho80 kinase under phosphate limiting conditions, Pho81 may be regulated by post-translational or allosteric activations. Inositol polyphosphate species play a particular role in Pho81 regulation: an isoform synthesized by Vip1 (the signaling molecule myo-D-inositol-heptakisphosphate, IP_7) is found to bind non-covalently with Pho85-Pho85-Pho81, thereby inducing additional interactions between Pho81 and Pho80-Pho85 that prevent substrates from accessing the kinase active site. Intriguingly, under phosphate starvation conditions, Pho4-dependent antisense and intragenic RNA transcription of the *KCS1* locus induces downregulation of the Kcs1 activity, leading to a positive feedback loop for activation of the PHO genes (Lee et al., 2007; Nishizawa et al., 2008). Furthermore, mutants lacking the adenosine kinase Ado1, required for the synthesis of AMP from adenosine and ATP (Lecoq et al., 2001), display a Pho81-dependent nuclear translocation of Pho4 (Huang and O'Shea, 2005). This signaling pathway is represented in Fig. I14.

S. cerevisiae encodes for two different phosphate systems: a low-affinity system consisting of Pho87, Pho90 and Pho91 and a high-affinity system containing Pho84 and Pho89. Under phosphate limiting conditions, genes encoding for high-affinity phosphate transporters are induced (Auesukaree et al., 2003). It has been recently demonstrated by O'Shea and colleagues that high-affinity transporters are regulated through a negative feedback loop induced by Pho4, which helps to bring phosphate into the cell and inactivate the PHO pathway. On the other hand, low-affinity transporters are inhibited by Pho4 through a positive feedback loop which tends to reduce phosphate uptake leading to further pathway activation. Because the feedback loops are both controlled by the PHO pathway, they create mutually exclusive states in which cells either activate the PHO pathway expressing high-affinity transporters and down-regulating low-affinity transport system or, instead, keep the PHO pathway turned off and utilize low-affinity transporters for phosphate uptake. They speculate that the bimodality in high-affinity transporters expression arises under intermediate phosphate conditions, because there are phenotypical differences between genetically identical cells. Some cells activate the PHO pathway and uses high-affinity transporters, whereas others keep the pathway repressed and instead take up phosphate using low-affinity transporters. This population heterogeneity is generated by the feedback loops and may provide a strategy for anticipating changes in environmental phosphate levels (Wykoff et al., 2007) (See Fig. I15).

In addition, several repressible acid phosphatases, encoded by *PHO5*, *PHO11* and *PHO12* as well as the repressible alkaline phosphatase *PHO8* are induced under phosphate starvation. The acid phosphatases are secreted into the periplasmic space where they act on a multitude of phospho-ester substrates, whereas the alkaline phosphatase Pho8, localized at the vacuole, acts on other phosphate-containing substrates to liberate free phosphate (Klionsky and Emr, 1989).

Genes involved in catabolism of alternative phosphorous sources (*GIT1*, *GDE1* and *HOR2*) are also induced under phosphate limiting conditions, as well as genes involved in accumulation of polyphosphate in the vacuole (*VTC1*,*2*,*3*,*4*). And finally, *PHO81* itself is induced under these conditions, comprising a positive feedback loop for constitutive activation of the PHO pathway (Ogawa et al., 2000).

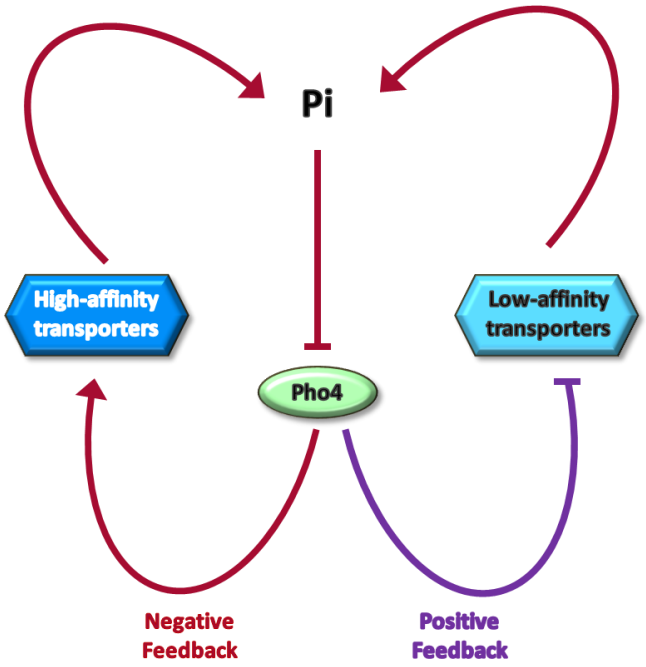

Figure I15. Switching of phosphate transporters. Pho4, which is regulated by internal phosphate concentrations, controls the transcription of high- and low- affinity transporters of phosphate. High-affinity transporters serve as a negative feedback element in the network, whereas low-affinity transporters are modulated by positive feedback, reducing internal phosphate levels and leading to further activation of Pho4 (adapted from Wykoff et al., 2007).

2.3.2. Pho85 in phosphate starvation-induced G_0 entry

During the phosphate starvation-induced stress responses, the Pho81 CKI has a central role in the activation of Rim15. Indeed, the *pho81*Δ mutant strain, like *rim15*Δ strain has a reduced expression of STRE and PDS genes. And, interestingly, a *PHO85* deletion revert all the observed phenotypes of these strains. Importantly, regulation of PDS genes requires the protein kinase Sch9, so it is logical to think that this kinase could play a role in controlling these genes in response to phosphate availability in

parallel with Pho85. Indeed a combined deletion of *PHO85* and *SCH9* is synthetic lethal (Swinnen et al., 2005).

It is known that Pho85-Pho80 complex promotes the nuclear exclusion of Rim15 through Msn5 when phosphate is abundantly available. Conversely, TORC1 inactivation triggers dephosphorylation of Rim15, thereby favoring accumulation of Rim15 in the nucleus. Since Pho85-Pho80 is mainly localized within the nucleus and TORC1 is predominantly associated with the vacuolar membrane, both complexes may act on different pools of Rim15 (Wanke et al., 2005).

2.3.3. Other functions of Pho85 in nutrients sensing

2.3.3.1. Calcium absence stress

Pho80-Pho85 negatively regulates the nuclear localization of another transcription factor, Crz1. Crz1 is activated by the calcium-dependent phosphatase calcineurin in stress conditions that trigger Ca^{2+} signaling to drive programs of gene expression and promote cell survival. Calcineurin dephosphorylates Crz1 and causes its rapid translocation from the cytosol to the nucleus. Contrary, Pho80-Pho85 phosphorylates this transcription factor retaining it at the cytoplasm. In addition to Pho85, Crz1 is also phosphorylated by other kinases such as PKA (Cyert, 2003).

The involvement of Pho85 in the regulation of both calcium and phosphate (chapter 2.3.1) signaling is logical because these nutrient sources are both generally found together in nature as Calcium phosphate ($Ca_3(PO_4)_2$). Therefore, it makes sense that their sensing effects act in parallel under similar pathways.

2.3.3.2. Amino acids and Carbon Source absence stress

Gcn4, a transcription factor that activates amino acid biosynthesis genes in response to amino acid starvation, is targeted for degradation through a Pcl5-Pho85-dependent phosphorylation when amino acids are abundant. Under starvation conditions, only a small amount of Pcl5 is synthesized, Gcn4 is thus hypophosphorylated, and the reduced phosphorylation results in Gcn4 stabilization (Shemer et al., 2002; Bömeke et al., 2006).

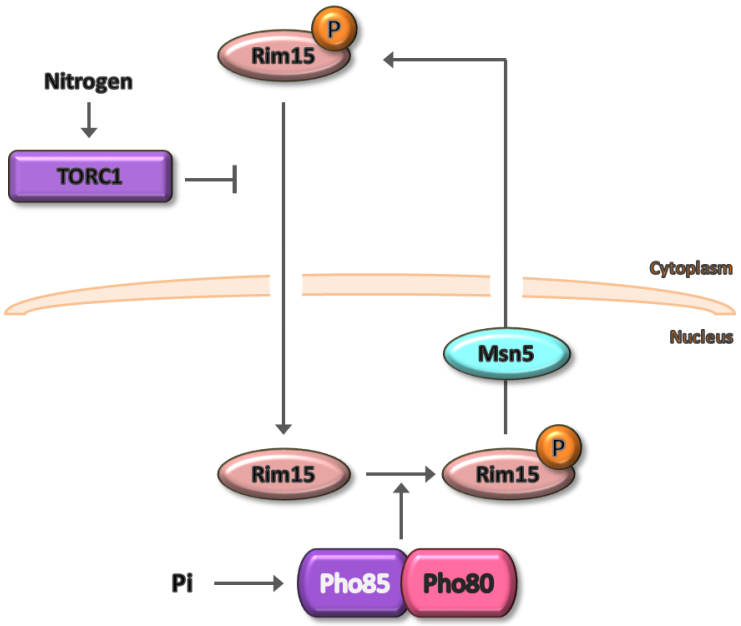

Figure I16. Regulation of Rim15 by nutrient-sensing kinases. Cytoplasmic Rim15 is kept inactivate by TORC1. In absence of nitrogen source, through TORC1 inactivation, Rim15 is dephosphorylated with concomitant abrogation of its cytoplasmic retention. Following its nuclear import active Rim15 induces the G_0 program, initiates an autophosphorylation process and accelerates its Msn5-mediated nuclear export. The Pho80–Pho85 cyclin–CDK complex phosphorylates Rim15, thereby promoting export.

On the contrary, Gcn4 is induced in the presence of nitrogen source as explained in chapter 2.2.1. Nitrogen promotes TORC1 activation that leads to the Gcn4 translocation to nucleus, where induces gene expression for amino acid biosynthesis. This process supports the idea that when external amino acids are high enough, its synthesis is inhibited via Pho85-Pcl5, whereas when nitrogen is present cells induce new synthesis of amino acids via TORC1, since this nutrient source is needed for this process.

3. Cln3 REGULATION BY NUTRIENTS AVAILABILITY AND AGING

Cln3 is a potent G_1 cyclin regarding the timely activation of SBF- and MBF-dependent genes with an extremely short half-life. Hence, Cln3 levels may be hardly affected by any input that alters its expression, translation or stability. Thus, several studies postulate Cln3 as a target for nutrient signaling pathways. In fact, some of the different proteins involved in this signaling, converge in Cln3 as a cell cycle regulator depending on the presence or absence of various nutrients.

As explained before, *CLN3* transcription is modulated by the presence of carbon sources via Azf1, which acts as a glucose-dependent transcription factor that interacts with the *CLN3* A_2GA_5 repeats to play a positive role in the regulation of *CLN3* mRNA expression by glucose (Newcomb et al., 2002).

Moreover, *CLN3* translation seems to be affected by nutrients as well. TORC, phosphatidylinositol 3-kinase and the RAS/GPA2/cyclin AMP (cAMP) pathways are thought to regulate Cln3 translation through effects on protein synthesis rates depending on the abundance of amino acids (Polymenis and Schmidt, 1997). In addition to this mechanism, it has been suggested that nutritional signals mediated by the TOR pathway inhibit the degradation of eIF4G and increase the association of Tap42 with Sit4 which then, presumably, promote the translation of *CLN3* (Berset et al., 1998).

Contrary to this general Cln3 regulation pathway, Gallego and colleagues postulate that not only changes in the overall protein synthesis rate modulate Cln3 levels. They propose that the down-regulation of the cyclin at a translational level by the absence of a nutrient like nitrogen is the main factor that regulates this protein. And that the faster turnover observed under these nitrogen deprivation conditions would help to decrease the Cln3 levels more rapidly.

Whether phosphate, recently claimed as an important nutrient source, regulates Cln3 levels at either transcriptional or translational levels, or in terms of stability and localization, is still unknown.

It is clear that the lack of some nutrients promotes Cln3 downregulation and the consequent cell cycle arrest. On this way, Burhans and colleagues have demonstrated that ectopic expression of Cln3 in nutrient depleted cells increases the frequency of arrest of these cells in S phase instead of G_1. Moreover, ectopic expression of Cln3 shortens chronological lifespan in concert with age-dependent increases in genome instability and apoptosis (Weinberg et al., 2007). Chronological lifespan (CLS) is determined by measuring the length of time that cells maintain

viability or reproductive capacity after nutrient depletion induces a stationary phase growth arrest.

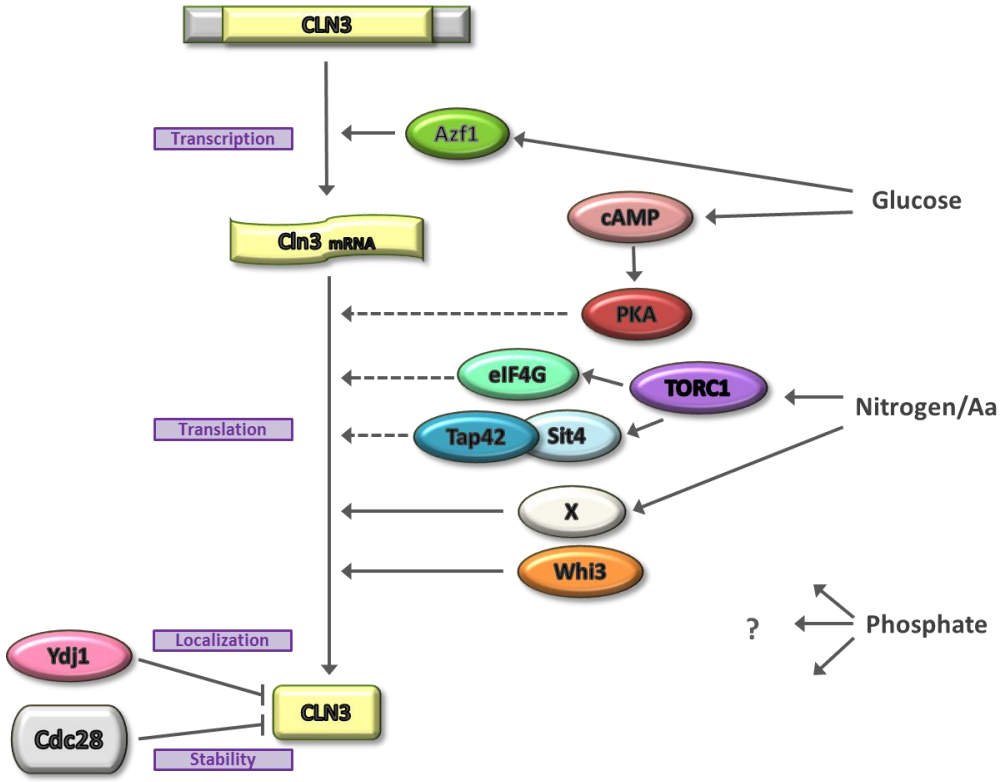

Figure I17. Cln3 regulation by nutrient availability. Cln3 is regulated at different levels: transcription is induced by glucose presence via Azf1 transcription factor; Translation is regulated by glucose also through cAMP-PKA pathway, and by nitrogen and amino acid signaling via TORC1 in an indirect way depending on protein synthesis rates, but in a direct way through an unknown effector; some other proteins as Whi3 modulate Cln3 translation by ER retention; Finally, Cln3 protein is locally restricted by Ydj1 chaperon and is destabilized by Cdc28. Phosphate source regulation to Cln3 is still uncovered. Dashed lines represent indirect interactions.

Several studies have investigated the impact of conserved growth signaling pathways on CLS. It has been observed that alterations in growth signaling pathways inhibit growth arrest of stationary phase cells in G_0. For example, CLS is extended by inactivation of *SCH9* gene, Ras signaling or TOR pathways. When they are active, TORC1, Sch9 and Ras2 inhibit the Rim15 kinase and its induction of oxidative stress defenses, whereas reduced signaling through these pathways in nutrient-restricted cells extends CLS in a Rim15-dependent manner (Fabrizio et al., 2003; Pan and Shadel,

2009; Bonawitz et al., 2007; Wei et al., 2008). Hence, constant high glucose or mutations that activate growth signaling accelerate chronological aging in parallel with reduced efficiency of stationary phase G_0 arrest (Weinberger et al., 2010). Moreover, cells those fail to arrest in G_0 when nutrients are not high enough show increased DNA replication stress that decreases their CLS or viability. The entry of these cells into S phase in the absence of nutrients required for efficient DNA replication is a recipe for stress and mutations. Therefore, downregulation of Cln3 is required for G_1 arrest and long-term survival in nutrient-depleted cells. See summary represented on Fig. I18.

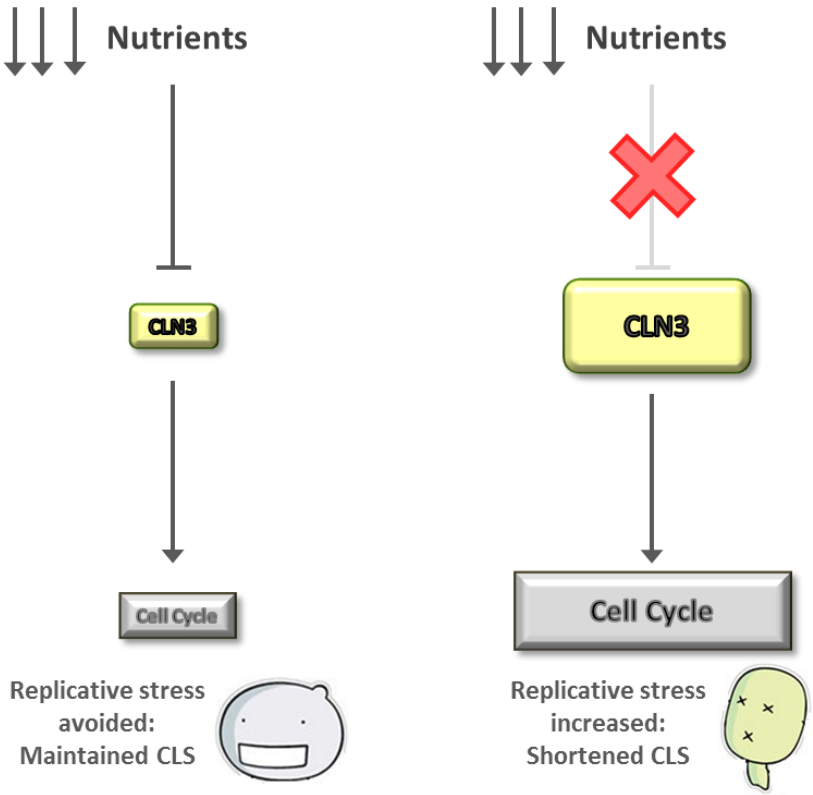

Figure I18. Cln3 regulation by nutrient is important for proper cell cycle arrest and CLS. Lack of nutrients induces Cln3 downregulation that leads to cell cycle arrest in order to avoid replicative stress, resulting in a long CLS. Hence, if nutrient signaling downregulation to Cln3 fails, cells do not arrest cell cycle and enter S phase where, due to the absence of needed nutrients to replicate DNA, stress increases and CLS gets shortened.

OBJECTIVES

Cell cycle control by trophic factors has a key role in regulation of cell proliferation in all organisms. Nutrients are one of this important factors needed by cells to reproduce, so very well regulated mechanisms must exist that connect nutrient availability to cell cycle. Hence the importance on studying how exactly nutrient-dependent signaling pathways work.

For several years, it has been thought that nutrient signalling was composed by linear pathways acting in parallel, but nowadays this concept has changed towards a nutrient-dependent signalling network with extensive cross-talk between pathways at different levels: It is now clear that pathways converge on common effectors.

One well demonstrated common effector of multiple nutrient-dependent signaling pathways is the G_1 cyclin Cln3. Moreover, its role in cell cycle is crucial, so it is a good candidate as being a protein affected by the nutrient sensing and modulating the cell cycle progression depending on its availability.

One important question is what protein could directly modulate Cln3 levels in response to nutrient availability, or what kind of protein could act at the same time as a nutrient sensor and as a cell cycle regulator.

In this thesis, Pho85 is postulated as the perfect candidate that could run these two highly different tasks: the well characterized properties on sensing of phosphates and the well-known functions on modulating cell cycle as CDK, make Pho85 the best choice to start the study on finding the link between nutrient signalling and cell cycle regulation.

The general objective of the present thesis was:

- To better understand how nutrient availability regulates cell cycle.

The specific objectives of the present thesis were:

- To demonstrate the implication of Pho85 in the cell cycle regulation depending on nutrients availability.
- To check whether Pho85 controls cell cycle via Cln3.
- To find out the mechanisms by which Pho85 is affecting Cln3.
- To show a physiological role of the Cln3 regulation depending on the nutrients availability.

MATERIALS AND METHODS

1. Strains

The strains used are indicated in table M1. pho85-as strain (YAM67) was provided by Erin O'Shea and was used to inhibit Pho85 in presence of NaPP1 inhibitor (kindly provided by Shokat) as described (Carroll et al., 2001). The Pho85 inhibitor was checked out by following the entry of Pho4-GFP into the nucleus.

Strain	Background	Genotype	Source
BY4741	BY4741	MATa his3Δ1 leu2Δ200 met15Δ0 ura3Δ0	Euroscarf collection
YAM103	BY4741	pho80Δ::Leu2	This study
YAM111	BY4741	pho80Δ::Leu2 pRS416-CLN3-MYC::URA	This study
YAM113	BY4741	pRS416-CLN3-MYC::URA	This study
YAM114	BY4741	pho85Δ::Leu2 pRS416-CLN3-MYC::URA	This study
YAM115	BY4741	pcl1Δ::Leu2 pRS416-CLN3-MYC::URA	This study
YAM116	BY4741	pcl6Δ::KanMX pRS416-CLN3-MYC::URA	This study
YAM117	BY4741	pcl7Δ::KanMX pRS416-CLN3-MYC::URA	This study
YAM118	BY4741	pcl8Δ::KanMX pRS416-CLN3-MYC::URA	This study
YAM119	BY4741	pcl2::KanMX pcl1Δ::Leu2 pRS416-CLN3-MYC::URA	This study
YAM12	BY4741	whi5Δ::Leu2	This study
YAM14	BY4742	CLN3-HA::URA	This study
YAM142	BY4741	pho85Δ::URA ubc4Δ::Leu2 CLN3-MYC::KanMX	This study
YAM143	W303	dma1Δ::Leu2 dma2Δ::TRP pho85Δ::URA CLN3-MYC::KanMX	This study
YAM146	BY4741	PHO85-TAP::KanMX pRS416-CLN3-MYC::URA	This study
YAM147	BY4741	pho85Δ::Leu pRS416-CLN3-MYC::URA L226 and S227-->D	This study
YAM148	BY4741	pho85Δ::Leu pRS416-CLN3-MYC::URA T519 and T520-->D	This study
YAM149	BY4741	pho85Δ::Leu2 pRS416-CLN3-MYC::URA T519 and T520-->D / I448 and S449-->D	This study
YAM150	BY4741	vip1Δ::KanMX pRS416-CLN3-MYC::URA	This study
YAM151	BY4741	ubc4Δ::Leu2 CLN3-MYC::KanMX	This study
YAM161	BY4741	pRS416-CLN3-MYC::URA I448 and S449-->A	This study
YAM161	W304	WHI5-TMP-GFP::HIS pho85Δ::Leu2	This study
YAM162	BY4741	pRS416-CLN3-MYC::URA T519 i T520-->A	This study
YAM163	BY4741	pRS416-CLN3-MYC::URA I448 i S449-->A / T519 i T520-->A / L226 i S227-->A	This study
YAM43	BY4741	whi5Δ::Leu2 pho85Δ::URA	This study
YAM45	BY4741	pho81Δ::Leu2	This study
YAM57	W303	cdc28ts pRS416-Tet-CLN3-HA::URA	This study
YAM59	W303	cdc28ts pho85Δ::Leu2 pRS416-Tet-CLN3-HA::URA	This study
YAM63	BY4741	CLN3-MYC::KanMX pGEX-GAL-PHO85::URA	This study
YAM67	K699	pho85Δ::Leu2 pho3Δ::Ade2 PHO4-GFP trp::PHO85 F82G -TRP CLN3-MYC::KanMX	O'Shea E.
YAM74	BY4741	pRS416-Tet-CLN2-HA::URA	This study
YAM75	BY4741	pho85Δ::Leu2 pRS416-Tet-CLN2-HA::URA	This study
YAM76	BY4741	CLN3::KanMX pRS416-Tet-CLN2-HA::URA	This study
YAM78	BY4741	pho85Δ::Leu2	This study
YAM78	BY4741	pho85Δ::Leu2 CLN3-MYC::KanMX	This study
YAM91	BY4741	pRS416-CLN3-1-HA::URA	Aldea M.
YAM92	BY4741	pho85Δ::Leu2 pRS416-cln3-1-HA::URA	This study
YAM99	BY4741	pcl9Δ::KanMX pRS416-CLN3-MYC::URA	This study
YAN32	W303	CLN2-HA::URA SIC1-MYC::KanMX CLB5-TAP::Leu2	Posas F.
YNR11	BY4741	WHI5-TAP::KanMX	This study
YNR13	BY4741	PHO85-TAP::KanMX	This study
YNR26.4	W303	CLN2-HA::URA SIC1-MYC::KanMX CLB5-TAP::Leu2 pho85Δ::TRP	This study
YNR46	BY4741	vip1Δ::KanMX	This study
YNR55	BY4741	CLN3-MYC::KanMX	This study
YNR82	BY4741	pho81Δ::Leu2 CLN3-MYC::KanMX	This study
YPC603.7	W303	WHI5-TMP-GFP::HIS	Posas F.
YPC631	BY4741	cln3Δ::KanMX	This study
YPC702	BY4741	SWI6-TAP::KanMX	This study
YSH10	BY4741	atg1Δ::Leu2 CLN3-MYC::KanMX	This study
YSH11	BY4741	pho85Δ::URA atg1Δ::Leu2 CLN3-MYC::KanMX	This study

Table M1. Yeast strains used in this thesis.

2. Plasmids

CLN3-MYC was expressed, from its own promoter, in the centromeric plasmid pRS416. To express MYC-tagged mutated versions of *CLN3*, two substitutions were introduced. This double substitution to Asp was employed to mimic the double negative charge of the phosphate group. YAM69 strain contains a centromeric plasmid that overexpresses *PHO85* under the control of *GAL1* promoter (named *Gal-PHO85*). YAM91 carries a plasmid with *CLN3* HA-tagged protein lacking the PEST sequence of *CLN3*, (the *cln3-1-HA* allele). *ADH::CLN2* was used to constitutively express *CLN2* from a centromeric plasmid.

3. Growth conditions

3.1. Normal conditions

Cells were grown in either YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or in complete synthetic dextrose (SD) medium (0.67% yeast nitrogen base and 2% glucose) containing amino acids for auxotrophic requirements (100 mg/L leucine, 20 mg/L histidine, 50 mg/L tryptophan and 10 mg/ml tryptophan), prepared as previously described (Gallego et al., 1997).

However, protocol recipe was modified in case of BY4741 and K669 derived strains, which needed also methionine (20 mg/L) as auxotrophic amino acid. Figure 1 of Methodology (M1) shows that in absence of methionine, auxotrophic cells for this amino acid (i.e. BY4741 or K669) do not grow properly as observed in the absorbance measurement that is about OD = 0.2. Upon Methionine was added, these cells were able to exponentially grow, reaching OD = 2.4, compared to cells in presence of any other amino acid as Lysine.

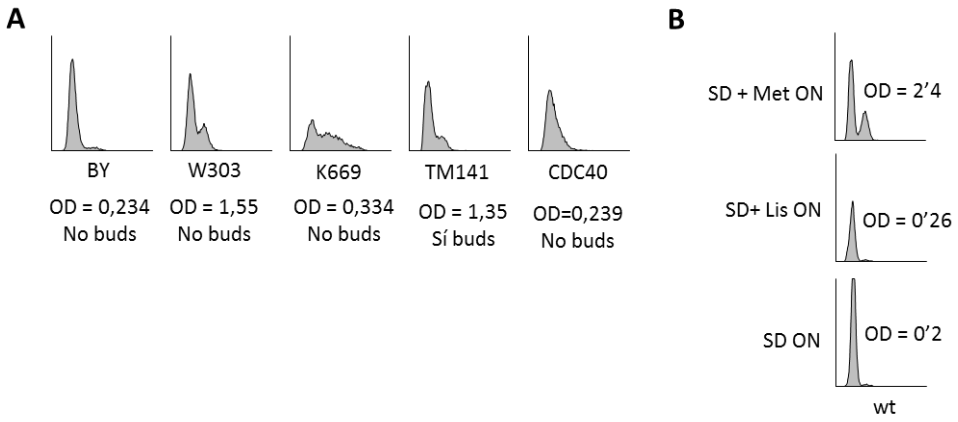

Figure M1. Improving Normal Growth conditions: Methionine is an auxotrophic requirement for BY4741. Wild type cells were grown exponentially in synthetic complete medium in absence (A) or in presence of Methionine as well as one amino acid (Lysine) chosen at random (B). Samples were collected and then subjected to DNA content analysis by FACS, to absorbance measurement (OD600) and to budding presence by light microscopy.

3.2. Nutrient depletion conditions

Yeast nitrogen base without phosphates was used as recommended by the manufacturer (MP Biomedicals) to prepare SD medium without the phosphate source. Phosphate deprivation experiments were done with cells growing exponentially in SD for 14 to 16 h until an OD600 of 0.3 to 0.4, at which point the cells were collected by filtration and, after a quick wash, resuspended at the same cellular concentration in pre-warmed medium lacking the phosphate source, as previously described (Gallego et al., 1997). The required time to proper arrest cell cycle in depletion of phosphates was over 7 hours (Fig. M2A), then cells were harvested and cultured in fresh YPD, where wild type cells normally restart cell cycle after over 2 hours (Fig. M2B).

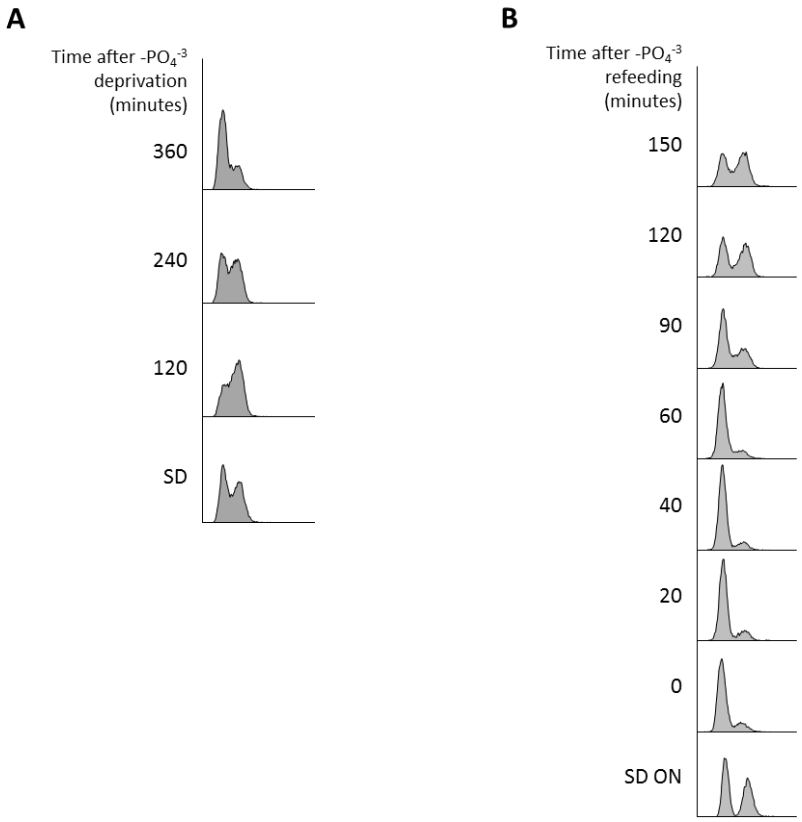

Figure M2. Improving nutrient depletion conditions. A) Phosphates lacking media arrests cells after 7 hours of depletion. Wild type cells were grown exponentially in synthetic complete medium (SD). At time "SD", cells were harvested and incubated in a medium without phosphates. At the indicated times, samples were collected and then subjected to DNA content analysis by FACS. B) Phosphates refeeding leads to a cell cyclerestart after 2 hours. After 6 hours, predepleted cells were refeeded in YPD and sampled to be analyzed by FACS.

Nitrogen deprivation experiments were performed in the same conditions using a yeast nitrogen base without ammonium sulphate (Difco).

3.3. Pheromone, Rapamycin and Nocodazole arrests conditions

α-factor cell synchrony experiments were done as previously described (Clotet et al., 2006). Cells were grown exponentially in YPD medium and α-factor was

added. Two hours later, cells were harvested, washed twice and cultured in fresh YPD medium to follow restart of cell cycle.

Rapamycin conditions were chosen as being 2 µg of TORC1 inhibitor per mL of media for reaching a proper G_1 arrest (Fig. M3A). Cells were cultured in presence of Rapamycin during 2 hours and then were harvested, washed twice and cultured in fresh YPD medium to follow restart of cell cycle (Fig. M3B).

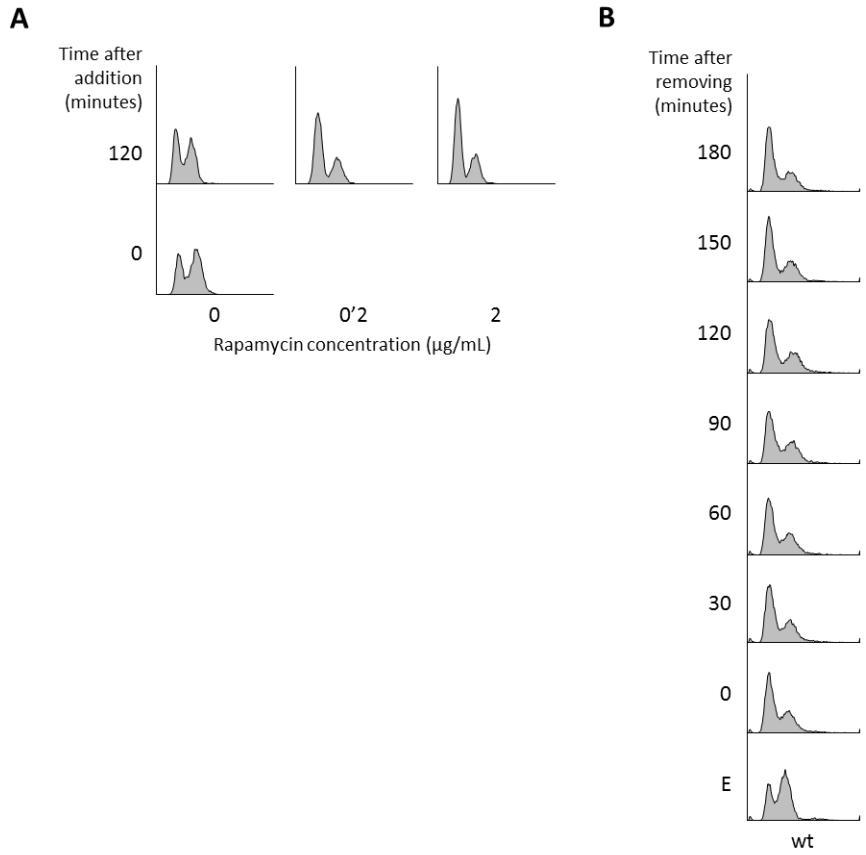

Figure M3. Improving Rapamycin arrest conditions. Wild type cells were grown exponentially in YPD medium in presence of different concentration of Rapamycin (absence, 0.2, or 2 µg/mL) (A). In B panel, at time E (exponential growth), cells were cultured in a medium containing 2 µg/mL of Rapamycin and after two hours (time 0), cells were harvested and cultured in fresh YPD media. Samples were collected and then subjected to DNA content analysis by FACS.

Cell cycle arrests induced by Nocodazole were performed by using 8 µg/mL of this microtubules polymerization inhibitor during 2 hours, as higher concentrations do not proper arrest cells (Fig. M4). A previous α-factor arrest at G_1 phase was needed to better synchronize cells and after the pheromone was removed, cells were cultured during 40 minutes in fresh YPD media in order to let them reach the M phase.

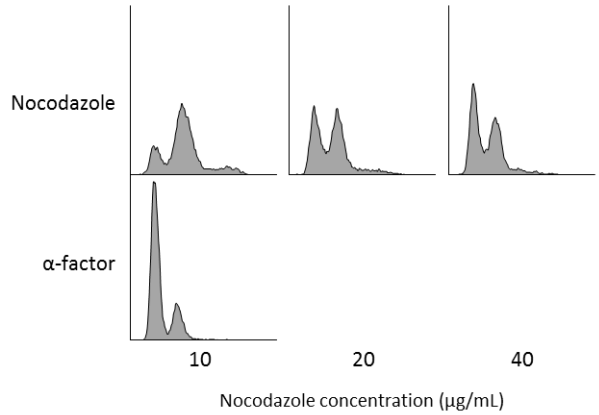

Figure M4. Improving Nocodazole arrest conditions. Wild type cells were grown exponentially in YPD medium in presence of α-factor during 2 hours. Then cells were harvested, washed twice and cultured in fresh YPD medium. After 40 minutes, 8 µg/mL of Nocodazole were added to medium. 2 hours later, cells were sampled and subjected to DNA content analysis by FACS.

4. DNA content, cell volume, cell number and budding index measurements

Flow cytometry analysis was performed on yeast cells stained with SBYR Green, as being the nucleic acid staining that gave the best FACS profiles (Fig. M5A). Cells were harvested, washed, fixed in 70% ethanol at 4 °C and then treated with RNase overnight at 30°C. Cells were incubated with Proteinase K for 1 h at 50°C, stained and then analyzed in a FACScalibur cytometer (Beckton Dickinson). The chosen concentration of Proteinase K was 0.02 mg/mL as being the lowest one that generates good FACS profiles (Fig. M5B).

Cell number and volume were quantified using a Scepter cell counter (Millipore). Budding was analyzed microscopically by scoring a minimum of 200 cells.

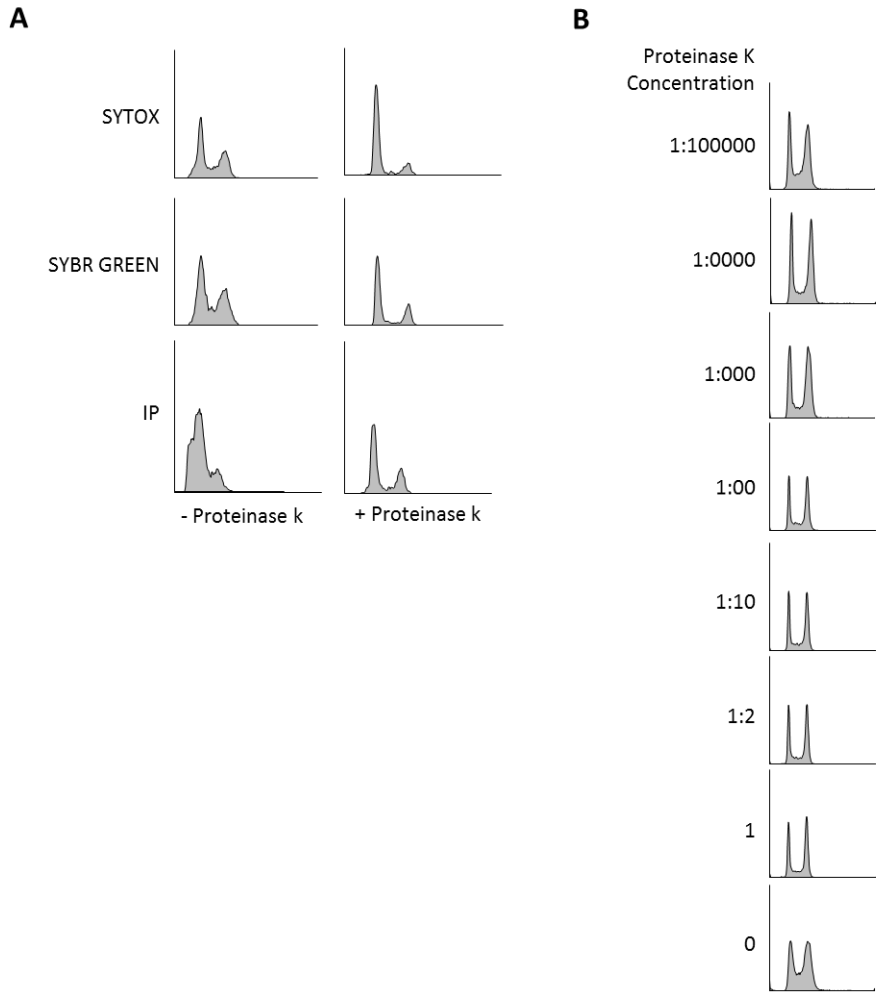

Figure M5. Improving FACS analysis. A) Wild type cells were grown exponentially in YPD medium. After 4 hours, cells were harvested, washed, fixed in 70% ethanol at 4 °C and then treated with RNase (0.1mg/mL) overnight at 30°C. Cells were incubated with Proteinase K (0.02 mg/mL) for 1 h at 50°C. Cells were stained with Propidium Iodide (IP; 2 µg/mL), SYBR Green (1 µg/mL) and SYTOX (1 µg/mL), and then analyzed in a FACScalibur cytometer (Beckton Dickinson). B) Same treatment as in A panel, but incubated at different concentrations of Proteinase K (1 = 2mg/mL) and stained with SYBR Green. The chosen concentration for Proteinase K was 1:100, or 0.02 mg/mL.

5. Immunoblot analysis

Cell culture samples were treated with trichloroacetic acid to a final concentration of 10%. 0.5 ml of glass beads was added, and the resulting suspension was shaked in a Fast-Prep (MPbio) for 3 min, and then centrifuged for 1 min at 10,000 *g*. Pellets were dissolved in 100 µl of 2% SDS, 125 mM Tris-HCI, pH6.8 and boiled for 5 min. 40 to 60 µg of protein from each sample were separated out (8% SDS gel), and transferred to nylon membranes. The proteins were detected by monoclonal antibodies followed by anti-HRP secondary antibodies, using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific).

6. Co-immunoprecipitation

Exponential phase yeast cells were harvested (500 ml OD600=0.7), and resuspended in 5 ml of cold extraction buffer A (50 mM Tris pH 8, 15 mM EDTA, 15 mM EGTA, 0.1% Triton x-100) containing protease inhibitors (2 µg/ml each of pepstatin, leupeptin, PMSF and benzamidine) and phosphatase inhibitors (10 mM sodium orthovanadate, 250 mM glycerophosphate). Cells were ruptured by vortexing with glass beads, and the resulting extract was centrifuged at 4 °C for 1 hour at 12,000 rpm. 3 mg of crude extracts were incubated for 2 h at 4 °C with 150 µl of IgG-Sepharose beads (Amersham Biosciences). After washing with extraction buffer, the proteins bound to the beads were resuspended in 30 µl of SDS-PAGE sample buffer, heated at 95 °C for 5 min and loaded onto SDS-PAGE gels.

7. Recombinant protein purification

For expression of GST or GST fusion proteins *Escherichia coli* strain BL21 (DE3) (Stratagene) was transformed with the corresponding plasmids. Protein expression was induced with 1 mM isopropyl-D thiogalactopyranoside for 3 h at 25 °C. Cells were collected by centrifugation, resuspended in 600 µl of phosphate-buffered saline with 0.1% Triton X-100 (PBST) supplemented with a protease inhibitor mixture (Roche Applied Science), and subjected to mechanical rupture using glass beads. The cell debris was removed by centrifugation, and the supernatants were purified using glutathione-Sepharose column chromatography, as described in the manufacturer's protocol. After incubation for 2 h at 4 °C with rotation, the beads were collected by centrifugation (4 °C, 1000 rpm for 1 min) and washed with PBST three times.

8. Kinase assays

Kinase assays were performed essentially as described previously (Jeffery et al., 2001). Pho85 was immunopurified from yeast extracts (Pho85-TAP) or from bacteria (Pho85-GST) as described above. Pho80-GST, Pcl9-GST, Whi5-GST and Cln3-Cter2/3-GST were expressed and purified from bacteria. Cln3-Cter2/3-GST is a partial Cln3 construct that encompasses S228 to the end of the coding sequence. Phosphorylated proteins were detected using the Pro-Q Diamond phosphoprotein gel stain kit (Invitrogen). This fluorescent stain allows direct in-gel detection of phosphate groups attached to tyrosine, serine, or threonine residues.

9. Viability assays

The viability experiments were done as described in Fabrizio et al., 2007. Stationary phase cells were diluted and plated in YPD, and incubated for 2 days.

10. RNA isolation and analysis

Cells were harvested and stored at -80°C. Total RNA was isolated by hot phenol extraction and quantified spectrophotometrically. For Real Time analysis, 2 mg of total RNA was incubated with DNase and reverse-transcribed using Quanta qScript cDNA SuperMix following the manufacturer's conditions. The cDNA was subjected to RT210 PCR on a C1000 thermal cycler CFX96 RT system, and expression was normalized to *CDC28*. For Northern Blot analysis, RNA samples were separated out (agarose - 6.7% formaldehyde gel), and transferred by capillary to nylon membranes. RNAs were detected through specific probes previously marked with digoxigenine, following the instructions of the manufacturer (DIG Kit, from Roche) and using the CDP-Star chemiluminescent substrate.

RESULTS

In the experimental approach of this thesis, the aim was to better understand how nutrient availability may modulate cell cycle progression. The first question to answer was whether cell cycle progression was affected by the presence or absence of a not well studied nutrient source: phosphate.

Once this was shown to be true, the involvement of the Pho85 CDK in linking these 2 processes (nutrient sensing and cell cycle regulation) was studied with the Cln3 G_1 cyclin as the candidate target. The mechanism by which Pho85 controls Cln3 stability was then identified.

Finally, the role of Pho85 in M phase progression was studied to allow for a greater understanding of how this kinase affects cell cycle regulation.

1. PHOSPHATE AVAILABILITY REGULATES CELL CYCLE THROUGH Pho85 CDK

1.1. Nitrogen-deprived cells arrest at G_1 when using a specific protocol

Nutrients are important trophic factors for all organisms. Without a nitrogen or carbon source, yeast cells use accumulated reserves to complete the current cell cycle and arrest at the next G_1 phase. To understand how cells regulate the cell cycle according to nutrient availability, nitrogen, a well-studied nutrient source known to arrest the cell cycle specifically at G_1 when absent, was used to compare and validate the results obtained with phosphate.

Exponentially growing yeast cells were transferred from complete medium (SD) to a medium lacking the nitrogen source ($-N_2$) or to a fresh complete medium. The DNA content underwent FACS analysis to determine the cell cycle phase in which the cells were collected (Fig. 1A). FACS profiling showed that cells cultured in SD media continued to divide because they were mainly asynchronous, while cells cultured in $-N_2$ media were arrested at the G_1 phase after about 6 hours.

The capability of the cells to detect new nutrient availability following depletion was then studied. Cells were transferred to YPD media after a previous nitrogen depletion ($-N_2$ + YPD). Figure 1B shows arrested cells due to absence of nitrogen and a restart of the cell cycle 90 minutes after refeeding.

Because cyclins and CKIs are required for the G_1 phase to occur, western blot analysis (Fig. 1C) was be used to determine if the absence and refeeding of nitrogen

could affect the protein levels controlling the G_1/S transition, thus validating the protocol used in this study. The protein level profile showed very low levels of Cln2 cyclin and almost nonexistent levels of Cln3 protein at time 0, but increased levels of the CKI Sic1. When cells were recultured in YPD, Cln3 and Cln2 levels increased and Sic1 levels decreased. Glucose 6-phosphate dehydrogenase (G6PDH), an enzyme involved in glucose metabolism that maintains a constant level, was used as a loading control.

Rapid changes in media osmolarity can cause osmotic shock in cells, leading to cell cycle arrest through the activation of signaling pathways. In order to differentiate between an arrest due to nutrient scarcity and an arrest due to osmotic stress, the osmolarity of the different media used in nitrogen depletion was measured (Fig. 2). SD and -N_2 media were identical (233) and YPD media osmolarities were slightly higher (260) in comparison. This result showed that there were no significant differences among media osmolarities to trigger an osmostress response. Thus, cell cycle arrest was shown to be induced by a nutrient signaling pathway such as the TOR pathway, known to be involved in nitrogen sensing.

1.2. TOR inhibited cells arrest the cell cycle at G_1

In *S. cerevisiae*, environmental nitrogen is known to be detected by the TOR signaling pathway. TOR is inactive in the absence of this nutrient or in the presence of rapamycin, which mimics this inactivation because it is able to restrain the TORC1 complex activity.

To confirm that the observed cell cycle arrest in this model (Fig. R1) was due to the sensing of nitrogen deprivation via the TOR pathway, a comparison was made between nutrient depletion and synthetic inactivation by rapamycin. To compare normally dividing cells with arrested cells, cultures were treated with DMSO in parallel with the rapamycin cultures.

The cultures were elutriated at 20°C, and G_1 phase cells were collected and transferred to YPD with either DMSO or rapamycin at 30°C. Cell cycle progression was then monitored by analyzing DNA content and determining the budding index (Fig. R3A), as well as examining the protein level profile (Figs. R3B and R3C). The proteins that were analyzed, the cyclins Cln2 and Clb5, and the CKI Sic1, were known to be key regulators of G_1.

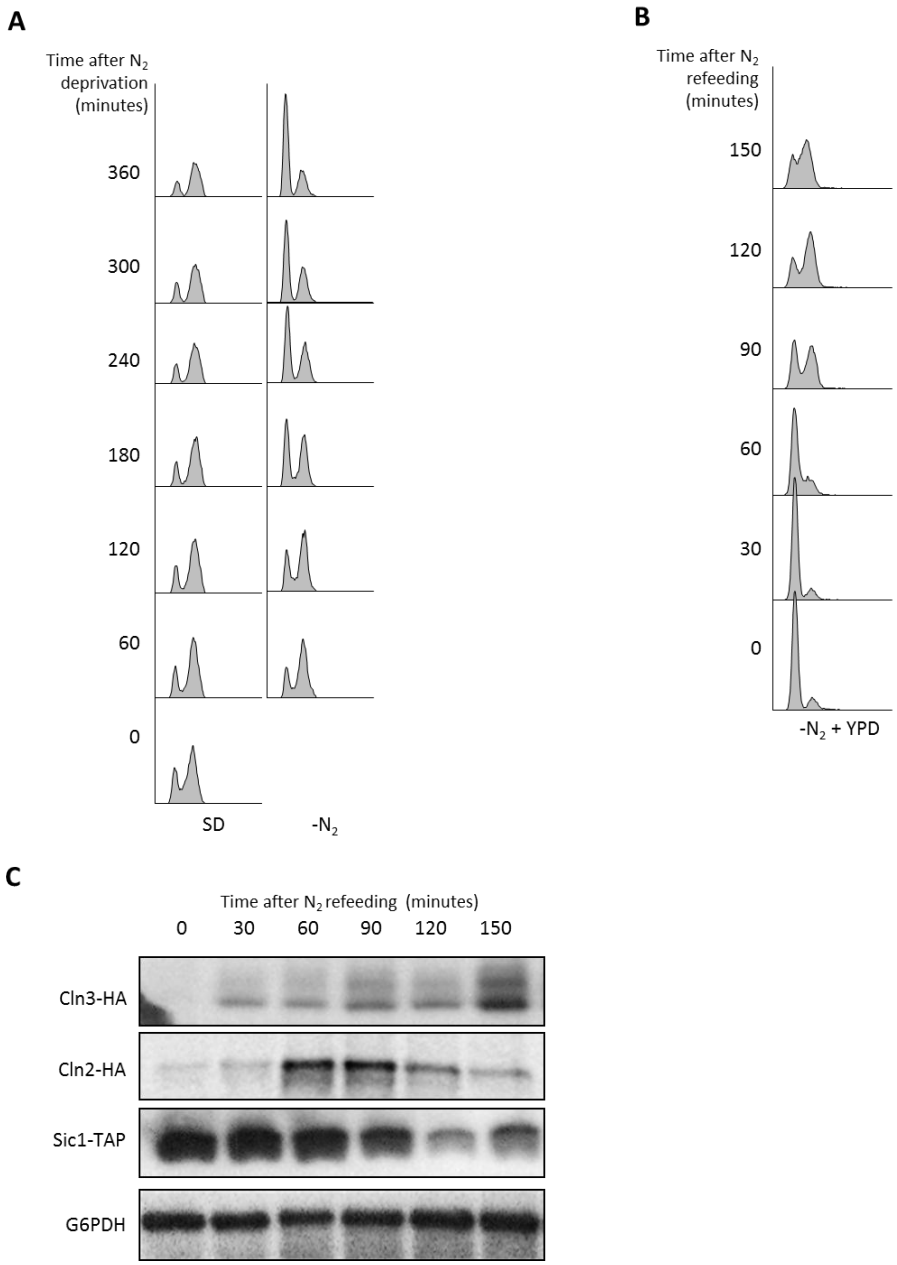

Figure R1. Nitrogen deprived cells arrest the cell cycle by modulation of G_1 regulators. A) Wild-type tagged cells (*YAM14* and *YAN32* strains) were grown exponentially in synthetic complete medium. At 0 minutes cells were harvested and incubated in either fresh complete medium (SD) or medium without nitrogen (-N_2). At the indicated times, samples were collected and then underwent DNA content analysis using FACS. (B and C) After 6 hours, predepleted cells were refed and sampled for FACS and western blot analysis.

Media	Osmolarity
YPD	260
SD	233
SD-N_2	233

Figure R2. Similar osmolarities among medium types. The osmolarities of the different media used were measured and expressed in milliosmoles per liter (mOsm/L).

As shown in the DNA profile (Fig. 3A), cells cultured in media with rapamycin arrested their cell cycle at G_1 and did not bud, while cells cultured in media with DMSO progressed normally through the different phases of the cycle.

Western blot analysis of Cln2 and Clb5 in the presence of DMSO showed almost nonexistent levels at time 0 (early G_1), but a rapid increase in these cyclins took place 50 minutes later. In addition, the Sic1 inhibitor was quickly degraded.

Meanwhile, cells cultured in media with rapamycin did not show an important increase in Cln2; this protein expressed a unique band that could correspond to its inactive fraction. Clb5 cyclin, on the other hand, was not detected. In these cells, Sic1 was not degraded and remained constant.

In cells cultured in DMSO media, Cln2 accumulated and was able to phosphorylate and thus degrade Sic1, leading to an increase in Clb5 that triggered entry into the S phase at 70 to 80 minutes. However, when cells arrested the cell cycle in the presence of rapamycin, Clb2 levels were shown to be insufficient; Sic1 could not be targeted and thus continued to inhibit Clb5. These results were consistent with the DNA content profile.

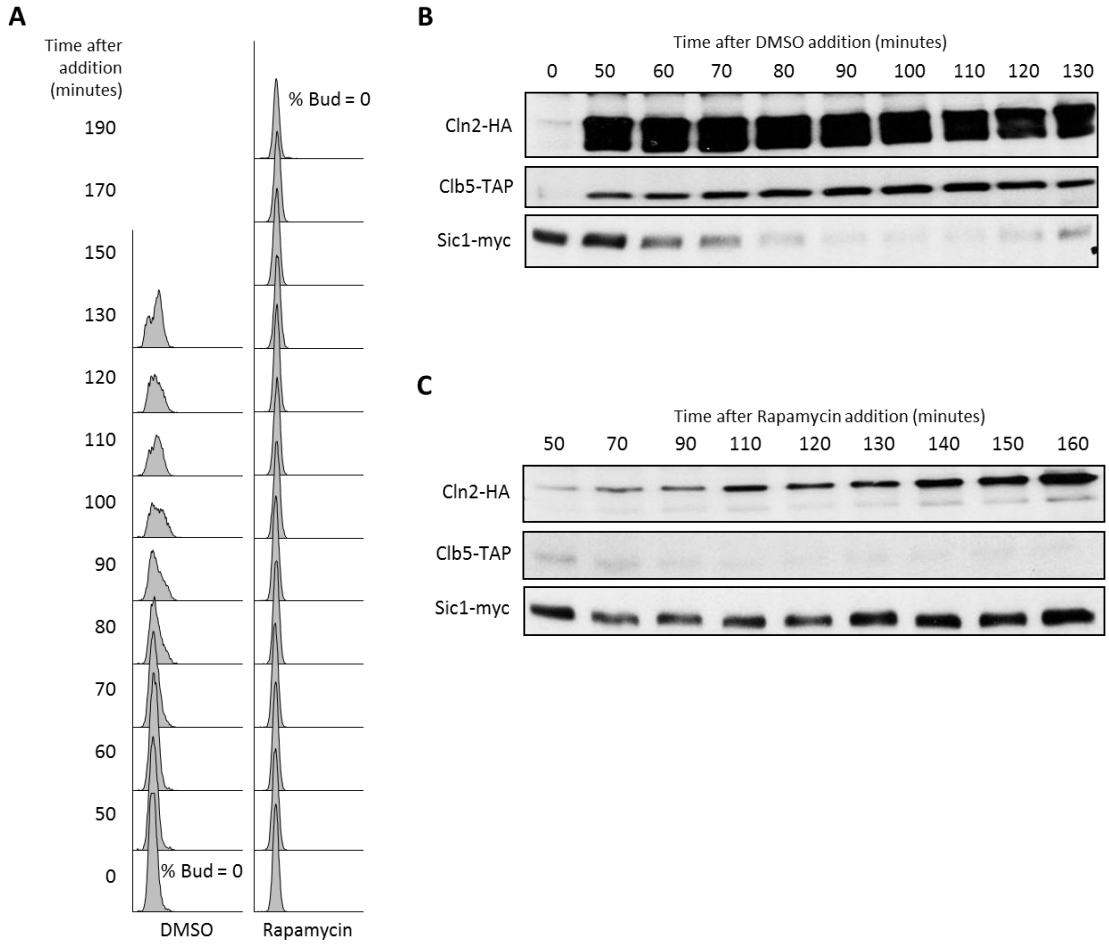

Figure R3. TOR inhibited cells arresting the cell cycle by modulating G_1 regulators. Wild-type tagged cells (*YAN32* strain) were grown exponentially in YPD media and elutriated at 20°C. G_1 phase cells were collected and transferred to fresh YPD media containing either DMSO or Rapamycin (0'2µg/mL) at 30°C. At the indicated times, samples were subjected to DNA content analysis by FACS and the budding index was determined (A). Levels of Cln2, Clb5, and Sic1 proteins underwent simultaneous western blot analysis (B and C).

Nitrogen depletion, sensed by TOR requires a protein to link nutrient availability and cell cycle regulation. This protein, Cln3, was identified in the present thesis.

1.3. Cell cycle restart after nitrogen depletion depends on Cln3 cyclin via regulation of Pho85 CDK

It is known that some exogenous nutrients activate Pho85, a CDK that has been shown to be involved in cell cycle restart (Kaffman et al., 1994; O'Neill et al., 1996). Therefore, it is logical to examine whether Pho85 is implicated in cell cycle restart following nutrient refeeding.

To test whether Pho85 is involved in cell cycle restart after nutrient depletion, cells were refed by transferring them to YPD medium after 4 hours of nitrogen deprivation. After sampling, DNA content was analyzed and the budding index was determined (Figs. R4A and R4B).

DNA content analysis and budding index showed that wild-type cells restarted the cell cycle at 90 minutes after nitrogen refeeding; *pho85Δ* cells did not restart until 1 hour later.

Some previous results (Fig. 1C) showed that cell cycle restart required Cln3 as a first cyclin to trigger normal progression. Cln3 levels would therefore have appeared to have a different profile in cells lacking *PHO85*, explaining the cell cycle restart delay.

Figure 4C shows how Cln3 started to increase 30 minutes after nitrogen refeeding in wild-type cells, but Cln3 levels in the *pho85Δ* strain did not increase until 150 minutes after refeeding, and never reached the same levels as those of the wild-type strain.

These results suggested that Pho85 activity was involved in the cell cycle restart after nitrogen depletion by sufficiently raising Cln3 levels to trigger G_1 progression. However, some other downstream proteins may also have been involved.

Whi5 is an inhibitor of G_1 SBF-dependent genes such as Cln1 and Cln2. A delay in cell cycle restart after nitrogen depletion in *pho85Δ* cells could involve both Cln3 and Whi5 proteins, and Pho85 could inhibit the Whi5 CKI to promote cell cycle reentry.

In order to determine whether Pho85 affects Whi5, a kinase assay was performed using Pho85 as kinase, Pcl9 (because of its role in early G_1) and Pho80 (because of its role in nutrient sensing) as cyclins, and Whi5 as substrate. Pho85-Pcl9, but not Pho85-Pho80, was shown to phosphorylate Whi5 *in vitro* (Fig. R5A and R5B).

To verify this finding, a co-immunoprecipitation assay was carried out by pulling out Pho85 and identifying Whi5 through western blot analysis. It was observed that Pho85 and Whi5 interacted *in vivo* (Figure R5B).

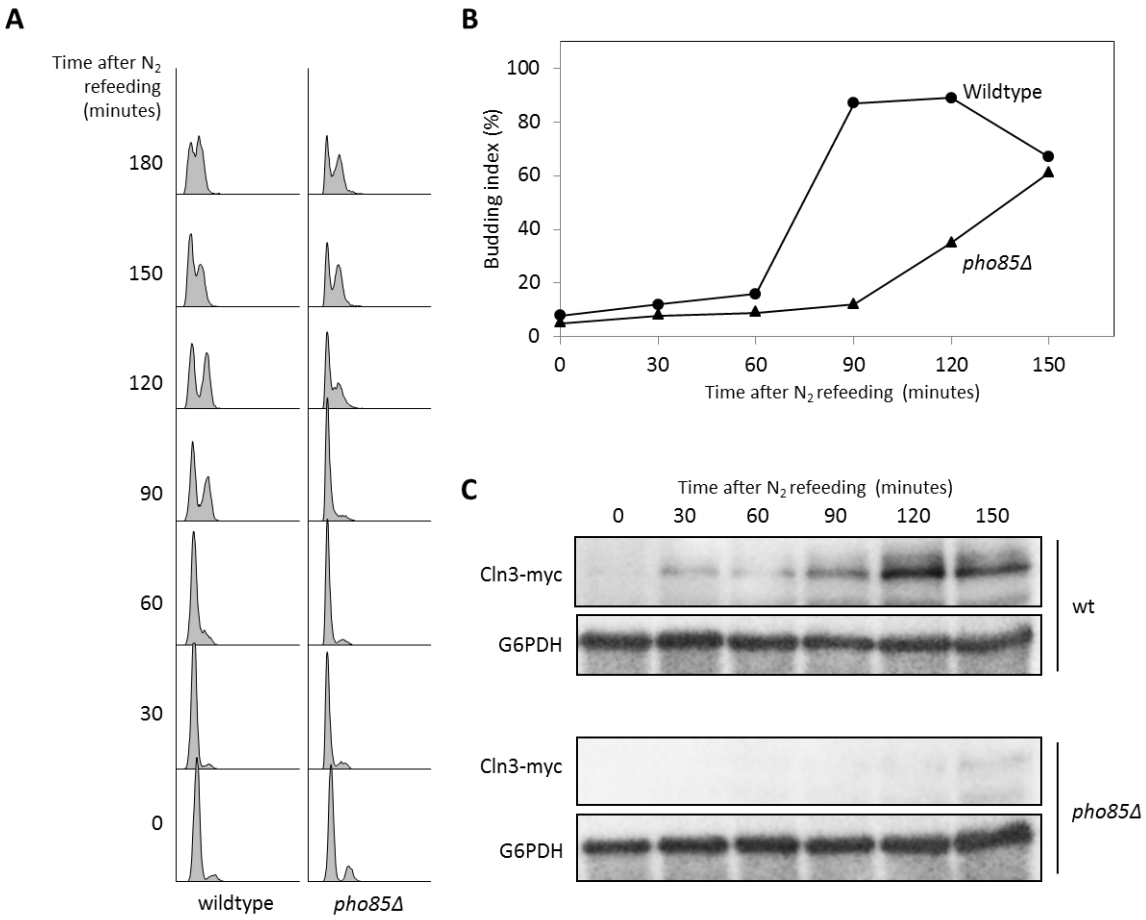

Figure R4. Cell cycle restarts after nitrogen depletion through Pho85 CDK. Wild-type and *pho85Δ* tagged cells (*YNR55* and *YAM78* strains) were grown exponentially in synthetic complete medium. At 0 minutes, cells were harvested and incubated in a nitrogen-free medium. At time 0, cells were refed by transferring to YPD medium. Samples underwent DNA content analysis (FACS) and the budding index was determined (A and B). At the same time, Cln3 protein levels were examined using western blot analysis (C). G6PDH was used as a loading control.

It is known that when Whi5 is phosphorylated by Cdc28, it is excluded from the nucleus. GFP-tagged Whi5 was seen to exit from the nucleus during a nitrogen refeeding. Pho85 could have the same effect on this protein.

Fluorescence microscopy imaging revealed that the Whi5 exit from the nucleus is delayed in *pho85Δ* cells (Fig. R5C). Whi5 was exported 60 minutes after refeeding in wild-type cells, but not in *pho85Δ* cells.

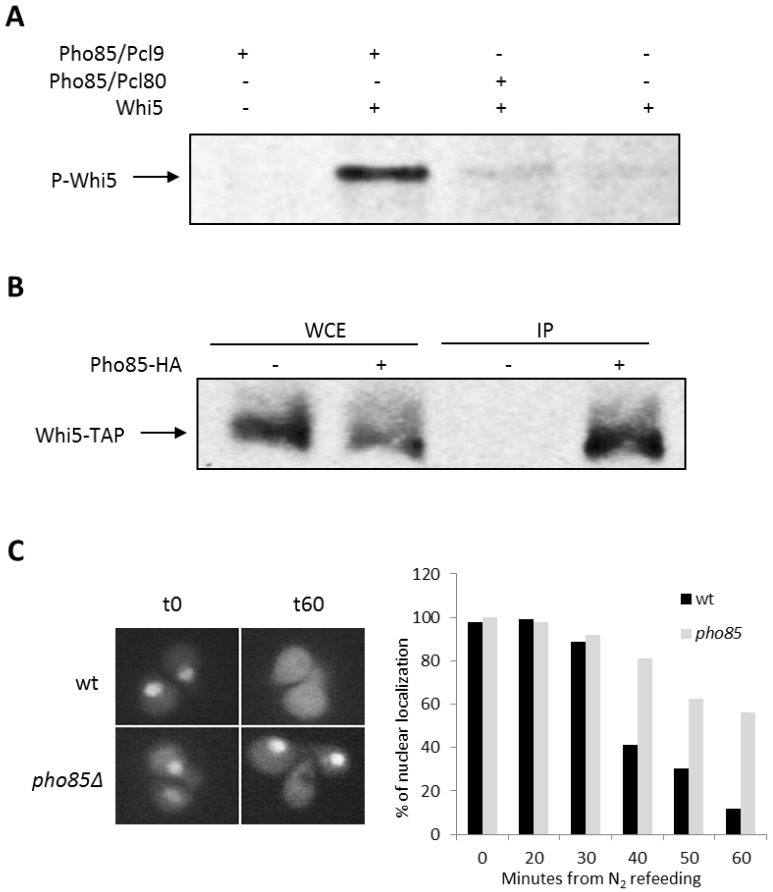

Figure R5. Pho85 affects Whi5 activity. Pho85-PCl9 complexes phosphorylate Whi5 *in vitro*. Recombinant GST-Pcl9, GST-Pho80, and GST-Pho85, purified from bacteria, were incubated with the GST-Whi5 protein (A). Pho85 and Whi5 interact *in vivo*. Yeast extracts containing untagged *PHO85* or HA-*PHO85* and tagged TAP-Whi5 were co-immunoprecipitated with the protein A-sepharose and probed using specific antibodies (B). Wild-type and *pho85Δ* Whi5-GFP tagged cells (*YPC603.7* and *YAM161* strains) were grown exponentially in synthetic complete medium then harvested and incubated in a medium without nitrogen. At 0 minutes, cells were refed by transferring them to YPD medium. Samples underwent fluorescence microscopy observation at the indicated intervals (C).

As a whole, these results suggested that Pho85 phosphorylated Whi5 in order to force its nuclear exclusion and trigger the release of the SBF promoter.

This model indicated that cell cycle delay in the restart after nitrogen deprivation observed in *pho85Δ* cells may have been partially avoided by the double knock-out of *pho85Δwhi5Δ.*

However, FACS analysis showed that no phenotypic recovery of *pho85Δwhi5Δ* occurred during a cell cycle restart (Fig. R6). Moreover, these cells maintained arrest 1 hour longer than wild-type cells such as the *pho85Δ* strain.

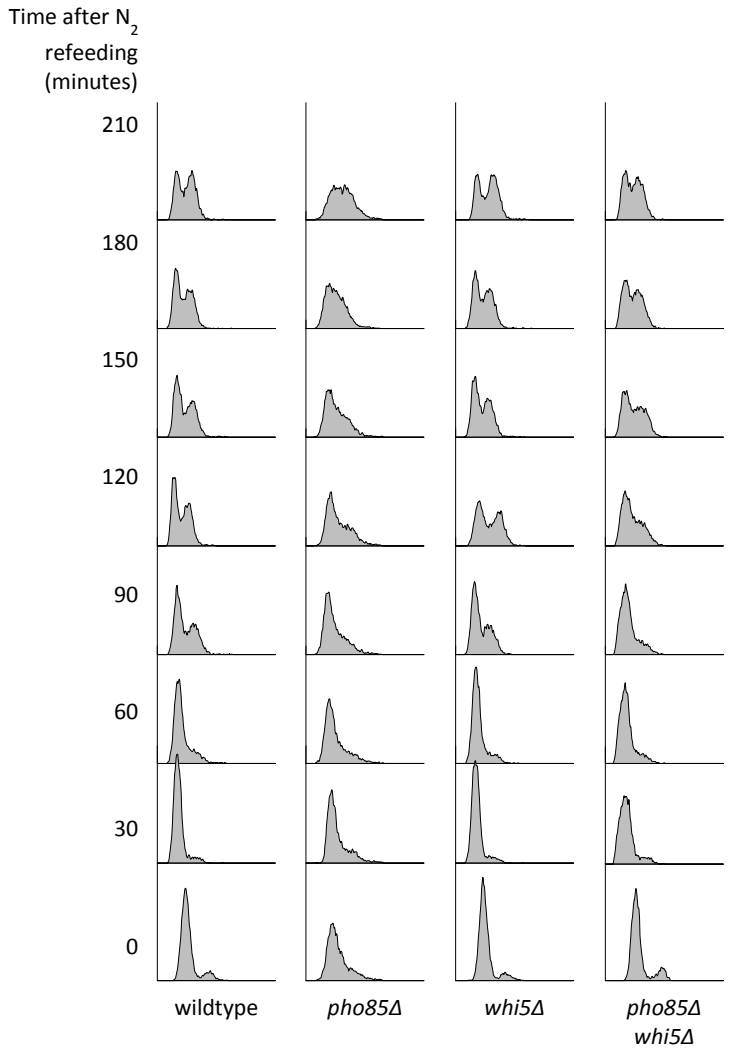

Figure R6. Cell cycle restart after nitrogen depletion depends on Pho85 CDK, but not only via Whi5. Wild-type, *pho85Δ, whi5Δ,* and *pho85Δwhi5Δ* cells (BY4771, YAM78, YAM12 and YAM43) were grown exponentially in synthetic complete medium, then harvested and incubated in a medium without nitrogen. At 0 minutes, cells were refed by transferring them to YPD medium. Samples underwent FACS analysis at the indicated intervals.

The studies conducted for this thesis showed that the role of Whi5 in cell cycle regulation through the presence of nutrients appeared to be less crucial than that of Cln3. In addition, the Brenda Andrew group reported that Whi5 was affected by Pho85 (Huang et al., 2009); this is also described in the present thesis. Consequently, Whi5 characterization was abandoned as one of the main goals of this thesis, and the Cln3 line of study was followed.

The first results of this thesis suggested that nitrogen sensing pathways appeared to be linked to cell cycle regulation by Pho85 through Cln3 modulation; another important nutrient was thus chosen for further examination of the mechanisms controlling cell cycle during its absence: phosphate.

1.4. G_1 arrest induced by phosphate depletion may involve active mechanisms to down-regulate Cln activity

Phosphate is a good candidate for the study of the relationship between Pho85, nutrients and cell cycle due to the high regulation of phosphate metabolism by this CDK.

This line of research was aimed at determining whether phosphate depletion also induces G_1 arrest. For this purpose, exponentially growing yeast cells were transferred from complete media ($+PO_4^{-3}$) to media lacking the phosphate source ($-PO_4^{-3}$). The cell division rate was seen to decline slowly over the next 3 hours (Fig. 7B), leading to cell arrest in G_1, confirmed by DNA content analysis and the budding index (Figs. R7A and R7D).

The average cell volume did not decrease under phosphate deprivation but was instead even higher compared to cells in SD media, suggesting that the cell cycle arrest was not merely a consequence of the inability to reach the growth requirements for G_1/S transition (Fig. R7C).

Because cyclins are essential to progression through G_1, the arrest caused by phosphate deprivation may have required down-regulation of G_1 cyclin activity. Cells expressing *CLN2* from a mild constitutive promoter continued to grow in size and, more importantly, crossed START despite the absence of phosphates, as shown by the budding index (Fig. R7C). This indicates that the down-regulation of Cln activity may have been essential in achieving a proper G_1 arrest during phosphate scarcity.

Taken together, these results strongly suggest that G_1 arrest induced by phosphate deprivation is mediated by specific mechanisms, some of which involve the down-regulation of Cln proteins; it cannot be explained as a simple passive consequence of growth arrest that prevents cells from reaching the critical cell mass necessary to bud and initiate the S phase.

As in nitrogen depletion arrest studies, it was necessary to characterize the protein levels in the main G_1 regulators.

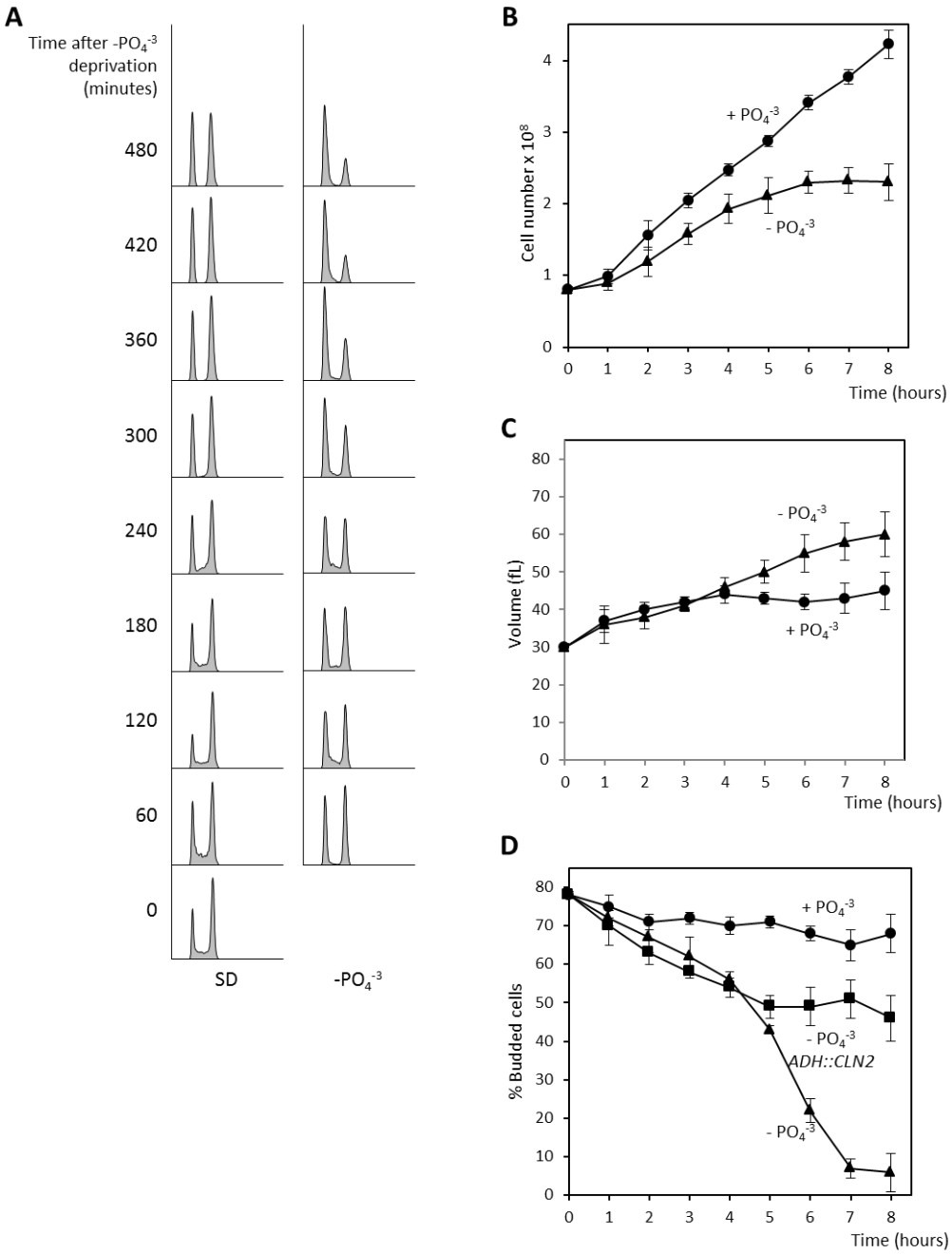

Figure R7. Phosphate starvation leads to G_1 arrest. Wild-type cells were grown exponentially in synthetic complete media. At 0 minutes, cells were harvested and incubated either in the same media ($+PO_4^{2+}$) or in media without phosphates ($-PO_4^{2+}$). Samples were removed and underwent different analyses: DNA content (A), total cell number (B), cell volume (C), and percentage of budding (D). D also shows the percentage of budding in wild-type cells carrying a centromeric plasmid with *CLN2* expressed by an ADH promoter. Data ± s.d. from 3 independent experiments is shown.

1.5. External phosphate controls Cln3 cyclin levels

Because the constitutive expression of *CLN2* was able to partially rescue G_1 arrest, phosphate absence was examined to determine whether it affected the protein levels controlling the G_1/S transition. Figure R8A clearly shows that Cln2 and Clb5 protein levels (essential for budding and S phase entry) decreased after 4 hours of growth in phosphate-free medium, whereas levels of the protein Sic1 (essential for stopping the cell cycle before the S phase) remained constant.

At this time, mRNA levels of all the genes were analyzed (Fig. 8B). mRNA levels were also shown to be progressively depleted, but the transcripts of *SIC1* remained constant. This result suggests that the absence of phosphate may inhibit the transcription of genes required for G_1/S transition.

The G_1/S transcriptional wave is controlled by the SBF and MBF complexes, which may also be regulated by phosphate levels. As observed in Figure R8A, the Swi6 protein levels are not affected during the first 6 hours after phosphate deprivation. This data rules out the possibility that SBF and MBF are controlled at the mRNA expression level.

Whi5 and Cln3 are known to play a key role in the activation of SBF and MBF complexes in late G_1. For this reason, Whi5 and Cln3 levels were also studied. Figure R8A shows how after 3 hours of phosphate depletion, the Cln3 protein levels decreased rapidly (this did not occur in complete medium, Fig. R8C), while Whi5 repressor levels remained fairly constant.

These results support showed that phosphate deprivation, in addition to nitrogen depletion, down-regulated SBF and MBF activity as a result of the loss of Cln3. Because a triple *cln* mutant cannot undergo G_1/S transition unless Sic1 is removed, the absence of Cln proteins and the sustained presence of Sic1 may explain the G_1 arrest produced by phosphate deprivation in terms of molecular requirements.

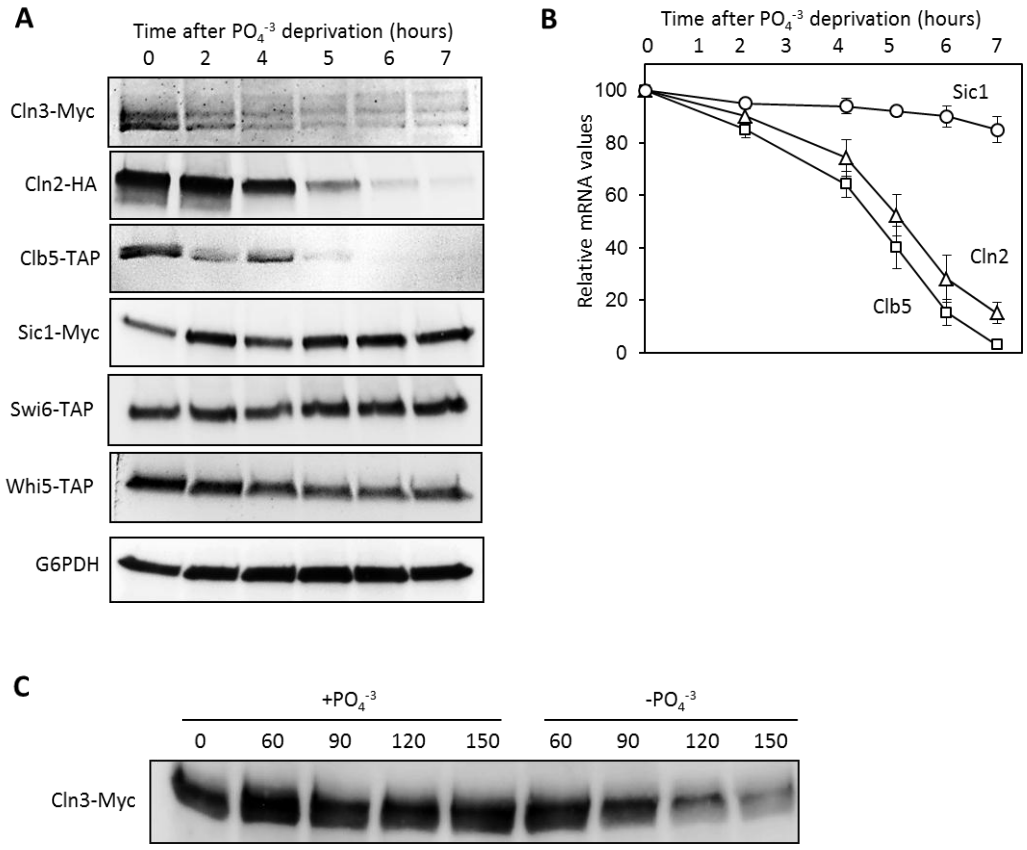

Figure R8. Phosphate starvation leads Cln3 downregulation. Wild-type tagged cells (YNR55, YAN32, YPC702, and YNR11) were grown exponentially in synthetic complete media. At 0 minutes cells were harvested and incubated in phosphate-free media. Samples underwent western blot (A,C) and real-time PCR (B) analyses at the indicated times.

1.6. Pho85 regulates cell cycle reentry after phosphate deprivation

It has been shown that exogenous phosphates activate Pho85, which in turn controls the homeostasis of this nutrient; Pho85 CDK could therefore be involved in cell cycle arrest and reentry after phosphate depletion.

Figure R9B shows how Pho85 was needed for a proper restart after phosphate deprivation because *pho85Δ* exited G_1 arrest 1 hour later than wild-type cells. However, FACS analysis showed that Pho85 did not appear to have a relevant role in stopping cell cycle in the absence of phosphates (Fig. R9A). This issue was later

examined more closely (Fig. R23) and Pho85 was shown to affect G_1 arrest in terms of cell viability.

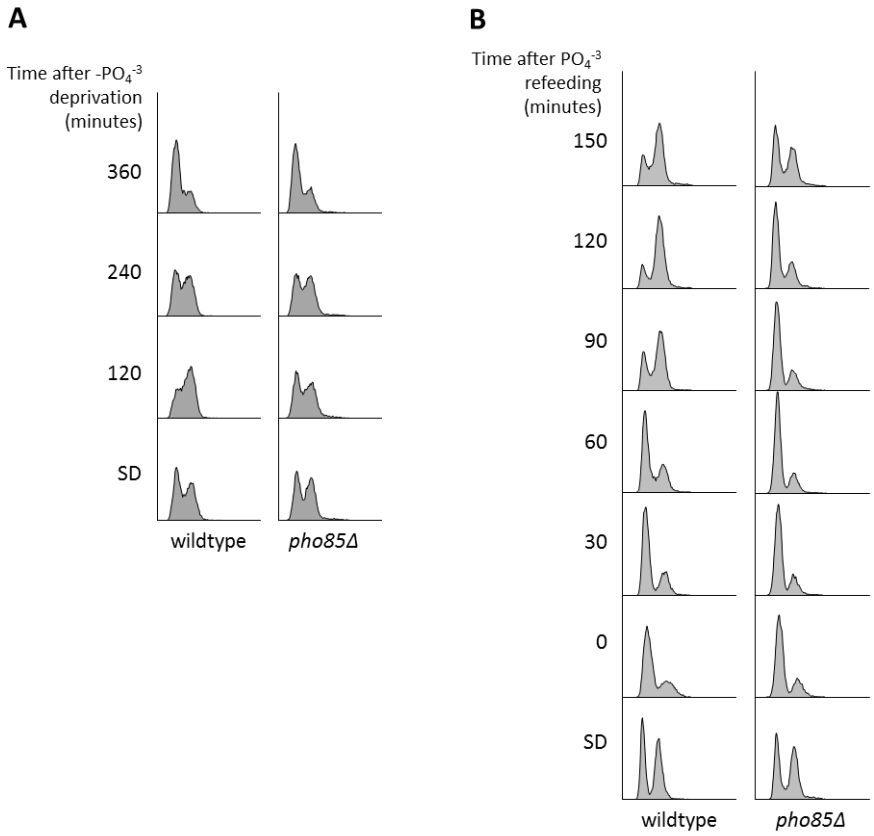

Figure R9. Pho85-regulated cell cycle restart. Wild-type and *pho85Δ* cells were grown exponentially in synthetic complete media (SD). Cells were then harvested and incubated in media without phosphates (A), and refed in YPD medium (B). Samples underwent FACS analysis at the indicated times.

2. Pho85 CDK REGULATES Cln3 CYCLIN LEVELS

2.1. Pho85 activity regulates the levels of Cln3 protein

As with nitrogen deprivation, Cln3 levels during phosphate depletion were studied and compared with Cln3 levels in a *pho85Δ* strain to check for similarities. Figure R10A shows how *PHO85*-depleted cells growing exponentially in rich medium exhibited low levels of Cln3. This decrease is very similar to that observed in wild-type cells grown in phosphate-free media.

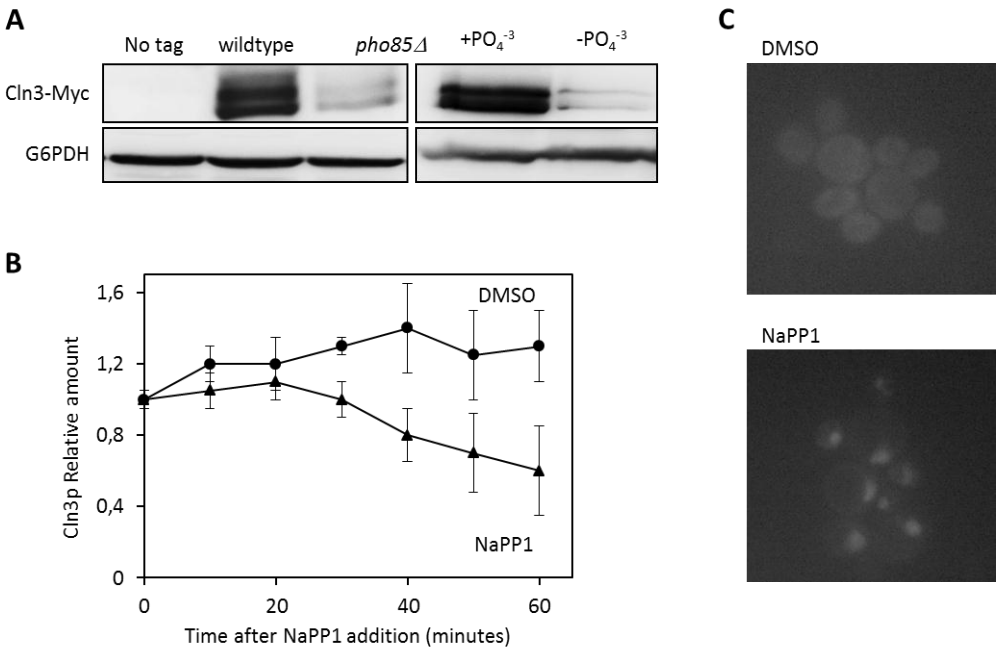

Figure R10. Pho85 inactivation led Cln3 downregulation. A) Left panel: Wild-type and *pho85Δ* cells were grown exponentially in YPD and Cln3-myc levels were evaluated with monoclonal antibodies. Right panel: wild-type cells were grown in synthetic complete media with ($+PO_4^{2-}$) or without ($-PO_4^{2-}$) phosphates. At 6 hours, samples were taken and Cln3-myc levels were monitored (left panel). B) YAM67 (a *pho85-AS* strain) was incubated with NaPP1 (a specific inhibitor of *pho85-AS*) or with a drug vehicle (DMSO), samples were taken, and Cln3-myc was analyzed by western blot analysis using monoclonal antibodies. Data ± s.d. from the 2 independent experiments are shown. C) These same samples were analyzed by fluorescence microscopy at 40 minutes after DMSO or NaPP1 addition to observe Pho4 localization.

PHO85 gene deletion results in a broad spectrum of defects; the *pho85Δ* strain used may have carried suppressor mutations that alleviate such defects. To avoid this problem, the *pho85-AS* strain, which carries an analog-sensitive allele of *PHO85,* was used. These cells showed a behavior that was quite similar to wild-type cells until the specific inhibitor NaPP1 was added to the culture. NaPP1 is a reversible cell-permeable inhibitor of tyrosine kinases that have been mutated by a single-base substitution to become "analog sensitive". As observed in Figure R10B, addition of NaPP1 to the exponentially growing cells resulted in a progressive depletion of Cln3 in comparison with DMSO-treated cells.

A read-out of Pho4-GFP localization was used to evaluate Pho85 activity. Cln3 protein levels started to decrease at the same time that Pho4-GFP entered the nucleus (Fig. R10C). This data suggested that a good correlation existed between Pho85 inactivation and Cln3 reduction. Pho85 activity, rather than the physical presence of the protein itself, seemed to be a key factor in controlling Cln3 protein levels.

2.2. Pho85 regulation is specific to Cln3

To rule out that Pho85 was affecting other cyclins apart from Cln3, a strain containing the *CLN2* gene regulated by a Tet-off promoter was constructed. This strain avoided the negative effects that Cln3 scarcity had in *pho85Δ* cells because *CLN2* would be expressed independently from SBF promoter regulators.

As seen in Figure R11A, Cln2 protein levels were not affected by the depletion of PHO85; Cln2 maintained the same half-life in both wild-type and *pho85Δ* strains (Fig. R11B).

Taken together, this data indicated that Pho85 regulates cell cycle restart after nutrient depletion specifically via Cln3 and not via any other G_1 cyclin. This specific mechanism could have involved a concrete Pho85 complex in which only 1 particular cyclin belonging to the Pho85 family would bind to the CDK to trigger Cln3 regulation.

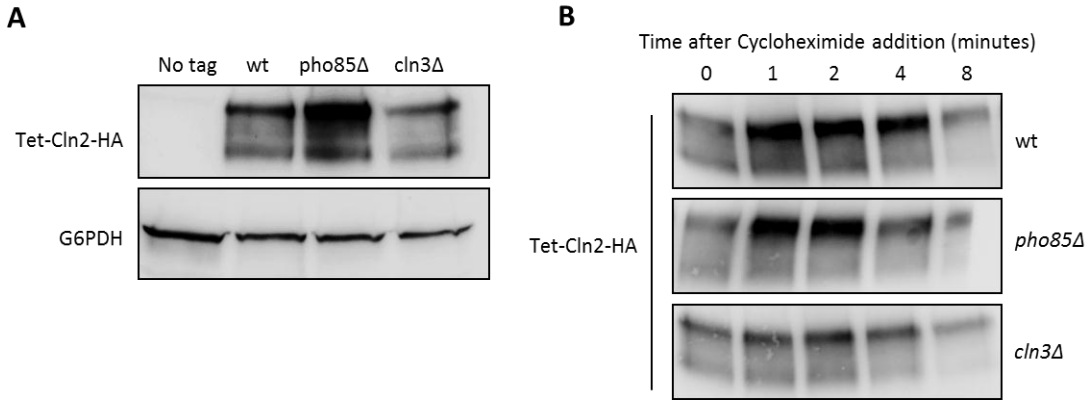

Figure R11. Pho85 does not regulate Cln2 levels. Wild-type, *pho85Δ,* and *cln3Δ* cells containing a plasmid expressing tagged *CLN2* under the Tet-off promoter (YAM74, YAM75 and YAM76) were grown exponentially in YPD for 4 hours. Samples then underwent western blot analysis (A). These same cells were treated with cycloheximide and sampled at the indicated times for western blot analysis (B).

2.3. Pho80 is the cyclin that modulates Cln3 via Pho85 activity

The loss of a particular Pho85 cyclin, can phenocopy some aspects of a *pho85Δ* mutant. In order to find the specific cyclin involved in cell cycle restart by Pho85, 2 independent experiments were performed with different cyclin mutants: cell cycle progression after phosphate depletion (Fig. R12A) and Cln3 protein levels under normal growing conditions (Fig. R12B).

Cells lacking the *PHO80* gene were delayed at cell cycle reentry following phosphate depletion, as were *pho85Δ* and *cln3Δ* cells (Fig. R12A). A battery of knock-out strains for several Pho85 cyclins was analyzed by western to check Cln3 levels; only *pho80Δ* cells showed low Cln3 protein levels comparable to the *pho85Δ* strain.

These results suggested that the effect of Pho85 CDK on Cln3 protein amount was mediated by Pho80 cyclin, reinforcing the idea that Pho80-Pho85 complexes are inhibited during phosphate scarcity and that phosphates control Cln3 levels by modulating Pho85 kinase activity. What was not yet clear was whether the overexpression of this complex induces Cln3 accumulation.

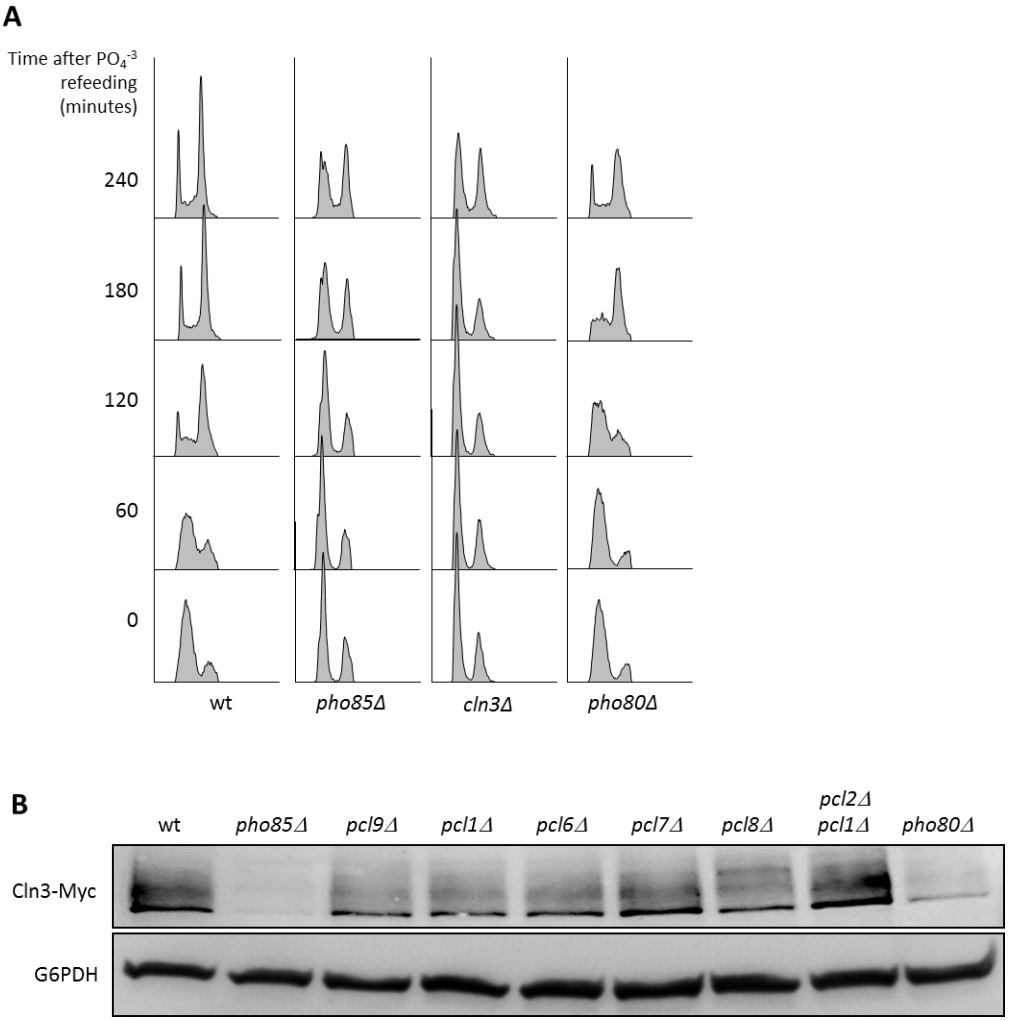

Figure R12. Pho85-Pho80 complex regulates Cln3 levels. Wild-type, *pho85Δ*, *cln3Δ,* and *pho80Δ* (BY4741, YAM78, YPC631 and YAM103) cells were grown exponentially in synthetic complete media, harvested, and incubated in media without phosphates. At 7 hours cells were refed in YPD medium, and sampled at the indicated times to analyze DNA content using FACS (A).Wild-type, *pho85Δ, pcl9Δ, pcl1Δ, pcl6Δ, pcl7Δ, pcl8Δ, pcl1Δ pcl2Δ* and *pho80Δ* cells containing a plasmid expressing tagged *CLN3* under its own promoter (YAM113, YAM114, YAM99, YAM115, YAM116, YAM117, YAM118, YAM119, and YAM111) were grown exponentially in YPD for 4 hours and then sampled for western blot analysis (B).

2.4. Pho85 overexpression leads to Cln3 accumulation regardless of phosphate availability

Pho85 is inactivated by Pho81 CKI during phosphate depletion, leading to a downregulation of Cln3 levels. In *PHO85* overexpression with a *GAL* promoter, this effect could be reverted and Cln3 levels could be maintained even during nutrient depletion because Pho85 protein levels would be at such high levels that it would be difficult to inhibit the CDK.

When *PHO85* was overexpressed (Gal-Pho85 cells), Cln3 levels were high, even during phosphate scarcity. In cells containing an empty plasmid (Gal-Ø), Cln3 decreased upon phosphate depletion. *PHO85* overexpression was followed by the accumulation of Pho85-GST.

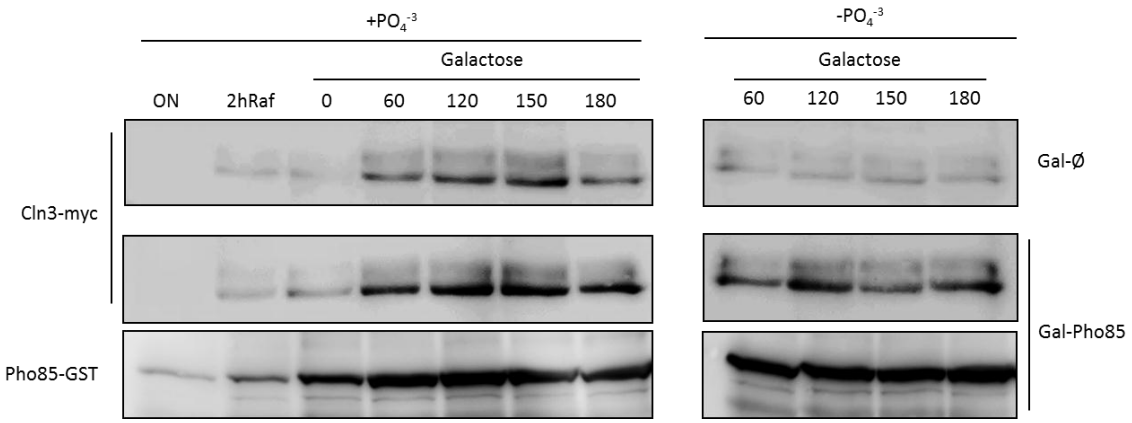

Figure R13. Pho85-Pho80 complex regulates Cln3 levels depending on phosphate availability. *Gal-PHO85-GST* (a wild-type strain that carries a centromeric plasmid with *PHO85* expressed under a Gal promoter and with GST tagging) and Gal-Ø (similar to the same *Gal-PHO85-GST* but containing an empty plasmid) were grown for 5 hours in synthetic complete media with or without phosphates, with galactose as a carbon source. At the indicated times, cells were sampled and analyzed by western blot analysis.

The increase of Pho85 protein levels by GAL induction allowed Cln3 cyclin to be maintained, even during phosphate depletion. As seen previously, both the presence of Pho85 and its activated state modulated Cln3 levels. Based on this finding, Pho85 hyperactivation could give more clues about this mechanism.

2.5. Upstream elements of the PHO pathway also affect Cln3 levels

Pho85 activity is controlled by external phosphates through an upstream signaling transduction pathway (Fig. 14A). The strains vip1Δ and pho81Δ are not able to respond to changes in the external concentration of phosphate and therefore cannot inhibit Pho85 during phosphate deprivation.

Using experimental models, it was confirmed that these strains could not control Cln3 protein levels in response to phosphate depletion (Fig. R14C and R14D). These results, together with data from Figure R13, were represented in a graph (Fig. R14B), which shows that *PHO85* overexpression has the same effect than the absence of the inhibitors.

The results as a whole strongly suggested that phosphates controlled Cln3 protein levels by modulating Pho85 activity. However, more experiments were necessary to identify which step in Cln3 synthesis is regulated by Pho85.

2.6. Pho85 does not regulate *CLN3* gene expression

Pho85 may modulate Cln3 accumulation at different stages of gene expression: transcription, translation or stability. Cyclin transcription is a tightly-controlled process and Pho85-Pho80 complexes control the transcription of many genes. Thus, it was reasonable to question whether Pho85 modulates the transcription of *CLN3* in response to changes in phosphate levels.

However, northern blot analysis showed no differences in *CLN3* mRNA when comparing wild-type and *pho85Δ* strains in cell cycle arrest induced by phosphates depletion (Fig. R15B). Cln3 protein levels, meanwhile, were nonexistent in cells lacking *PHO85* (Fig. R15C). Restart of cell cycle was followed by FACS analysis (Fig. R15A).

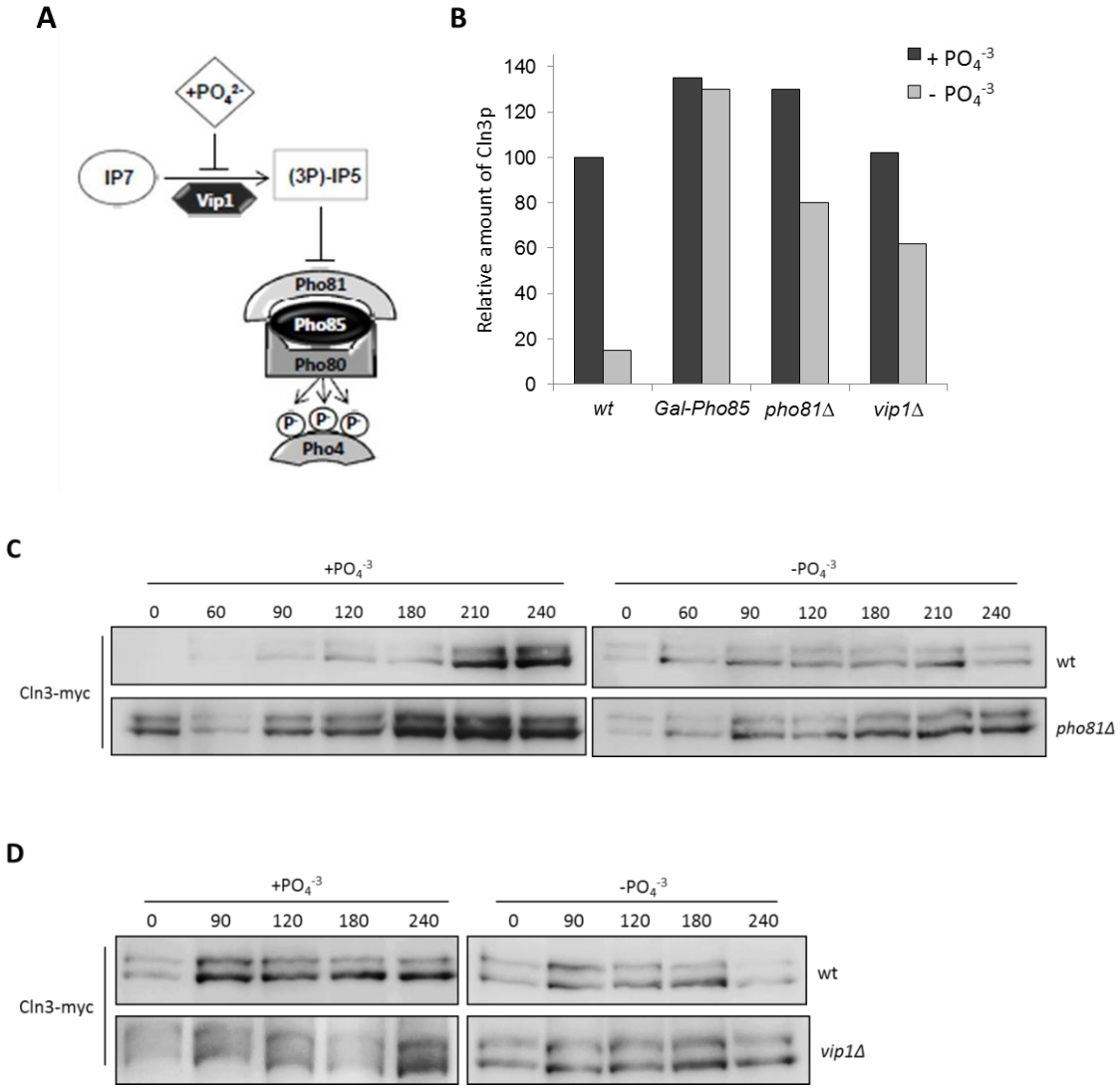

Figure R14. Phosphate controls cellular amount of Cln3 through PHO pathway modulation. A) The PHO pathway. During phosphate starvation, Vip1 increases the levels of the inositol heptakisphosphate (IP7), which binds and changes the conformation state of Pho81, leading to an inactivation of Pho85/Pho80 complexes. B) Relative amounts of Cln3 in several strains. Wild-type, *pho81*Δ, and *vip1*Δ cells were grown in a synthetic complete media either with ($+PO_4^{2-}$) or without ($-PO_4^{2-}$) phosphates and after 5 hours the levels of Cln3-myc were evaluated by immunoblotting using monoclonal antibodies. The strain *Gal-PHO85* (a wild-type strain that carries a centromeric plasmid with *PHO85* expressed under the GAL promoter) was grown during 5 hours in synthetic complete media either with or without phosphates, with galactose as a carbon source. C, D) Wild-type, *pho81*Δ, and *vip1*Δ tagged cells (YNR55, YNR82, and YAM150) were cultured as in Panel B but different sampling times were used for western blot analysis.

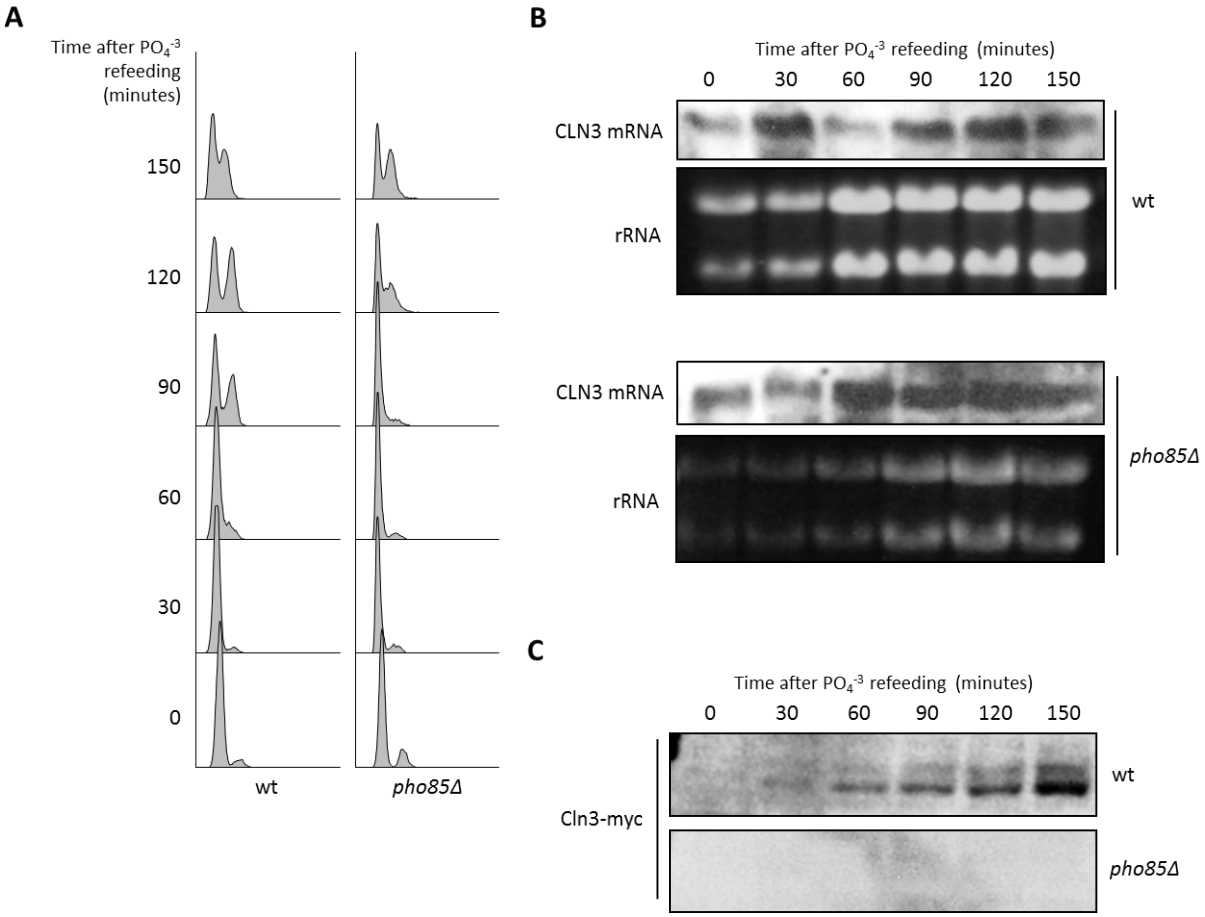

Figure R15. *CLN3* mRNA levels are not affected by Pho85.Wild-type and *pho85Δ* (BY4741 and YAM78) cells were grown exponentially in synthetic complete media. Cells were harvested and incubated in media without phosphate. Seven hours later cells were refed in YPD medium and sampled at the indicated times to analyze DNA content by FACS analysis (A), *CLN3* mRNA levels by northern blot analysis (B), and Cln3 protein levels by western blot analysis(C).

Ribosomal amount (rRNA) was found to decrease in the *pho85Δ* strain. This result is consistent with the findings of other groups, who have postulated that CLN3 translation is extremely sensitive to the decreased rate of overall protein synthesis in nutrient-depleted conditions. If *PHO85* absence mimics sustained phosphate depletion, this drop in rRNA could be predicted.

Real-time PCR analysis these results (Fig. R16A) and compared them to CLN2 expression under the same conditions (Fig. R16B). *CLN3* mRNA levels did not significantly change when comparing wild-type and *pho85Δ* strains, but levels of *CLN2*

transcript showed a delay in cells lacking *PHO85*. *CLN2* mRNA levels 60 minutes after refeeding were higher in wild-type cells compared to *CLN2* mRNA levels in *pho85Δ* cells, and the levels in *pho85Δ* cells did not reach the same high value at any later time.

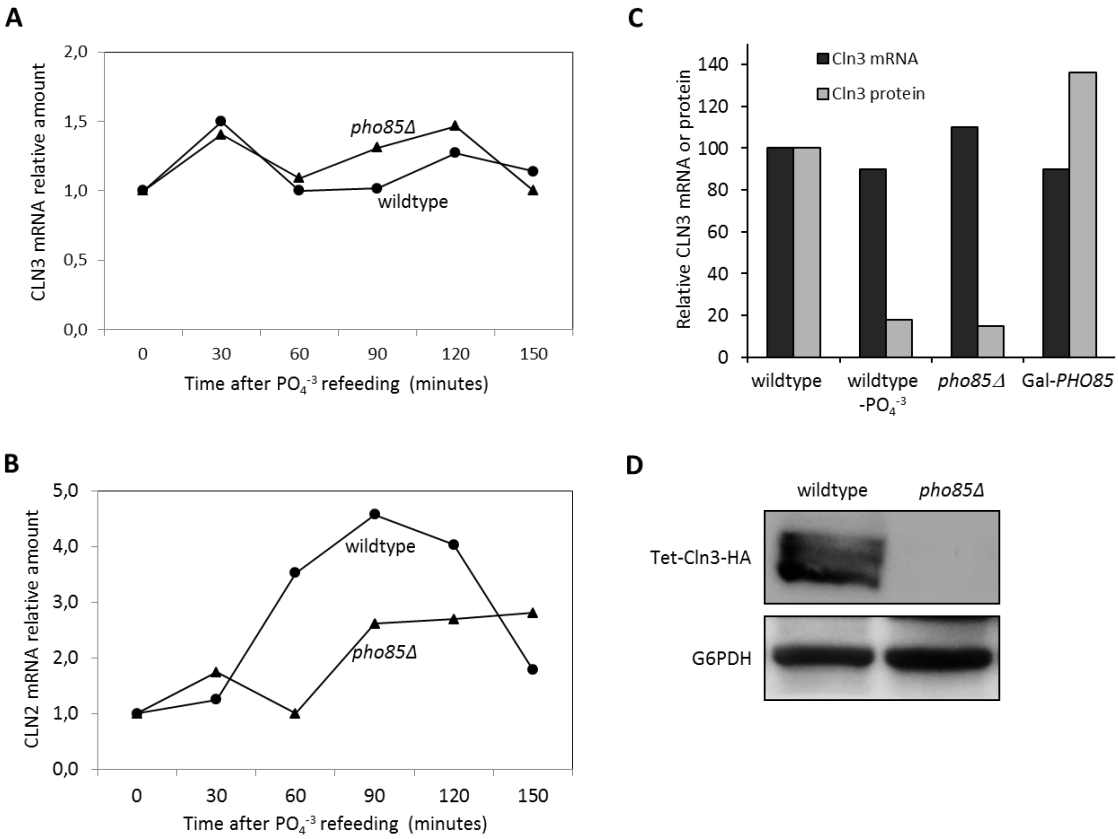

Figure R16. *CLN3* mRNA levels are not affected by Pho85. *CLN3* (A) and *CLN2* (B) mRNA from the samples in Figure R15A were analyzed by real-time PCR and normalized to *CDC28* levels. C) The different strains were sampled; the mRNA of *CLN3* was analyzed with real-time-PCR and Cln3 protein levels were analyzed with immunoblotting. Wild-type cells were grown exponentially in synthetic complete media with ($+PO_4^{2-}$) or without ($-PO_4^{2-}$) phosphates, *pho85Δ* cells were grown in complete media with phosphates, and GAL-*PHO85* was grown for 5 hours in synthetic complete media with galactose as a carbon source. D) Wild-type cells containing a *CLN3-HA* plasmid expressed under the control of a Tet-off promoter were cultured in YPD without tetracycline for 5 hours.

Figure R16C shows how *CLN3* mRNA levels compared to those of Cln3 protein with different strains and under various conditions. Differences in *CLN3* mRNA were not found in wild-type cells cultured in synthetic medium in the presence or absence of phosphates. Similarly, no changes in *CLN3* mRNA levels were found when comparing wild-type cells, *pho85Δ* cells, and cells that overexpressed *PHO85*. Despite the similar

levels of mRNA, differences in Cln3 protein levels were observed under these new experimental conditions and thus confirmed.

Finally, in order to express *CLN3* independently from regulator proteins such as Pho85, a strain containing the tagged *CLN3-HA* cyclin under the control of a Tet-off promoter was created. In *CLN3* mRNA sustained expression due to a lack of Tetracycline, levels of Cln3 protein were dramatically low in *pho85Δ* cells compared to wild-type cells (Fig. R16D).

These results suggested that Cln3 was being down-regulated by Pho85 kinase through post-transcriptional mechanisms.

2.7. Pho85 affects Cln3 protein stability

The possible effect of Pho85 activity on Cln3 stability was examined by studying Cln3 levels in cycloheximide-treated cells.

In wild-type cells, the estimated half-life of the Cln3 cyclin was about 8 minutes (Fig. R17), which was reflective of values reported previously (Gallego et al., 1997). Unfortunately, it was not possible to measure the Cln3 half-life in *pho85Δ* cells because of the low starting levels of Cln3 at the beginning of the time course.

For this reason, Cln3 stability was monitored in the pho85-AS strain in the presence or absence of the NaPP1 inhibitor.

As shown in Figure R18, although the AS strain probably still retains some residual levels of Pho85 activity, it exhibits an apparent reduction in the half-life of Cln3 ; the half-life of Cln3 in the control (DMSO) was about 6 minutes, but the NaPP1-treated cells showed a very unstable Cln3 protein (half-life = 3.2 min.).

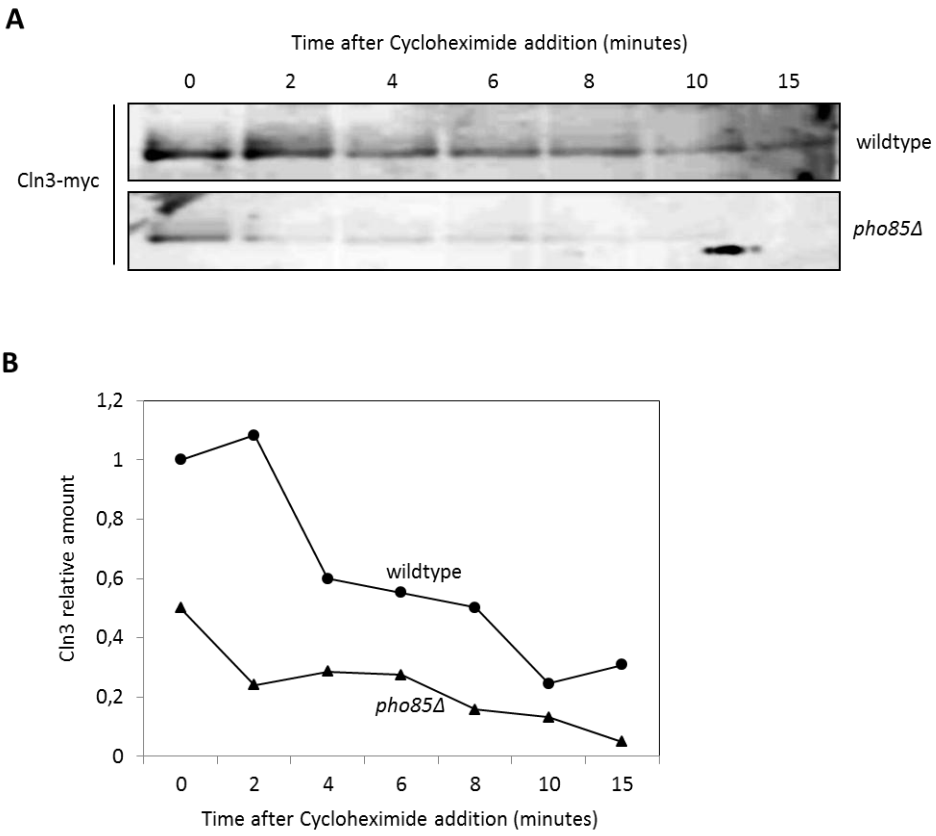

Figure R17. The Cln3 protein has 8 minute half-life in wild-type cells. Wild-type and *pho85Δ* tagged cells (YNR55 and YAM78) were grown exponentially in YPD for 4 hours, treated with cycloheximide and sampled at the indicated times for western blot analysis (A). Panel B shows a graph of the quantified results from A.

Although this result does not preclude the existence of other regulatory mechanisms (such as translation control of Cln3 mRNA), it strongly suggested that Pho85 activity increased the protein stability of the Cln3 cyclin.

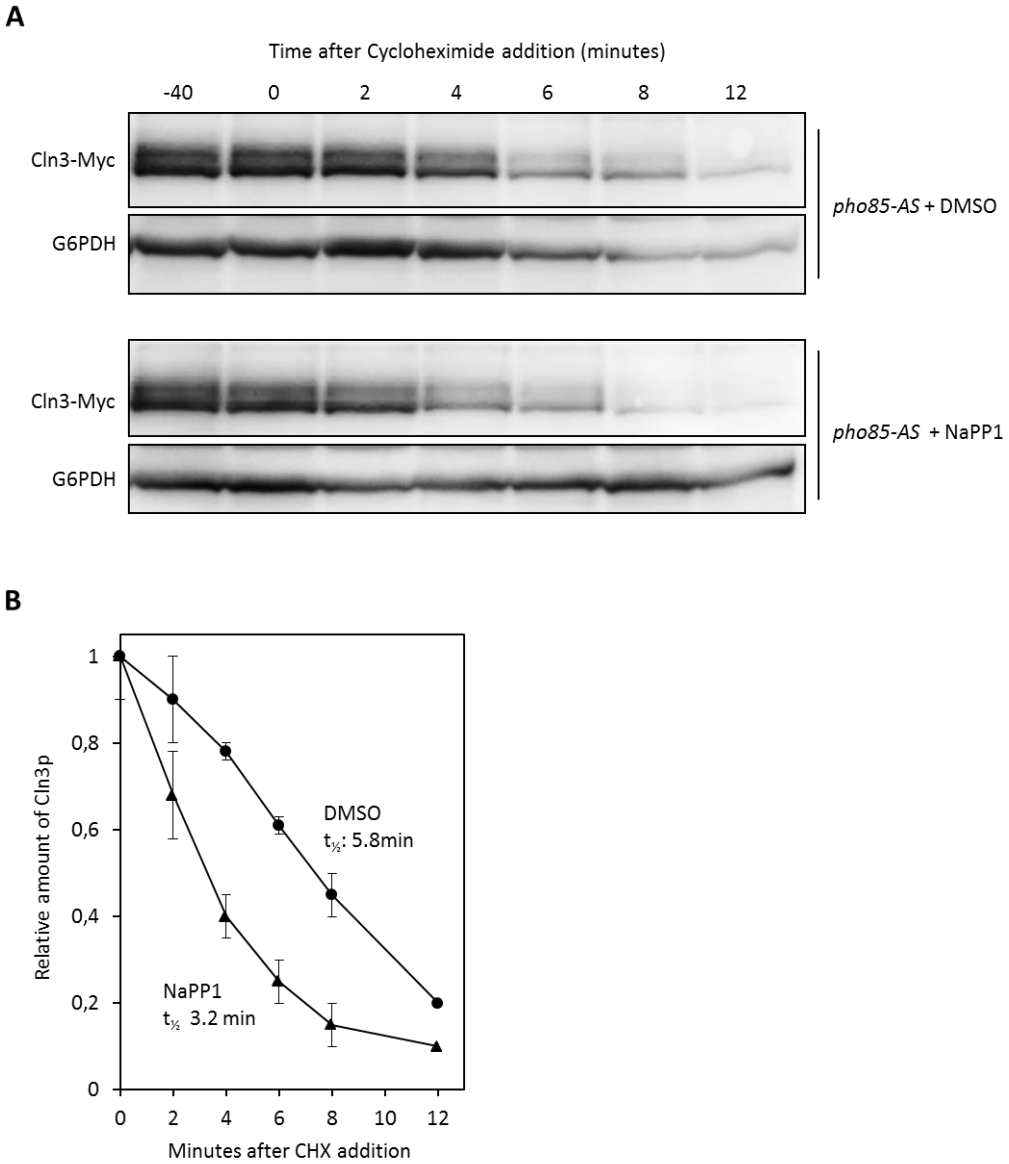

Figure R18. Pho85 activity increases Cln3 half-life. *pho85-AS* cells (YAM67) were incubated with NaPP1 (a specific inhibitor of *pho85-AS*) or with drug vehicle (DMSO). Forty minutes later (time zero) cycloheximide was added to the cultures, samples were taken at the indicated times, and Cln3-myc was analyzed with immunoblotting using monoclonal antibodies (A), and quantified and represented on a graph (B). Data ± s.d. from 4 independent experiments are shown.

2.8. Pho85 does not control Cln3 protein stability through Cdc28 regulation

The degradation of Cln3 has been shown to involve *CDC28*-dependent phosphorylation events (Yaglom et al., 1995). Therefore, Cln3 proteolysis may be controlled by Pho85 via Cdc28 CDK.

Cln3 stability assays were performed by using a *cdc28ts* strain, which is able to inactivate the CDK at 37°C. When Cdc28 CDK was inactivated at 37°C, Cln3 protein levels in *cdc28ts* cells greatly increased when compared to those in the active form at 25°C (Fig. R19). Despite this upregulation, *pho85Δ* cells did not show a recovery of Cln3 levels, even when Cdc28 was inactivated.

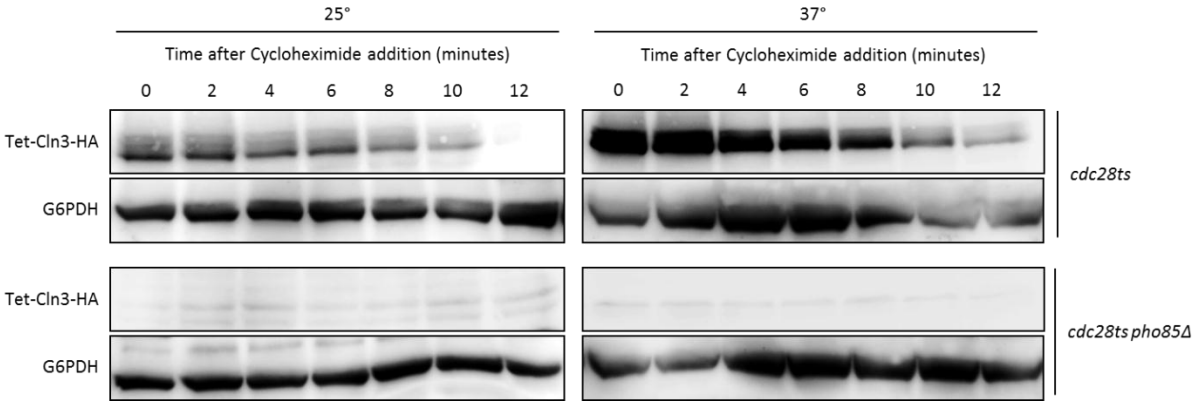

Figure R19. Diminished Cln3 levels of *pho85Δ* mutants are not restored by Cdc28 CDK inactivation. The cells types *cdc28ts* and *cdc28ts pho85Δ* (YAM57 and YAM59) containing a *CLN3-HA* plasmid expressed under the control of a Tet-off promoter were cultured in YPD without tetracycline for 5 hours. At 0 minutes, cycloheximide was added to the cultures, samples were taken at the indicated times, and Cln3-HA was analyzed with immunoblotting using monoclonal antibodies.

The failure to recover Cln3 protein levels in *pho85Δ* cells despite the inactivation of Cdc28 suggested that Pho85 modulated Cln3 stability independently of Cdc28. Pho85 could therefore have been directly controlling this cyclin. Identifying the specific mechanism used by Pho85 regulate its half-life required more study. Cln3 ubiquitination was examined to determine whether it was affected by this phosphate-sensing CDK.

2.9 Pho85 affects Cln3 ubiquitination

It is known that Cln3 is constitutively degraded by an ubiquitination-dependent mechanism in exponentially growing cells (Yaglom et al., 1995). The results of the present thesis indicate that Pho85 in fact interferes with this process.

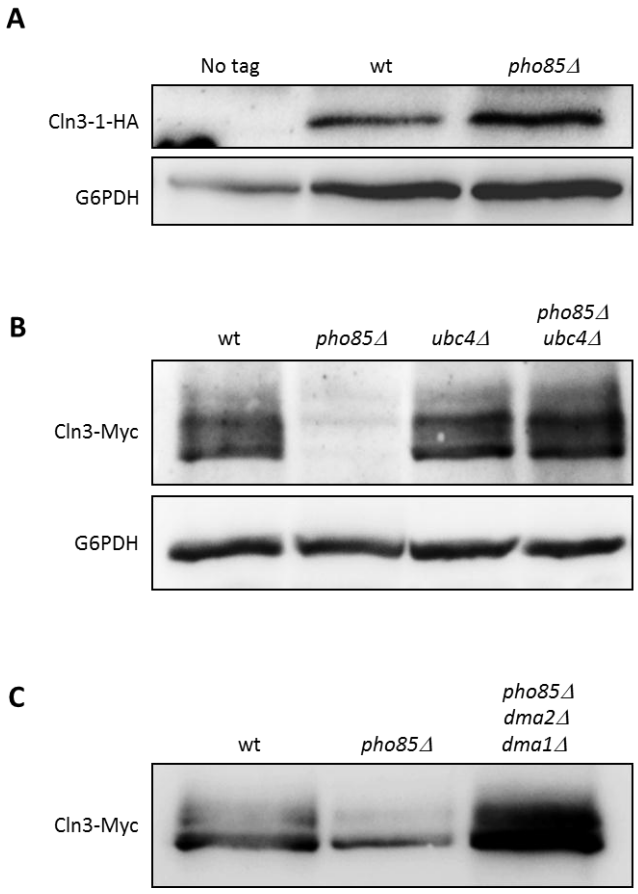

Figure R20. Pho85 controls Cln3 stability via ubiquitination. The strains carrying a genomic Myc-tagged version of Cln3 (YAM91, YAM92, YNR55, YAM78, YAM151, YAM142, and YAM143) were grown exponentially in rich media. Samples were taken and Cln3p levels were evaluated with immunoblotting using monoclonal antibodies against the Myc epitope.

First, deletion of the PEST region of *CLN3* (the *cln3-1* allele), which is known to avoid ubiquitination, stabilized the protein even in the absence of *PHO85* (Fig. R20A). Deletion of the E2 ubiquitin-conjugating enzyme Ubc4 [important for nutrient homeostasis and involved in Cln3 ubiquitination of Cln3 (Yaglom et al., 1995)] then

maintained Cln3 levels in the absence of Pho85 activity (Fig. R20B). Finally, strains lacking the E3 ubiquitin ligases Dma1 and Dma2 recovered Cln3 levels despite the absence of Pho85 (Fig. R20C).

These data suggested that Pho85 interferes with Cln3 ubiquitination. Because Pho85 is a kinase, Pho85 may have phosphorylated Cln3 and interfered with the ubiquitination process.

2.10. Cln3 may be a substrate of Pho80/Pho85

The question of whether Pho85 phosphorylates Cln3 was examined using *in vitro* phosphorylation assays. For these studies, purification of full-length Cln3 protein was necessary. Wild-type yeast cells were used in an attempt to obtain Purified Cln3. However, the cyclin was phosphorylated by the tightly associated Cdc28 kinase and it was very difficult to use as a substrate for phosphorylation assays. Attempts were also made to purify Cln3 as a recombinant protein, but expression of the full-length protein was highly toxic for *E. coli* cells, resulting in very poor expression levels. Finally, a fragment of recombinant GST-Cln3 protein was successfully expressed and purified. This fragment was incubated with either reconstituted Pho80/Pho85 complexes, also purified from *E. coli* cells, or with the Pho85-TAP protein purified from *S. cerevisiae* cell extracts.

Figure R21A shows how in the presence of ATP Cln3 was phosphorylated by Pho80/Pho85 recombinant complexes or by Pho85 purified from yeast. In addition, Cln3 co-immunoprecipitated with Pho85 purified from yeast cells grown in rich medium (Fig. R21B), suggesting that Cln3 and Pho85 interacted *in vivo*.

The canonical site for the Pho80/Pho85 kinase is S/TPXHyd ("Hyd" is any hydrophobic amino acid); Cln3 has only 3 types of this site, 2 of which are located within the PEST region of Cln3 (Fig. R22A) that is necessary for targeting and destroying this G_1 cyclin (the *cln3-1* allele described above lacks this region). In order to test the *in vivo* effects of phosphorylation at these 3 sites, protein levels of several Cln3 versions in which the residues at the canonical site were Aspartic acid (D) or Alanine (A) were analyzed. Aspartic acid substitutions mimic the effect of phosphorylation, and it was predicted that they would at least partially restore Cln3 levels in a *pho85Δ* strain. To better simulate the presence of phosphate, 2 amino acids were substituted because 1 aspartic acid alone has only half the negative charge of a phosphate group. Alanine substitution was used to avoid phosphorylation at a specific site, and it was postulated that this would lead to a decrease in Cln3 levels in wild-type cells.

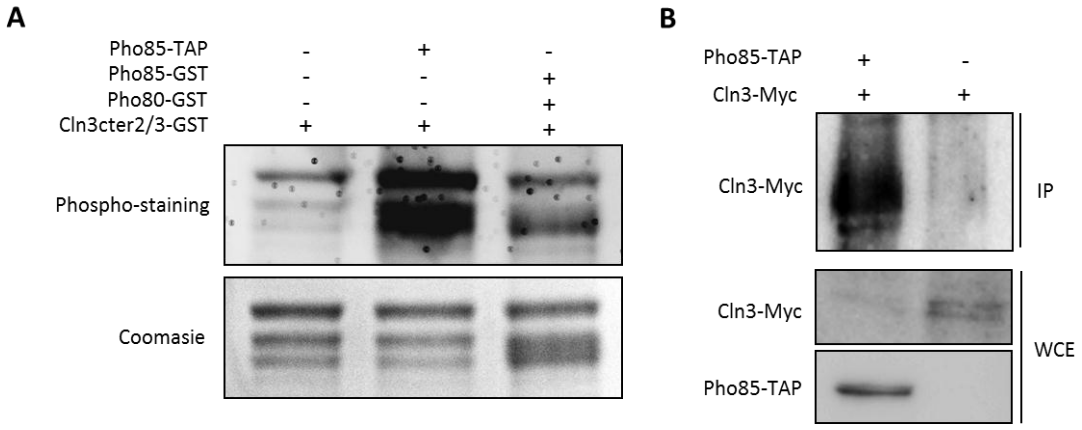

Figure R21. Cln3 phosphorylated by Pho85/Pho80. A) Pho85/Pho80 complexes phosphorylate Cln3p *invitro*. Recombinant GST-Pho80 and GST-Pho85 purified from bacteria or TAP-Pho85 purified from yeast cells were incubated with a fragment of Cln3 protein (without the third N-terminal part, see Materials and Methods). Tap-purification from untagged wild-type extract cells was used as a negative control. B) Pho85 interacts with Cln3 *in vivo*. Yeast extracts containing untagged *PHO85* or TAP-*PHO85* and tagged Myc-Cln3 (from its chromosomal locus) were co-immunoprecipitated with protein A-sepharose and probed using specific antibodies.

In *pho85Δ* cells, protein levels in Cln3 carrying the aspartic acid substitutions were higher than those of the Cln3 wild-type allele (Fig. R22B and R22C). This data suggested that *in vivo* phosphorylation at these sites was essential for maintaining high levels of Cln3. However, Alanine substitutions did not decrease Cln3 levels in wild-type cells as expected (Fig. R22D and R22E).

A possible scenario emerging from these results is that in the absence of phosphates Pho85 becomes inactive and Cln3 is no longer phosphorylated, which in turn elicits an increase in the rates of ubiquitination and destruction of the cyclin (Fig. R23A). At this point, the proposed model of this thesis required testing for physiological validity.

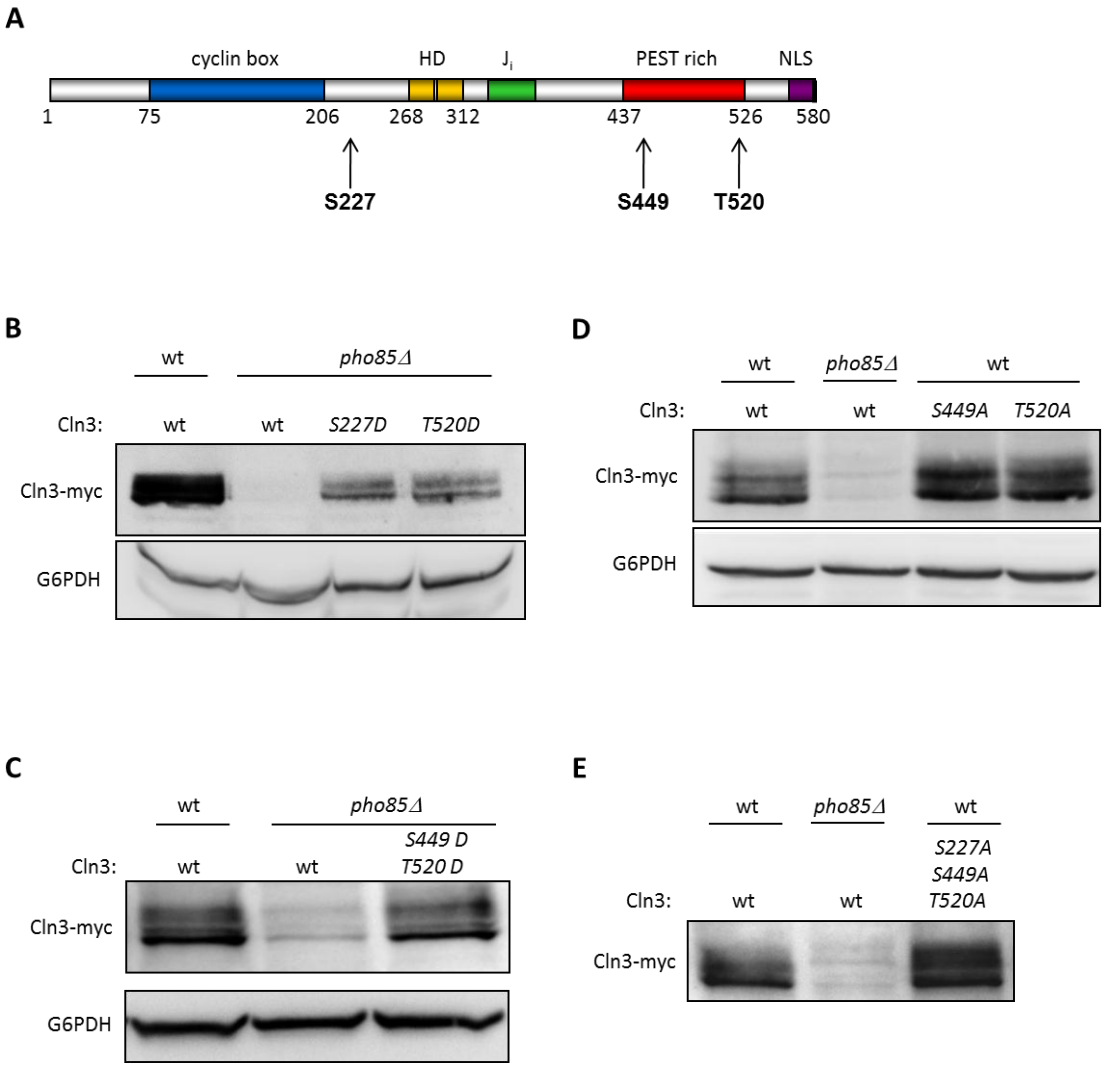

Figure R22. Cln3 levels are recovered in pho85 cells when canonical sites for Pho85/Pho80 are phosphorylated. A) The most relevant features of Cln3. The 3 Ser and Thr canonical residues for the Pho80/Pho85phosphorylation are indicated using arrows. B to E) wild-type and *pho85Δ* cells carrying a plasmid with different versions of *CLN3* (YAM113, YAM114, YAM147, YAM148, YAM149, YAM161, YAM162, and YAM163) were grown exponentially in rich media for 4 hours. Cln3 levels were analyzed by immunoblotting using monoclonal antibodies against the Myc epitope. Nomenclature was simplified; for instance, S449D means a double substitution of Asp into Ser 449 and its previous respective residues to mimic the double negative charge that represents the phosphate group (see Materials and Methods).

3. Pho85 ACTIVITY VIA Cln3 IS CRUCIAL UNDER SPECIFIC CONDITIONS

3.1. Pho85 inactivation is essential for proper G_1 arrest

Ectopic expression of Cln3 in G_1 arrested cells (due to rapamycin or nutrient depletion) leads to accidental entry into the S phase and diminished cell viability (Zinzalla et al., 2007; Weinberger et al., 2007). To ascertain the relevance of the results shown in this thesis, cells of different strains were arrested in phosphate-depleted medium; as expected, a hyperactive form of *CLN3* (the *cln3-1* allele) reduced cell viability. This correlated with an increase in the number of cells that crossed START (indicated by the high percentage of budded cells) (Fig. R23B). Thus, downregulation of CDK activity appears to be essential for proper G_1 arrest during phosphate deprivation.

According to this model, hyperactivation of Pho85 should also be detrimental to cell viability in G_1-arrested cells; this was demonstrated by experimental results. Figure R23B shows that 91% of wild-type cells incubated in phosphate-free medium arrested at G_1 and that after 7 days they showed a viability rate of 55%. On the other hand, *vip1Δ* and *pho81Δ* cells, which should retain Pho85/Pho80 activity under the same conditions of deprivation, showed an increase in improperly arrested cells (16% to 18% of budded cells) and a significant loss in viability (a total of 20%). Furthermore, strains carrying Cln3 along with Asp mutations that mimic Pho85 phosphorylation also showed a reduced viability during phosphate depletion.

These results supported the idea that in the absence of phosphate, downregulation of Pho85 activity was essential to decreasing Cln3 levels and properly arresting at G_1.

In addition, Pho85 was shown to be essential for proper cell cycle reentry, as seen in Figures R9B and R12A.

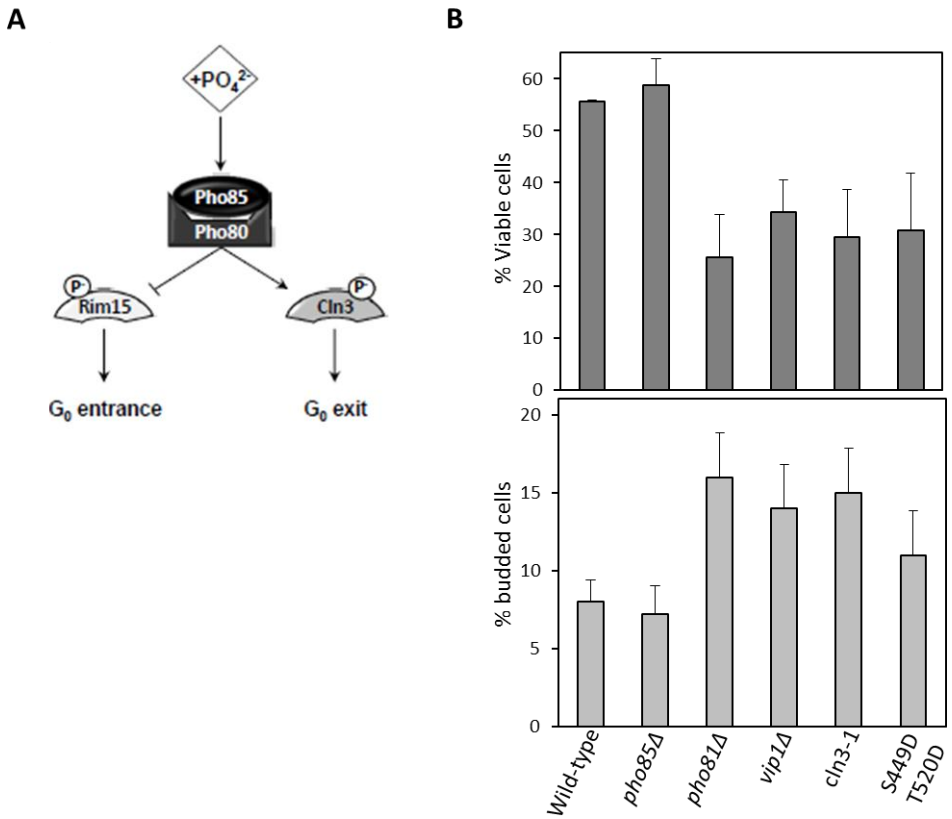

Figure R23. Cln3 phosphorylation is essential for proper arrest in G_0. A) A proposed model of Pho85 role. In rich media, the presence of phosphate maintains high-activity Pho85/Pho80 complexes. Pho85 then phosphorylates and inactivates Pho4 and Rim15 (Wanke et al., 2005), and activates cyclin Cln3. This process could be relevant to wild-type cells, where nutrient availability often fluctuates. B) Proper regulation of Pho85 activity was seen to be essential to maintaining viability during phosphate deprivation. Cells were incubated in synthetic complete media for 7 days, then viability was assessed using colony counting (upper panel), and budding percentages were analyzed by counting 200 cells under a microscope (lower panel). Data ± s.d. from 3 independent experiments is shown.

3.2. Pho85 activation is essential for proper cell cycle reentry after nutrient deficiency arrest

It has been postulated that Pho85 activity is essential in situations with no Cdc28 activity, for example, when restarting the cell cycle (Huang et al., 2007). This idea is consistent with the proposed model of this thesis; if true, cells with low Pho85 activity would have difficulties reentering the cell cycle from G_0.

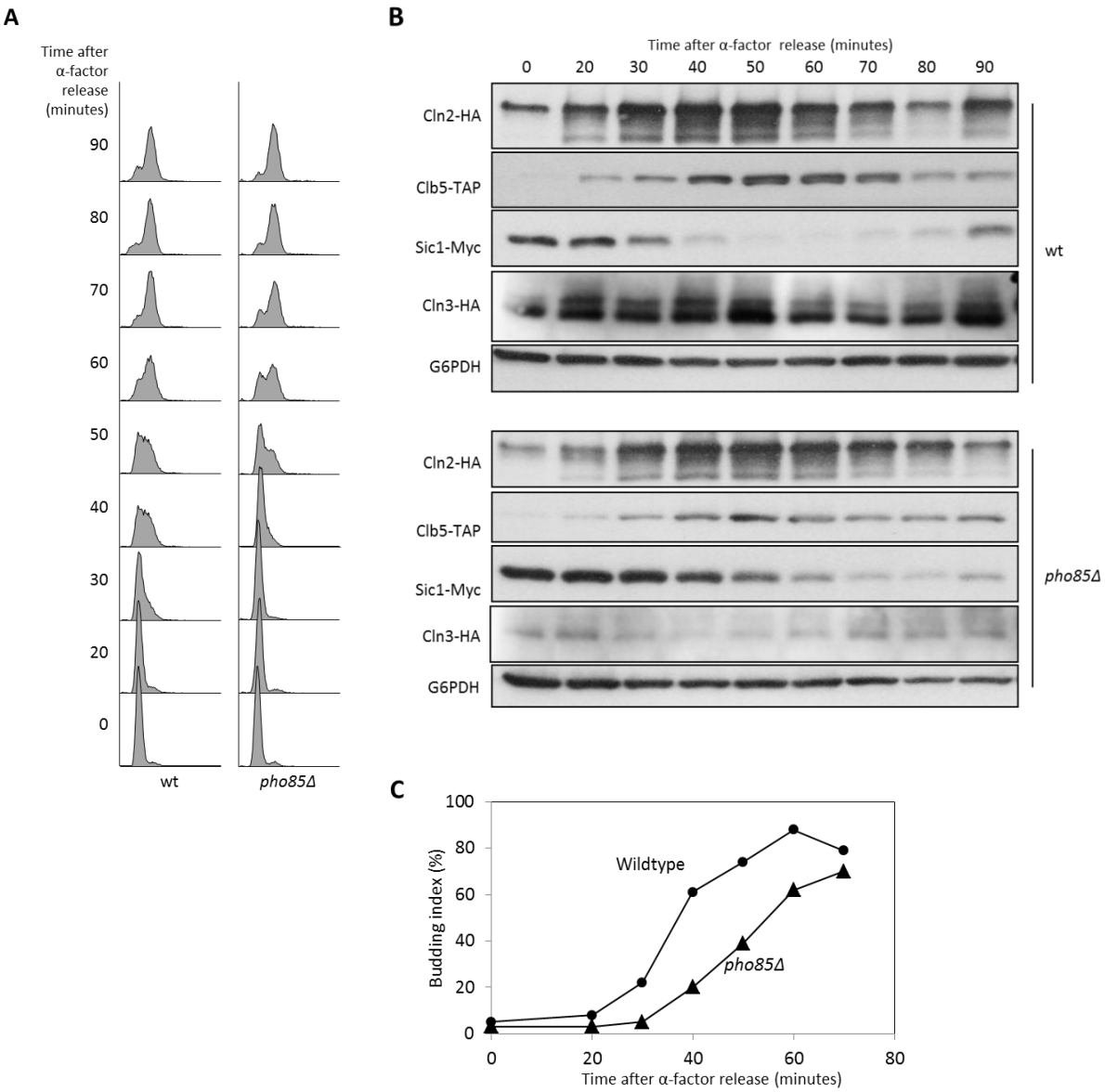

Figure R24. *pho85Δ* cells progress through G_1 after α-factor exit. Wild-type and *pho85Δ* tagged cells (YAN32 and YNR26.2) were synchronized with alpha factor for 2 hours, and released into fresh media at 30°C. Samples were taken at the indicated times. A) Total DNA content was measured (see Materials and Methods) using propidium iodide instead of SYBR green. B) The budding index was determined with microscopy. C) Western blot analysis of Cln2, Clb5, and Sic1 was performed.

The results shown in Figure R12A indicate that *cln3Δ* cells are clearly delayed in reentry; this confirms the role of Cln3 in cell cycle reentry. After 4 hours of incubation in phosphate-rich medium, cells with low Pho85 activity (e.g. *pho85Δ* and

pho80Δ) remained arrested, while wild-type cells were already undergoing mitosis. It is important to note that when cell cycle reentry from an α-factor arrest, the differences between strains are minor (Fig. R24).

In Figure R24, a slight 10-minute delay occurred in *pho85Δ* cells when restarting the cell cycle after an α-factor arrest, as demonstrated by DNA content (Fig. R24A) and budding index (Fig. R24C). As previously seen, the biochemical profile showed very low levels of Cln2 and Clb5 cyclins and in the Sic1 inhibitor when comparing wild-type and *pho85Δ* strains (Fig.R24B).

Although only Cln3 levels showed significant differences, these studies demonstrated that *pho85Δ* cells have very low levels of this cyclin, even when exponentially growing. It is important to note that the lack of Cln3 in an α-factor release is not determinant because Cln3 is crucial at the early G_1 phase (i.e. after a nutrient depletion), but not at the late G_1 phase. Some publications uphold the theory that different cell cycle events require different cyclin levels (Oikonomou and Cross, 2011). Hence, a mid-to-late G_1 event may require very low levels of Cln3 when compared to early G_1 events.

These findings suggested that the observed delay in cell cycle reentry after a phosphate-depletion arrest was specific rather than a general defect of G_1 phase regulation. However, these results reinforced the idea that Pho85 activity plays an indispensable role in exiting from G_0 arrest. The regulation of other cell cycle phases by this CDK had to be considered.

4. Pho85 MODULATES OTHER PHASES OF THE CELL CYCLE

As a final line of study, the present thesis examined new Pho85 functions in cell cycle regulation.

With the purpose of determining whether Pho85 was involved in the regulation of other cell cycle phases, cell cycle arrest was observed in the presence of nocodazole, an agent that interferes with the polymerization of microtubules, leading to arrest in the M phase. To better synchronize cells, a pre-arrest with α-factor was induced, and after 2 hours cells were grown again without the pheromone but with nocodazole. Figure R25A shows a FACS profile in which *pho85Δ* cells did not restart cell cycle after almost 30 minutes following nocodazole release compared to a wild-type strain. These data were confirmed by light microscopy (Fig. R25B). At 20 minutes, a

high percentage of wild-type cells (61.5%) had already finished anaphase, but only 28.5% of *pho85Δ* cells had completed mitosis.

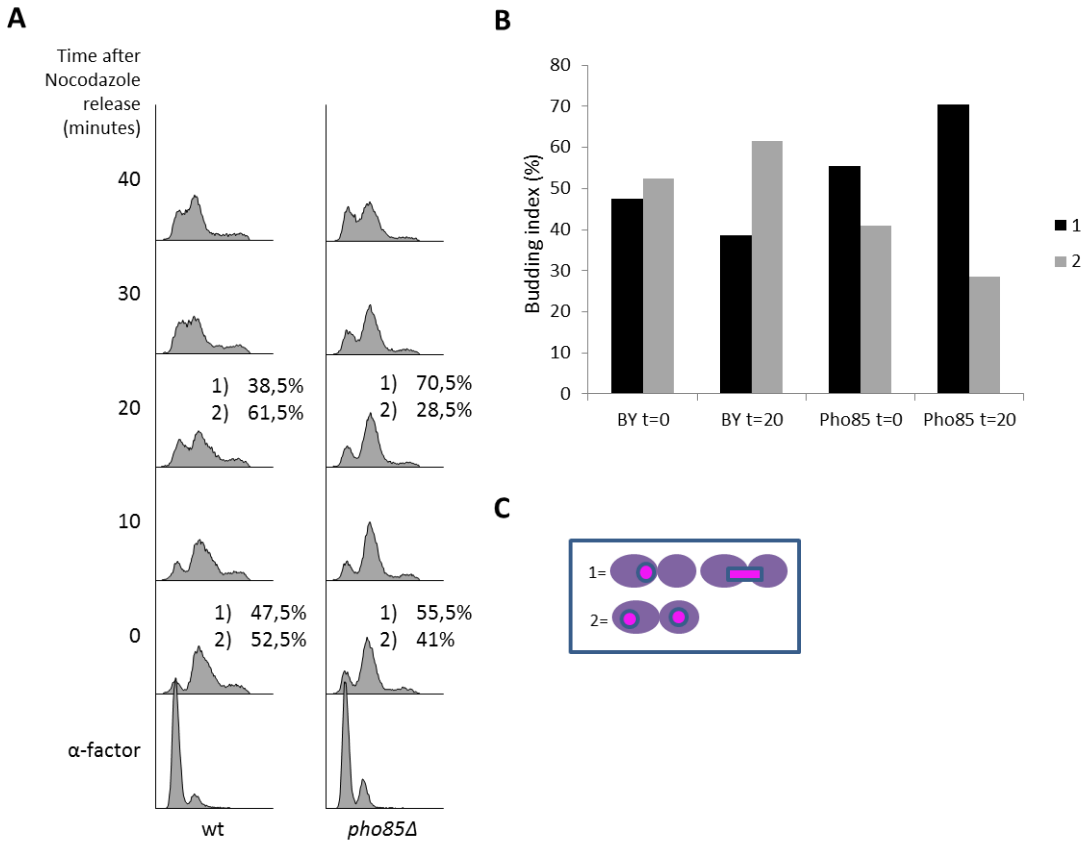

Figure R25. Cell cycle delay in *pho85Δ* after nocodazole arrest. Wild-type and *pho85Δ* cells were synchronized with α-factor for 2 hours and released into fresh media with nocodazole at 30°C. After 1 hour, cells were harvested and cultured in new YPD (time 0 minutes). Samples were taken at the indicated times. A) Total DNA content was measured (see Materials and Methods) using propidium iodide instead of SYBR green. B) Cell cycle phases were analyzed by microscopy. Legend is explained at C pannel. C) To count cells at different phases, samples were divided into 2 groups: cells that did not complete anaphase (1) and cells that completed anaphase (2).

These final results indicated that Pho85 CDK may have been involved in the regulation of both the M phase and the G_1 phase.

DISCUSSION

Nutrient signaling pathways enable yeast to benefit from rich nutrient conditions by stimulating cell proliferation while at the same time survive periods of nutrient scarceness by inducing the entry into a quiescent G_0 phase. This switching between proliferation and quiescent must be highly regulated by cell cycle mechanisms that may be directly influenced by nutrient availability.

Glucose and nitrogen sources have been described as critical elements that modulate cell cycle progression; their absence leads to a G_1 arrest. However, most of these nutrient signaling pathways are poorly understood. For example, the first sensor to translocate the signal to the effectors, the glucose sensor that activates the Snf1 pathway, and the mechanism that allows the presence of nitrogen to be transmitted to TORC1 have yet to be identified. Knowledge about nutrient sensing and its involvement in the cell cycle is greatly lacking.

1. PHOSPHATE LEVELS REGULATE CELL CYCLE PROGRESSION VIA Pho85

The present thesis examines whether the availability of phosphate, the latest nutrient found to participate in cell cycle regulation (Gray et al., 2004; Klosinska et al., 2010), interacts directly with cell cycle control and, when demonstrated, describes the precise mechanisms involved.

The findings of this thesis show that the absence of phosphate stops cell growth (Fig. R7) in a pattern that is similar to nitrogen or rapamycin (Figs. R1 and R3), and that cell cycle restart after refeeding, regardless of the nutrient, depends on Pho85 CDK (Figs. R4 and R9).

In the case of phosphate depletion, one might assume that this defect is merely a nonspecific consequence arising from the reduction in the level of a particular metabolite (e.g. ATP). However, metabolomic studies have shown that cells growing under phosphate-limiting conditions maintain a relatively constant free energy of ATP hydrolysis (Boer et al., 2010), suggesting that at the beginning of phosphate deprivation, cells might have enough energy to initiate an adaptive response such as finishing 1 round of division until the next G_1 phase. On the other hand, the present thesis confirms that the observed arrest is a controlled process based on the following findings:

1) When the source of phosphate is depleted, cells are mostly unbudded with 1N DNA content. If they arrest accidentally, cells at different stages of the cell cycle

would be observed (Fig. R7). It is important to note that in the depletion experiments cells did not arrest immediately, but gradually moved into G_1 and finally, within approximately 6 hours of deprivation, stopped dividing (90% of cells). This gradual response to the culture is probably due to polyphosphate reserves in cells (Thomas and O'Shea, 2005), which serve to buffer the sudden external changes.

2) The volume of arrested cells continues to increase, indicating that they are metabolically active and not in a collapsed state (Fig. R7).

3) A phosphate depletion-based arrest can be reversed with an increase in CDK/Cln2 activity. This finding suggests that the delay is a specific inactivation of the G_1/S transcriptional machinery, leading to a lack of *CLN1,2* and *CLB5,6* gene expression.

4) Under the same conditions, the biochemical markers that were observed also indicate G_1 arrest (Fig. R8). It is clearly shown that *CLN2* and *CLB5* protein levels decrease after 4 hours of growth in phosphate-free medium, whereas levels of Sic1 protein remain constant. All mRNA levels in these proteins follow a similar pattern, indicating that phosphate depletion may inhibit the transcription of genes required for G_1/S transition.

Thus, phosphate is shown to regulate START through the control of G_1/S transcriptional machinery in *S. cerevisiae*, as before suggested by Hartwell (Johnston et al., 1997).

Cell cycle regulation based on phosphate availability appears to take place through Pho85 CDK. As shown in Figure R9B, Pho85 is needed for a successful cell cycle restart after phosphate deprivation because *pho85Δ* cells exit later from the G_1 arrest than wild-type cells. Pho85 could link these 2 processes (nutrient sensing and cell cycle progression) because exogenous phosphates are known to activate Pho85, and one of its multiple functions is to regulate the G_1 phase as CDK.

2. Pho85-Pho80 COMPLEX ACTIVITY SPECIFICALLY REGULATES THE LEVELS OF THE Cln3 PROTEIN

The central idea of this thesis is that phosphate availability modulates Cln3 levels by regulating the activity of the Pho85-Pho80 complex.

Whi5 and Cln3 are known to play a key role in the activation of SBF and MBF complexes in late G_1. However, only Cln3 protein levels drop dramatically in the absence of phosphates; the levels of the repressor Whi5 remain fairly constant (Fig. R8). Although it has been demonstrated that Pho85 phosphorylates Whi5 in order to promote its exit from the nucleus and trigger the release of the SBF promoter (Fig. R5), there is no phenotypic recovery of *pho85Δwhi5Δ* cells during cell cycle restart after nitrogen depletion (Fig. R6). Therefore, Whi5 appears to have an important role in the regulation of the cell cycle based on the presence of nutrients, but together with some other key regulators.

These results support the idea that phosphate deprivation, like nitrogen depletion, downregulates SBF and MBF activity due to loss of Cln3. It is known that a triple *cln* mutant cannot undergo G_1/S transition unless Sic1 is removed. Therefore, the absence of Cln3 cyclin appears to lead to a downregulation of Cln1,2 proteins that promote the sustained presence of Sic1. This could explain the G_1 arrest produced by phosphate deprivation in terms of molecular requirements.

The involvement of Pho85 in Cln3 downregulation during phosphate absence is confirmed by the almost inexistent Cln3 levels in *pho85Δ* cells, in addition to the gradually decreasing levels of Cln3 protein found when Pho85 was inactivated by NaPP1 (Fig. R10). Pho85 activity, rather than the physical presence of the protein itself, seems to be a key factor in controlling Cln3 protein levels.

The regulation of Cln3 also appears to be specific because Pho85 does not affect the control of levels of other cyclins such as Cln2 (Fig. R11). This highly specific mechanism indicates that a specific Pho85 complex must be involved, with only 1 particular cyclin belonging to the Pho85 family binding to the CDK to trigger Cln3 regulation.

Experiments show that the deletion of *PHO80* alone causes a decrease in Cln3 similar to that in the deletion of *PHO85*, and that cell cycle restart after phosphate refeeding is similarly slow in *pho80Δ* and *pho85Δ* cells when compared to wild-type cells (Fig. R12). These results suggest that the effect of Pho85 CDK on Cln3 protein levels is mediated by Pho80 cyclin, and are consistent with the idea that Pho80-Pho85

complexes are inhibited in the absence of phosphates. This further demonstrates that phosphates control Cln3 levels by modulating the activity of the Pho85 kinase.

Pho85 appears to regulate Cln3, not only through *PHO85* deletion directly affecting Cln3 levels, but also through Pho85 overexpression or overactivation via PHO pathway modulation. This has an important impact on the G_1 cyclin: the maintenance of Cln3 levels despite phosphate depletion (Figs. R13 and R14).

Demonstrating that Cln3 is affected by Pho85/Pho80 activity could be relevant for 2 reasons. For the first time, the Pho85-Pho80 route is shown to be involved in directly controlling the cell cycle machinery, suggesting coordination between phosphate homeostasis and the cell cycle. At the same time, this is the first reported case showing how 1 CDK controls the activity of another. At present, this appears to be a case of crosstalk between CDK kinases, with Pho85 reporting to Cdc28 on the nutritional status in yeast. In this regard, the Pho85-Pho80 complex could be considered a CAK, but one that affects the cyclin partner of Cdc28.

3. Pho85 AFFECTS Cln3 IN TERMS OF STABILITY

The absence of phosphate has long been known to inactivate the Pho85-Pho80 complex, causing a large transcriptional response to adapt to the new situation. A critical element in this response is the transcription factor Pho4 which, under high phosphate concentration, is phosphorylated by Pho85-Pho80 and retained in the cytoplasm (O'Neill et al., 1996). Currently, there are no known *in vivo* substrates of Pho85-Pho80 other than Pho4 (and Rim15). Thus, it seems reasonable to assume that Pho85 also has a transcriptional effect on *CLN3*. However, the results shown in this thesis demonstrate that the mRNA levels of *CLN3* seem to be unaffected by changes in phosphate levels (Figs. R15 and R16), and regulation therefore occurs at some post-transcriptional level.

Several studies indicate that Cln3 is downregulated by translational repression during nutrient deprivation (Gallego et al., 1997; Polymenis et al., 1997; Hall et al., 1998). Although it cannot be ruled out that phosphate scarcity may have the same effect, the results of the present thesis show that Cln3 stability is affected by phosphate availability, demonstrated by using a *pho85-AS* strain, as levels of Cln3 in *pho85Δ* cells were too low to follow the half-life of the cyclin (Fig. R18). An analogous

destabilization phenomenon has been described in nitrogen-deprived cells, although its mechanism was left unexplained (Gallego et al., 1997). Aldea and colleagues showed that the half-life of Cln3 was also reduced by half. Thus, the destabilization of Cln3 (and possible translational repression) could be a general response by the cell to the limited availability of different nutrients. This response would limit the Cdc28-Cln3 complex activity necessary to pass START in order to slow down the cell cycle and adapt to the new conditions.

The findings of this thesis show that Pho85 activity affects the stability of the Cln3 cyclin in a direct way. It is important to note that Cln3 is an *in vitro* substrate of the Pho85-Pho80 complex (purified from yeast or *E. coli* cells) (Fig. R21). Due to several technical challenges, it cannot be confirmed that this phosphorylation occurs *in vivo* because Cln3 is already highly phosphorylated by Cdc28 (Yaglom et al., 1995); experimental detection of an additional phosphorylation by Pho85 remains a serious challenge. In fact, a differential mobility shift of Cln3 in *pho85Δ* cells compared to wild-type cells could not be seen despite the use of a *cdc28ts* strain; levels of Cln3 in *pho85Δ* cells remained almost inexistent and a clear band pattern could not be seen (Fig. R19).

Despite these findings, Cln3 and Pho85 were shown to interact *in vivo* (Fig. R21) and, more interestingly, simultaneous substitution of S449D and T520 was seen to largely recover the Cln3 levels in *pho85Δ* cells (Fig. R22), suggesting that phosphorylation of these 2 sites could be important for Cln3 stability. However, alanine substitutions did not decrease Cln3 levels in wild-type cells as expected (Fig. R22). The mutation sites were selected because they are the only sites in Cln3 to have the canonical sequence known to be phosphorylated by Pho85, and it is assumed that this CDK cannot phosphorylate any other site in the cyclin. However, recent studies show that about 50% of the Pho85 dependent phosphorylations are in non-canonical sites (Mok et al., 2010). Thus, a hypothetic model could explain the contradictory result of alanines mutations: the phosphorylation of the 3 canonical sites could be enough to maintain the stability of Cln3, even in *pho85Δ* cells, because Pho85 would phosphorylate them directly to stabilize the cyclin. Additionally, there could be an extra site (not canonical to Pho85 and therefore not detected in the sequence screening) in Cln3 that is phosphorylated by Pho85, resulting in the stable state of the cyclin. This hypothesis is outlined in Figure D1.

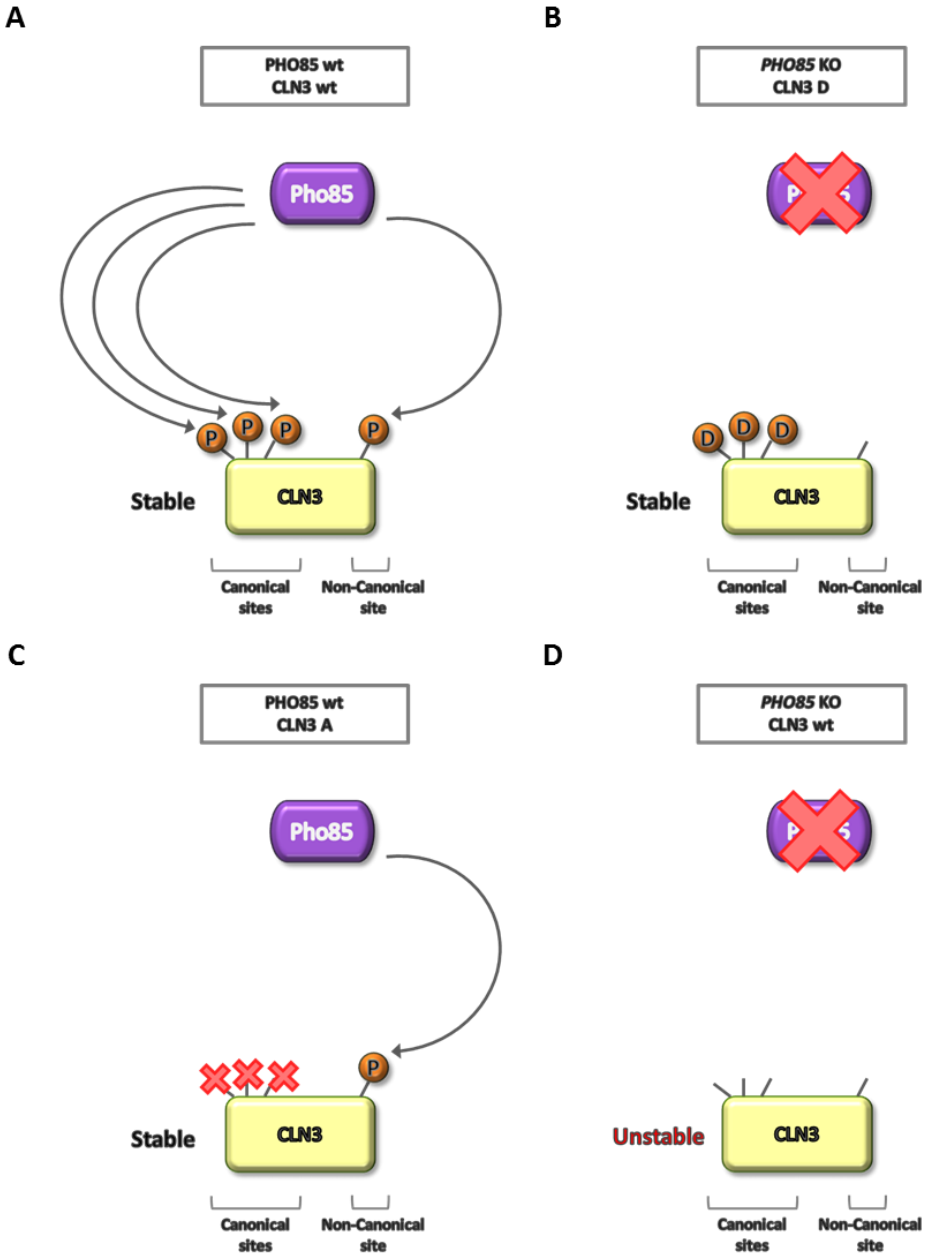

Figure D1. Hypothetical model of Cln3 stability in aspartic acid or alanine versions. Cln3 is stable when the 3 canonical sites are directly phosphorylated by Pho85 or when a non-canonical site is indirectly phosphorylated. A) Wild-type cells with a stable Cln3 directly phosphorylated at the 3 canonical sites by Pho85 and indirectly phosphorylated at a non-canonical site by an unknown protein that has been activated by Pho85. B) *pho85Δ* cells with a stable Cln3 due to aspartic acid mutations that permanently mimic phosphorylation at the canonical sites. C) Wild-type cells with nonphosphorylatable Cln3 that maintain cyclin stability through a different site phosphorylated by an unknown Pho85-activated protein. D) *pho85Δ* cells with unstable Cln3. Cln3 cannot be phosphorylated by Pho85 itself or by the other unknown protein, which cannot be activated by the CDK.

The scenario that is emerging from these results is that in the absence of phosphates, Pho85 becomes inactive and Cln3 is no longer phosphorylated, which in turn increases cyclin destabilization.

As previously discussed, Cln3 degradation depends on phosphorylation by its own CDK (Cdc28) in the PEST region; thus, mutants lacking this region (e.g. the *cln3-1* mutant) become hyperstable (Yaglom et al., 1995). Although the ubiquitin ligases involved have not been fully characterized, it is assumed that PEST phosphorylation enables degradation via ubiquitination (Willems et al., 1996). Bearing in mind the findings of the present thesis, Cln3 phosphorylation by Pho85 may somehow hinder the normal degradation of Cln3. This is suggested by the fact that the *cln3-1* protein is stable both in wild-type cells and in *pho85Δ* cells, and by the fact that the deletion of *UBC4* (an E2 ubiquitin conjugating enzyme associated with cellular response to nutrient deprivation) and *DMA1* and *DMA2* (two of its E3 ligases) also restore the Cln3 protein levels in *pho85Δ* cells (Fig. R20). Note that 2 of the 3 Pho85 phosphorylation sites (those mutated into aspartic acid) that control Cln3 stability frame the PEST region of the cyclin. This indicates that phosphorylation of Cln3 complicates recruitment by the ubiquitination system and consequently expands its lifetime. In the absence of phosphate, however, Cln3 would no longer be phosphorylated and would not be recognized by the ubiquitination machinery. Indeed, the idea that phosphorylation prevents interaction with E3 ligases has already been suggested in other multi-phosphorylated proteins (Escoté et al., 2004). The proposed model is represented below (Fig. D2).

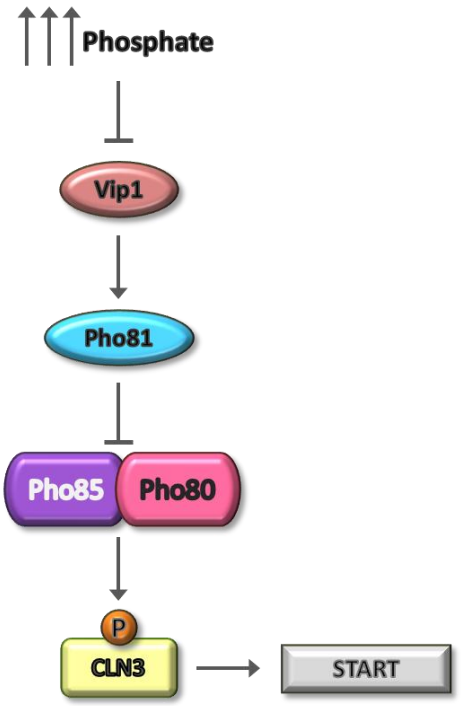

Figure D2. Phosphate regulation of Cln3 via Pho85. When phosphates are available, Vip1 is inhibited and Pho81 cannot inhibit the Pho85-Pho80 complex. This complex is then able to phosphorylate Cln3, leading to its stabilization and proper cell cycle progression.

4. THE PHYSIOLOGICAL SIGNIFICANCE OF Cln3 REGULATION

The underlying mechanisms that enable nutrient sensors to control cell cycle machinery remain unknown. Organisms may need to employ several convergent mechanisms to provoke complete G_1 arrest, making the identification of a single molecular target rather difficult. In this thesis, it is proposed that Cln3 stability modulation may be one of several principal targets in cell cycle regulation in terms of the adaptive response to phosphate depletion (proposed model in Fig. R23). Cln3 cyclin is a good candidate because it is the most upstream control point in the cell cycle and profoundly affects the cell's ability to move into the G_1 phase; Cln3 cyclin cellular content increases by either increased transcription or altered protein turnover (Nash et al., 1988; Yaglom et al., 1995). Thus, a simple model may be used to explain how in the G_1 arrest caused by phosphate deprivation , starved cells could not reach the Cln3 threshold level required to execute the START program due to the Cln3 half-life reduction. Moreover, it is demonstrated in this thesis that Cln3 is a nutrient-regulated target; this is consistent with several studies that demonstrate the regulation of this cyclin by different nutrient sources: 1) transcription is induced by glucose presence via the Azf1 transcription factor; 2) translation is regulated by glucose through the cAMP-PKA pathway, and by nitrogen and amino acid signaling indirectly via TORC1 according to protein synthesis rates, but directly through an unknown effector; 3) other proteins, such as Whi3 that modulates Cln3 translation by ER retention and is locally restricted by the Ydj1 chaperone and destabilized by Cdc28 under specific conditions or types of stress. Based on the results of the present thesis, phosphate source regulation of Cln3 may now be added to the list.

In the proposed model of this thesis, Cln3 destabilization may be crucial during G_1 arrest due to phosphate depletion. This downregulation could be important, for instance, in keeping Swi6-dependent transcription silenced in order to avoid the cell wall remodeling process during nutrient deprivation (Igual et al., 1996).

The Burhans group demonstrated that establishing and maintaining proper G_1 arrest is an important cellular response to nutrient deprivation (Weinberger et al., 2007); cells improperly halt at the S phase, suffer replication stress, and rapidly lose viability. This group also clearly demonstrated that the ectopic expression of *CLN3* increases the frequency with which nutrient-depleted cells arrest at the beginning of the S phase instead of the G_1 phase. The results shown in the present thesis confirm that the presence of a hyperstable Cln3 (*cln3-1*) also increases the number of S phase-arrested cells and decreases cell viability. Based on this finding, it is proposed that in the response to phosphate depletion Pho85 inactivation is critical for stopping the cycle at G_1, avoiding entry into S phase with low levels of nucleotides, and maintaining

chronological life span (CLS). Furthermore, evidence indicates that mutants with high Pho85 activity (*pho81Δ*, *vip1Δ,* and GAL-*PHO85*) incubated in phosphate-free medium maintain high levels of Cln3 (Fig. R13 and R14), have a high percentage of cells entering the cell cycle, and show decreased cell viability (Fig. R23). Under the same conditions of nutrient deprivation, cells carrying a *CLN3* allele with aspartic acid substitutions also die prematurely; these cellular defects may therefore be chiefly derived from sustained and unscheduled Cln3 phosphorylation (Fig. D3).

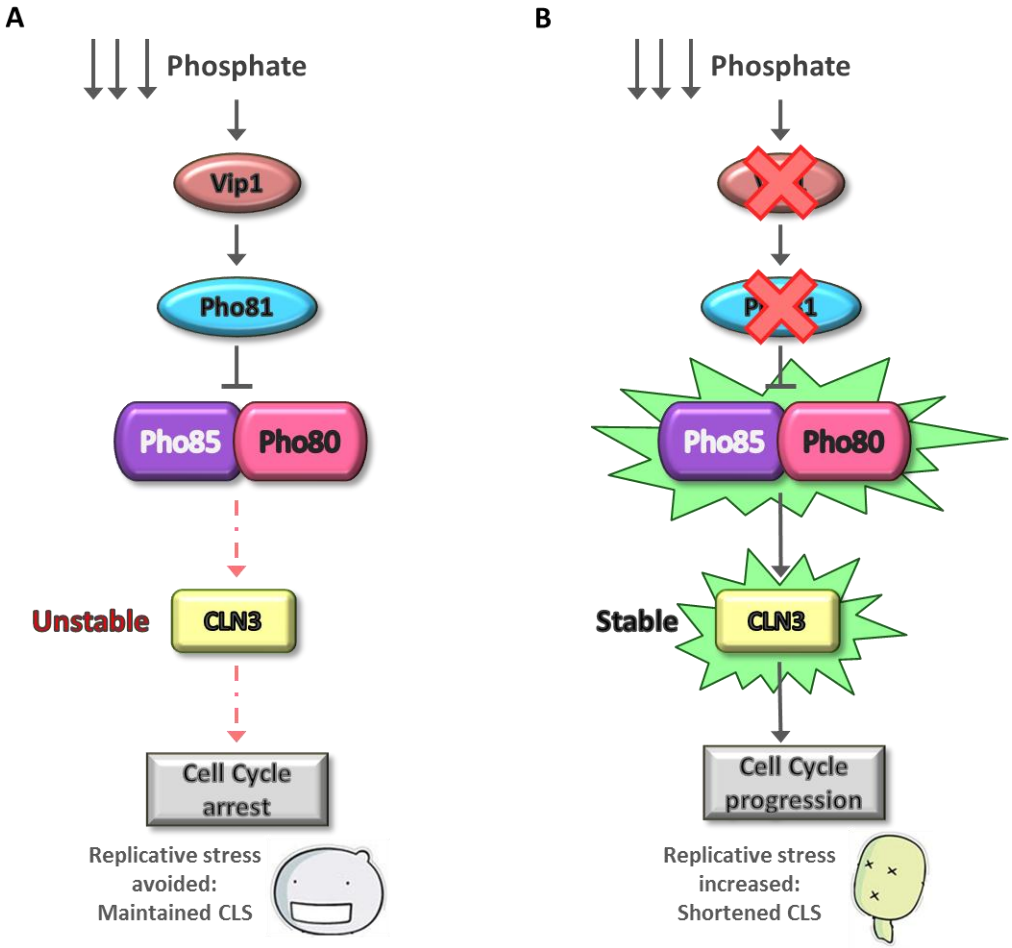

Figure D3. Phosphate-regulated Cln3 in proper cell cycle arrest and CLS. A) Phosphate depletion induces inactivation of Pho85, which is thus unable to stabilize Cln3. Cln3 absence leads to cell cycle arrest to avoid replicative stress, resulting in a long CLS. B) When nutrient signaling downregulation to Cln3 fails due to the absence of Vip1 or Pho81 inhibitors or, inversely, the overexpression of Pho85 under a Gal promoter or the hyperstabilization of Cln3 on *cln3-1* cells, proper cell cycle arrest does not take place and cells enter the S phase. At this point, stress increases and the CLS shortens due to the absence of nutrients needed to replicate DNA.

According to the proposed model (Fig. D3), Pho85 is important for restarting the cell cycle after refeeding in the presence of Pho85 via Cln3 (Figs. R9 and R12); either Cln3 absence or low Pho85 activity greatly hinders reentry into the cell cycle. The role of Pho85 in cell cycle reentry appears to be specific, reflected in the lack of significant differences between wild-type and *pho85Δ* cells released in alpha factor arrest (Fig. R24). These results are consistent with the hypothesis that Pho85 is essential when Cdc28 activity is lacking (Huang et al., 2007) and reinforce the idea that Pho85 activity is practically dispensable when yeast grows in nutrient-rich medium, whereas it is essential in other situations (e.g. exiting nutrient-induced G_0 arrest). Since wild yeasts need to thrive under diverse nutrient conditions (the availability of phosphate and other nutrients often varies widely), it is postulated in this thesis that Pho85 must be fundamental to controlling the constant cell cycle stalls and reentries that yeast cells are subjected to under natural conditions. Other well-known cases in which Pho85 is indispensable in the absence of Cdc28 include when DNA damage occurs in G1; Cln-Cdc28 is downregulated, leading to a delay in the cell cycle (Sidorova and Breeden, 1997) and Pho85 is required for a timely return to cell cycle progression following activation of the G_1 DNA damage checkpoint. Pho85 then antagonizes cell cycle arrest by phosphorylating Sic1 and promoting its degradation to reactivate Cdc28 (Wysocki et al., 2006).

Because Pho85 is essential only under specific conditions, this mechanism might not be important in rich medium, given that *cln3Δ* cells can still progress through the entire cell cycle without it, albeit with a longer G_1 phase. It has been shown that Cln1 and Cln2 have the potential to activate their own transcription in the absence of Cln3 because they can also phosphorylate Whi5 (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). However, this positive feedback activation is not thought to contribute greatly to the timing of *CLN1,2* induction, and cells significantly increase doubling time (Dirick et al., 1995; Stuart and Wittenberg, 1995; Bean et al., 2006). This scenario may explain why *pho85Δ* cells are viable as well.

Although the mechanism allowing *cln3Δ* and *pho85Δ* cells to progress through the cell cycle is not questioned in this model, an important point to examine is why these cells are able to detect phosphate absence and arrest the cell cycle even though the sensor (Pho85) and the effector (Cln3) are not present. This thesis clearly demonstrates that cell cycle arrest by phosphate depletion is mediated by Pho85 via Cln3, but the involvement of other signaling pathways is not ruled out. A pathway linking phosphate availability with downstream G_1 cyclins such as Cln1,2, or inhibitors such as Whi5 or Sic1, could be reinforcing Pho85-Cln3 regulation (Fig. D4).

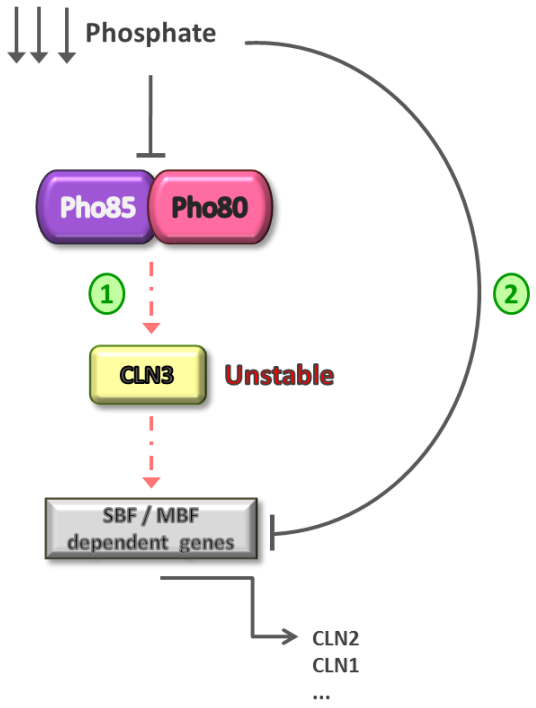

Figure D4. Phosphate regulation by Pho85 could be reinforced by other pathways. 1) Phosphate depletion leads to the inactivation of the Pho85-Pho80 complex, which cannot then stabilize Cln3, triggering a cell cycle arrest. 2) Phosphate absence activates an alternative pathway that directly targets cell cycle regulators downstream (e.g. Cln2 or Cln1).

Although the involvement of other pathways cannot be ruled out, the scenario that is emerging from these results is that in the absence of phosphates, Pho85 becomes inactive and Cln3 is no longer phosphorylated, which in turn elicits an increase in the rate of ubiquitination and destruction of the cyclin. This process would then promote proper cell cycle arrest and prevent cells from entering the S phase in an untimely way, preserving their viability.

As a final area of study, this thesis explores new Pho85 functions in cell cycle regulation, such as involvement in some other phases. The results from a restart after nocodazole arrest suggest that Pho85 CDK could be involved in M phase and G_1 phase regulation, and are consistent with the published results of Snyder and colleagues. These authors used proteome chip technology to locate many *in vitro* substrates recognized by most yeast protein kinases, among them Pho85 CDK. It has been shown that Pho85 phosphorylates *in vitro* the yeast securin Pds1 (Ptacek et al., 2005), which

inhibits anaphase by binding separin Esp1. Thus, Pho85 could phosphorylate Pds1, leading to the inhibition of securin in order to restart the cell cycle after mitotic arrest.

In conclusion, the present thesis identifies a new regulatory mechanism in which the availability of phosphate modulates the amount of Cln3 in yeast. Considering that cyclin D (the Cln3 homologue) is extremely conserved in mammals, the proposed mechanism may in fact be conserved across evolution; human cyclin D stability has been shown to be downregulated by serum deprivation through unknown mechanisms (Choi et al., 1997). Other cell cycle regulating functions of Pho85 (e.g. M phase control) have yet to be discovered, but it would seem that some other important cell activities require the multifunctional Pho85 CDK for proper cell cycle progression.

CONCLUSIONS

The following conclusions can be drawn from the results of the present thesis:

- Phosphate availability regulates cell cycle progression via Pho85.
- Pho85-Pho80 complex activity specifically regulates the levels of the Cln3 protein.
- Pho85 affects Cln3 in terms of stability through a direct phosphorylation.
- Cln3 proper regulation depending on nutrients availability maintains the cell viability.

RESUM EN CATALÀ

La senyalització de la presència de nutrients permet als llevats beneficiar-se de les condicions riques en nutrients, per una banda estimulant la proliferació cel·lular i per l'altra sobrevivint períodes de manca de nutrients, induint la entrada a la fase quiescent del cicle cel·lular anomenada G_0. Aquests canvis entre proliferació i quiescència han d'estar altament regulats per mecanismes que controlen el cicle cel·lular segons la disponibilitat de nutrients.

La glucosa i el nitrogen han estat descrits com elements crítics que modulen la progressió del cicle cel·lular; la seva absència porta a una aturada en la fase G_1. Tot i això, la majoria d'aquestes vies de senyalització de nutrients estan poc estudiades. Per exemple, el primer sensor que translocaria la senyal als efectors, el sensor de la glucosa que activa la via de Snf1, o el mecanisme que permet la transmissió de la presència de nitrogen al complex TORC1 encara romanen sense identificar. El coneixement sobre la detecció de nutrients i la seva participació en el control del cicle cel·lular està encara per resoldre.

1. El nivell de fosfat regula la progressió del cicle cel·lular via Pho85

Aquesta tesi examina si la disponibilitat de fosfat, l'últim nutrient que s'ha vist que participa en la regulació del cicle cel·lular (Gray et al., 2004; Klosinska et al., 2010), interacciona directament amb el control d'aquest procés i, un cop demostrat, descriu els mecanismes exactes pels quals el regeix.

Els resultats d'aquesta tesi mostren que l'absència de fosfat atura el cicle cel·lular (Fig. R7) de manera similar a com ho fa el nitrogen o la rapamicina (Figs. R1 i R3), i que la represa del cicle cel·lular un cop hi ha nutrients de nou, depèn de Pho85 CDK (Figs. R4 i R9).

En la situació de manca de fosfat, es pot assumir que aquest defecte és simplement una conseqüència no específica deguda a la reducció en el nivell d'un metabòlit particular (per exemple, l'ATP). Tanmateix, estudis metabolomics han demostrat que cèl·lules que creixen en condicions de manca de fosfats mantenen nivells energètics constants que provenen de la hidròlisi d'ATP (Boer et al., 2010), suggerint que a l'inici de la manca de fosfats les cèl·lules deuen tenir prou energia per iniciar una resposta adaptativa, com acabar una ronda de divisió fins la següent fase G_1. Per altra banda, aquesta tesi confirma que l'aturada observada és un procés controlat que es basa en les següents troballes:

1) Quan la font de fosfat s'acaba, les cèl·lules es troben majoritàriament sense gemma amb una dotació 1N de DNA. És important destacar que en els experiments de manca de fosfats, les cèl·lules no es varen aturar

immediatament, sinó que, de manera gradual es varen anar acumulant a G_1 i finalment, aproximadament 6 hores després de la manca del nutrient, varen parar de dividir-se (90% de les cèl·lules). Aquesta resposta gradual del cultiu és probablement deguda a les reserves de polifosfats que tenen les cèl·lules (Thomas i O'Shea, 2005), que serveixen per alleugerar els efectes dels canvis sobtats de l'ambient.

2) El volum de les cèl·lules aturades segueix augmentant, indicant que són metabòlicament actives i no es troben en un estat col·lapsat (Fig. R7).

3) Un arrest per manca de fosfats pot ésser revertit amb un increment d'activitat CDK-Cln2. Aquest resultat suggereix que el retard és una inactivació específica de la maquinaria transcripcional de la transició G_1/S, promovent una manca de l'expressió de *CLN1,2* i *CLB5,6*.

4) Sota les mateixes condicions, els marcadors bioquímics que foren observats també varen indicar una aturada a G_1 (Fig. R8). Es demostra clarament que els nivells proteics de Cln2 i Clb5 decreixen després de 4 hores de cultiu en un medi sense fosfats, contràriament a Sic1, que roman constant. Els nivells de mRNA d'aquestes proteïnes segueixen un patró similar, indicant que la manca de fosfat inhibiria la transcripció de gens requerits per la transició G_1/S.

Per tant, es demostra que el fosfat regula START mitjançant el control de la maquinaria transcripcional de G_1/S en *S. cerevisiae*, com fou suggerit per Hartwell (Johnston et al. 1997).

La regulació del cicle cel·lular basada en la disponibilitat de fosfat sembla estar regulada per la CDK Pho85. Tal i com mostra la figura R9B, Pho85 és necessària per una represa exitosa del cicle cel·lular després d'una privació de fosfats, ja que les cèl·lules *pho85Δ* surten més tard de l'aturada a G_1 comparat amb les cèl·lules salvatges. Pho85 podria unir aquests dos processos (la detecció de nutrients i la progressió del cicle cel·lular) perquè es sap que el fosfat exogen activa Pho85, i una de les seves múltiples funcions és regular la fase G_1 com a CDK.

2. L'activitat del complex Pho85-Pho80 regula específicament els nivells de Cln3

La idea central d'aquesta tesi és que la disponibilitat de fosfat modula els nivells de Cln3 mitjançant la regulació de l'activitat del complex Pho85-Pho80.

Se sap que Whi5 i Cln3 juguen un paper clau en l'activació dels complexos de SBF i MBF en la fase G_1 tardana. No obstant això, només els nivells de Cln3 cauen dramàticament en l'absència de fosfats, i en canvi, els nivells del repressor Whi5 romanen força constants (fig. R8). Encara que s'ha demostrat que Pho85 fosforila Whi5 per tal de promoure la seva sortida del nucli i provocar l'alliberament del promotor SBF (Fig. R5), no hi ha recuperació fenotípica de les cèl·lules pho85Δwhi5Δ en el reinici del cicle cel·lular després de la presència de nou de nitrogen (fig. R6). Per tant, Whi5 sembla tenir un paper important en la regulació del cicle cel·lular basat en la presència de nutrients, però juntament amb altres reguladors clau.

Aquests resultats recolzen la idea que tant la manca de fosfat, com l'esgotament de nitrogen, regulen l'activitat de SBF i MBF, a causa del decreixement dels nivells de Cln3. Se sap que un triple mutant *cln* no pot fer la transició G_1/S a menys que Sic1 s'elimini. Per tant, l'absència de ciclina CLN3 sembla conduir a un decreixement de les proteïnes CLN1,2, promovent la presència sostinguda de Sic1. Això podria explicar l'aturada a G_1 produïda per la privació de fosfat en termes de requisits moleculars.

La participació de Pho85 en la regulació de Cln3 durant l'absència de fosfat es confirma pels gairebé inexistents nivells de la ciclina en cèl·lules pho85Δ, a més dels nivells gradualment decreixents de Cln3 que es troben quan Pho85 es inactiu per NaPP1 (fig. R10). L'activitat de Pho85, en lloc de la presència física de la pròpia proteïna, sembla ser un factor clau en el control dels nivells de Cln3.

La regulació de Cln3 també sembla ser específica perquè Pho85 no afecta el control dels nivells d'altres ciclines com ara Cln2 (fig. R11). Aquest mecanisme molt específic indica que un determinat complex Pho85 hauria de participar, amb només una ciclina particular de la família Pho85, que s'uniria a la CDK per activar la regulació de Cln3. Els experiments demostren que únicament la supressió de *PHO80* causa una disminució Cln3 similar a la de la supressió de *PHO85*, i que reinicia el cicle cel·lular després de la presència de nou fosfat amb un retard igual en les cèl·lules pho80Δ i pho85Δ quan es compara amb cèl·lules de tipus salvatge (Figura R12). Aquests resultats suggereixen que l'efecte de Pho85 CDK sobre els nivells de la proteïna Cln3 està regulat per la ciclina Pho80, i són consistents amb la idea que els complexes Pho80-Pho85 són inhibits en absència de fosfats. Això demostra encara més que els fosfats modulen l'activitat de la quinasa Pho85, qui a la vegada regularia els nivells de Cln3.

No només a través de l'eliminació de PHO85 queden directament afectats els nivells de Cln3, sinó també a través de la sobreexpressió o sobreactivació de Pho85 mitjançant la via PHO. Això té un impacte important: el manteniment dels nivells de Cln3 malgrat l'absència de fosfat (Figures R13 i R14).

Demostrar que Cln3 es veu afectada per l'activitat Pho85/Pho80 podria ser rellevant per 2 raons. Per primera vegada, es demostra que la ruta Pho85-Pho80 està implicada en el control directe de la maquinària del cicle cel·lular, suggerint la coordinació entre l'homeòstasi de fosfat i el cicle cel·lular. Alhora, aquest és el primer cas que mostra com una CDK controla l'activitat d'una altra CDK. De fet, això sembla ser un cas d'interferència entre dues CDKs, a on Pho85 informés a Cdc28 de l'estat nutricional del llevat. En aquest sentit, el complex Pho85-Pho80 podria considerar-se una CAK, però una que afecta la ciclina de Cdc28.

3. Pho85 afecta Cln3 en termes d'estabilitat

L'absència de fosfat inactiva el complex Pho85-Pho80, causant una gran resposta transcripcional per adaptar-se a la nova situació. Un element crític en aquesta resposta és el factor de transcripció Pho4 que, quan la concentració de fosfat és alta, és fosforilat per Pho85-Pho80 i retingut al citoplasma (O'Neill et al., 1996). Actualment, no hi ha substrats de Pho85-Pho80 coneguts *in vivo* que no sigui Pho4 (o Rim15). Per tant, sembla raonable suposar que Pho85 també té un efecte sobre la transcripció de Cln3. No obstant això, els resultats mostrats en aquesta tesi demostren que els nivells de mRNA de *CLN3* semblen no estar afectats pels canvis en la concentració de fosfat (Figs. R15 i R16), i per tant, la regulació es produiria en un nivell post-transcripcional.

Diversos estudis indiquen que els nivells de Cln3 decauen per la repressió de la traducció durant la manca de nutrients (Gallego et al, 1997; Polymenis et al, 1997; Hall et al, 1998). Encara que no es pot descartar que l'escassetat de fosfat pot tenir el mateix efecte, els resultats d'aquesta tesi postulen que és l'estabilitat de Cln3 la que es veu afectada per la disponibilitat de fosfat. Això es va demostrar mitjançant cèl·lules pho85-AS, ja que els nivells de Cln3 en cèl·lules pho85Δ eren massa baixos per monitoritzar la vida mitjana de la ciclina (fig. R18).

Es va descriure un fenomen anàleg de desestabilització de Cln3 en cèl·lules privades de nitrogen, encara que el seu mecanisme es va deixar sense explicar (Gallego et al., 1997). Aldea i col·laboradors van demostrar que la vida mitjana de Cln3 també es redueix la meitat quan no hi ha disponibilitat de nitrogen. Per tant, la desestabilització de Cln3 (i la possible repressió de la seva traducció) podria ser una resposta general per la cèl·lula a la limitada disponibilitat de diferents nutrients. Aquesta resposta podria limitar l'activitat de complexes Cdc28-Cln3 necessaris per passar START i adaptar-se a les noves condicions.

Els resultats d'aquesta tesi mostren que l'activitat Pho85 afecta l'estabilitat de la ciclina Cln3 d'una manera directa. És important assenyalar que Cln3 és un substrat *in vitro* del complex Pho85-Pho80 (purificada a partir de llevats o cèl·lules d'*E. coli*) (fig.

R21). A causa de diversos problemes tècnics, no es pot confirmar que aquesta fosforilació es produeix *in vivo*, perquè Cln3 ja està molt fosforilada per Cdc28 (Yaglom et al, 1995.), i per tant la detecció experimental d'una fosforilació addicional per Pho85 és complicada. De fet, no s'ha pogut veure un canvi de mobilitat diferencial de Cln3 en cèl·lules pho85Δ en comparació amb cèl·lules de tipus salvatge malgrat l'ús d'una soca cdc28ts; els nivells de Cln3 en cèl·lules pho85Δ va romandre gairebé inexistent i no es pogué observar un patró clar de bandes (Fig. . R19).

Malgrat aquests resultats, s'ha demostrat que Pho85 i Cln3 interaccionen *in vivo* (Fig. R21) i, el més interessant, la substitució simultània de S449D i T520D va recuperar en gran mesura els nivells de Cln3 en les cèl·lules pho85Δ (Fig. R22), suggerint que la fosforilació d'aquests 2 llocs podria ser important per a l'estabilitat de Cln3. No obstant això, les substitucions d'alanina no va disminuir els nivells de Cln3 en cèl·lules de tipus salvatge com s'esperava (Figura R22). Els llocs de mutació van ser seleccionats perquè són els únics en Cln3 que tenen la seqüència canònica coneguda per ser fosforilada per Pho85, i se suposa que aquesta CDK no pot fosforilar cap altre lloc de la ciclina. No obstant això, estudis recents mostren que al voltant del 50% de les fosforilacions de Pho85 es troben en llocs no canònics (Mok et al., 2010). Per tant, un model hipotètic podria explicar el resultat contradictori de les mutacions a alanines: la fosforilació sostinguda (mutacions d'àcid aspàrtic) dels 3 llocs canònics podria ser suficient per mantenir l'estabilitat de Cln3, fins i tot en les cèl·lules pho85Δ, ja que imitaria una fosforilació directe de Pho85 per estabilitzar la ciclina. A més a més, podria haver-hi un lloc addicional (no canònic per Pho85 i per tant no detectable en l'estudi de la seqüència) en què Cln3 seria fosforilat per Pho85, resultant en l'estat estable de la ciclina. Aquesta hipòtesi es resumeix en la figura D1.

L'escenari que sorgeix d'aquests resultats és que, en absència de fosfats, Pho85 es torna inactiu i Cln3 ja no és fosforilat, augmentant la desestabilització de la ciclina. Com s'ha esmentat anteriorment, la degradació de Cln3 depèn de la fosforilació per la seva pròpia CDK (Cdc28) a la regió PEST, de manera que els mutants que no tenen aquesta regió (per exemple, els mutants *Cln3-1*) són hiperestables (Yaglom et al, 1995.). Encara que les ubiquitina lligases implicades no s'han caracteritzat completament, sembla que la fosforilació permet la degradació a través de la ubiquitinació (Willems et al., 1996). Tenint en compte els resultats d'aquesta tesi, la fosforilació de Cln3 per Pho85 podria dificultar d'alguna manera la degradació normal de Cln3. Això és suggerit pel fet que la proteïna *Cln3-1* és estable tant en cèl·lules de tipus salvatge com en cèl·lules *pho85Δ*, i pel fet que les supressions de *UBC4* (un enzim E2 ubiquitina conjugant associat amb la resposta cel·lular a la privació de nutrients) i *DMA1* i *DMA2* (dos de les seves E3 lligases) també varen restaurar els nivells de Cln3 en les cèl·lules *pho85Δ* (fig. R20). S'ha de tenir en compte que 2 dels 3 llocs de fosforilació per Pho85 es troben a la regió PEST de la ciclina Cln3. Això indica que la fosforilació de Cln3 complica la degradació pel sistema d'ubiquitinació i, en

conseqüència, amplia la seva vida mitjana. En absència de fosfat, però, Cln3 no seria fosforilada i seria reconeguda per la maquinària d'ubiquitinació. De fet, la idea que la fosforilació prevé la interacció amb lligases E3 ja s'ha suggerit en altres proteïnes amb múltiples fosforilacions (Escoté et al., 2004). El model proposat es representa a continuació (Fig. D2).

4. La importància fisiològica de la regulació de Cln3

Els mecanismes subjacents que permeten als sensors de nutrients controlar la maquinària del cicle cel·lular es desconeixen. És possible que els organismes hagin d'utilitzar diversos mecanismes convergents per provocar l'aturada completa en G_1, de manera que la identificació d'una diana molecular única és difícil. En aquesta tesi, es proposa que la modulació de l'estabilitat de Cln3 pot ser un dels diversos objectius principals en la regulació del cicle cel·lular en termes de la resposta adaptativa a la depleció de fosfat (model proposat a la figura. R23).

La ciclina és un bon candidat perquè és el primer punt de control en el cicle cel·lular i afecta profundament a la capacitat de la cèl·lula per passar a la fase G_1. Els nivells de Cln3 augmenten, ja sigui per l'augment de la transcripció o la alteració de la degradació proteínica (Nash et al, 1988; Yaglom et al, 1995) . Per tant, un model simple es pot utilitzar per explicar com en l'aturada a G_1 causada per la manca de fosfat, les cèl·lules no poden assolir els nivells de llindar de Cln3 necessaris per executar el programa START.

D'altra banda, en aquesta tesi es demostra que Cln3 és un diana regulada pels nutrients, la qual cosa és consistent amb diversos estudis que demostren que la regulació d'aquesta ciclina és afectada per diferents fonts de nutrients: 1) la seva transcripció és induïda per la presència de la glucosa a través del factor de transcripció Azf1, 2) la seva traducció està regulada per la glucosa a través de la via de AMPc-PKA, i pel nitrogen i pels aminoàcids indirectament a través d'TORC1 d'acord amb les taxes de síntesi de proteïnes, i també directament a través d'un efector desconegut; 3) altres proteïnes, com ara Whi3 que modula la traducció de Cln3 mitjançant la seva retenció al Reticle Endoplàsmic, localment alliberada per la xaperona Ydj1 i desestabilitzada per Cdc28 en condicions específiques o en situacions d'estrès. Basant-se en els resultats d'aquesta tesi, ara es pot afegir a la llista la regulació de l'estabilitat de Cln3 per la disponibilitat de fosfat.

En el model proposat a aquesta tesi, la desestabilització de Cln3 pot ser crucial durant l'aturada a G_1 a causa de la manca del fosfat. Aquesta regulació podria ser important, per exemple, per mantenir silenciada la transcripció de Swi6 per tal

d'evitar el procés de remodelació de la paret cel·lular durant la privació de nutrients (Igual et al., 1996).

El grup de Burhans va demostrar que l'establiment i el manteniment adequat de l'aturada a G_1 és una resposta important en condicions de manca de nutrients (Weinberger et al, 2007.); Les cèl·lules mal aturades que entren a fase S, pateixen estrès replicatiu, i perden ràpidament la seva viabilitat. Aquest grup també va demostrar clarament que l'expressió ectòpica de Cln3 augmenta la freqüència amb què, en situació de manca de nutrients, les cèl·lules s'aturen a fase S enlloc de a G_1. Els resultats mostrats en aquesta tesi confirmen que la presència d'un Cln3 hiperestable (*cln3-1*) també augmenta el nombre de cèl·lules S-aturades a fase S i disminueix la viabilitat cel·lular. Basant-se en aquesta troballa, es proposa que en la resposta a manca de fosfat, la inactivació de Pho85 és crítica per aturar el cicle a G_1, evitant l'entrada a fase S amb baixos nivells de nucleòtids, i mantenint la mitjana de vida cronològica. D'altra banda, es mostra que els mutants amb alta activitat Pho85 (*pho81Δ*, *vip1Δ*, i el *GAL-PHO85*) incubats en medi sense fosfat, mantenen uns nivells alts de Cln3 (Fig. R13 i R14), tenen un alt percentatge de les cèl·lules que entren en el cicle cel·lular, i mostren una disminució en la viabilitat cel·lular (Figura R23). Sota les mateixes condicions de manca de nutrients, les cèl·lules que porten un al·lel Cln3 amb substitucions d'àcid aspàrtic també moren prematurament; aquests defectes cel·lulars per tant, poden ser causats per la presència d'una sostinguda fosforilació de Cln3 que l'estabilitzaria (fig. D3).

D'acord amb el model proposat (Figura D3), Pho85 és important per reiniciar el cicle cel·lular després de la presència de nou de fosfats a través de Cln3 (Fig. R9 i R12), o bé l'absència de Cln3 o la baixa activitat de Pho85 dificulta en gran manera el reingrés en el cicle cel·lular. El paper de Pho85 en la reentrada del cicle cel·lular sembla ser específic, reflectit en l'absència de diferències significatives entre les cèl·lules de tipus salvatge i *pho85Δ* alliberades després d'una aturada d'α-factor (Fig. R24). Aquests resultats són consistents amb la hipòtesi que Pho85 és essencial quan manca l'activitat de Cdc28 (Huang et al., 2007) i reforça la idea que l'activitat de Pho85 és pràcticament prescindible quan el llevat creix en un medi ric en nutrients, però que és fonamental en altres situacions (per exemple, la sortida d'una aturada G_0 induïda per nutrients). Atès que els llevats salvatges necessiten sobreviure sota diverses condicions nutricionals (la disponibilitat de fosfat i altres nutrients sovint varia àmpliament), en aquesta tesi es postula que Pho85 ha de ser fonamental per al control de les parades i reentrades del cicle a les quals les cèl·lules estarien sotmeses en condicions naturals. Altres situacions ben conegudes en què Pho85 és indispensable en l'absència de Cdc28 inclouen per exemple el dany de DNA produït a G_1: en aquesta situació els complexes Cdc28-Clns decreixen, donant lloc a un retard en el cicle cel·lular (Sidorova i Breeden, 1997) i es requereix Pho85 per a una correcta reiniciació del cicle cel·lular. Així doncs,

Pho85 antagonitza l'aturada del cicle cel·lular mitjançant la fosforilació a Sic1 i la promoció de la seva degradació per reactivar Cdc28 (Wysocki et al., 2006).

Com que Pho85 és essencial només sota condicions específiques, aquest mecanisme podria no ser important en medi ric, atès que les cèl·lules *cln3Δ* encara poden progressar a través de tot el cicle cel·lular, encara que amb una fase G_1 més llarga. S'ha demostrat que Cln2 i Cln2 tenen el potencial per activar la seva pròpia transcripció en absència de Cln3 perquè també poden fosforilar Whi5 (Cross i Tinkelenberg, 1991; Dirick i Nasmyth, 1991). No obstant això, aquesta activació de retroalimentació positiva no es creu que contribueixi en gran mesura a la inducció de Cln1 i Cln2 en el moment oportú, i que les cèl·lules augmentin significativament el temps de duplicació (Dirick et al, 1995; Estuardo i Wittenberg, 1995; Bean et al, 2006). Aquest escenari pot explicar per què les cèl·lules *pho85Δ* també són viables.

Encara que el mecanisme que permet a les cèl·lules *pho85Δ* i *cln3Δ* progressar pel cicle cel·lular no es qüestiona en aquest model, un punt important a analitzar és per què aquestes cèl·lules són capaces de detectar l'absència de fosfat i aturar el cicle tot i que el sensor (Pho85) i el efector (Cln3) no estan presents. En aquesta tesi es demostra clarament que l'atura del cicle cel·lular per la manca de fosfat està regulada per Pho85 via Cln3, però no es descarta la participació d'altres vies de senyalització. Una via podria ser la que unís la disponibilitat de fosfat amb les ciclines de G_1 més tardanes, com ara Cln1,2, o inhibidors com Whi5 o Sic1, que podrien estar reforçant la regulació Pho85-Cln3 (Fig. D4).

Tot i que la participació d'aquestes altres vies no es pot descartar, l'escenari que està sorgint d'aquests resultats seria que, en absència de fosfats, Pho85 es torna inactiu i per tant Cln3 ja no pot ésser fosforilada, provocant-se un augment de la seva taxa d'ubiquitinació i destrucció. Aquest procés llavors promouria l'aturada del cicle cel·lular adequadament i evitaria que les cèl·lules entressin a fase S de manera prematura, preservant la seva viabilitat.

Com a última àrea d'estudi, aquesta tesi explora noves funcions de Pho85 en la regulació del cicle cel·lular, com ara la seva participació en algunes altres fases. Els resultats del reinici del cicle després de l'aturada amb nocodazole suggereixen que Pho85 podria estar involucrada en la regulació de la fase M i de la fase G_1, i són consistents amb els resultats publicats per Snyder i els seus companys. Aquests autors utilitzen la tecnologia de xip proteomic per localitzar molts substrats *in vitro* reconeguts per la majoria de les quinases de llevat, entre elles Pho85. S'ha demostrat que Pho85 fosforila *in vitro* la securin de llevat Pds1 (Ptacek et al., 2005), que inhibeix l' anafase unint-se a la separin Esp1. Per tant, Pho85 podria fosforilar Pds1, donant lloc a la inhibició de la securin per tal de reiniciar el cicle cel·lular després d'una aturada a mitosi.

En conclusió, aquesta tesi identifica un nou mecanisme de regulació en què la disponibilitat de fosfat modula la quantitat de la ciclina Cln3 en el llevat. Tenint en compte que la ciclina D (l'homòleg de Cln3) és conservada en els mamífers, el mecanisme proposat podria haver-se conservat en l'evolució. De fet, s'ha demostrat que l'estabilitat de la ciclina D humana disminueix per manca de nutrients a través de mecanismes desconeguts (Choi et al, 1997). Encara no s'han descobert altres funcions de Pho85 en el cicle (per exemple, el control de fase M), però sembla que algunes altres activitats cel·lulars importants requereixen la CDK multifuncional Pho85 per a la correcta progressió del cicle cel·lular.

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