Universitat Internacional de Catalunya

Control de la estabilidad de ciclinas de G1 por nutrientes

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Dipòsit Legal: B 18912-2014

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Memoria presentada para optar al título de Doctor por la

Universitat Internacional de Catalunya

Barcelona, 2014

Trabajo dirigido por el Dr. Josep Clotet Erra

Grupo de Ciclo Celular

Departamento de Ciencias Básicas

Programa de Doctorado en Investigación en Salud de la

Universitat Internacional de Catalunya

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INTRODUCCIÓN

CICLO CELULAR EUCARIOTA

El ciclo celular eucariota se compone de 4 fases: G1, S, G2 y M. En la fase G1, la célula debe asegurarse de disponer de suficientes nutrientes y todo el material para comprometerse a una nueva ronda de división celular. A este punto donde la célula toma la decisión, situado cerca del final de esta fase, se le llama START (1, 2), un punto de no retorno a partir del cual además las células deben haber alcanzado un tamaño mínimo.

Acabada la fase G1, las células entran en la fase S, en la cual se replica el DNA. G2 es la fase en la que la célula prepara la maquinaria necesaria para la fase M y comprueba que el material genético se haya duplicado correctamente. Finalmente, cuando la célula entra en M, se observa que el DNA se condensa durante la profase, se alinea con el huso mitótico en metafase, se separa en dos en anafase y se descondensa en telofase. Este proceso concluye con la separación celular (3).

Toda esta cascada de eventos está perfectamente regulada por diferentes CDKs (*Cyclin Dependent Kinases*) asociadas a sus correspondientes ciclinas, las cuales les dan especificidad para reconocer un sustrato y fosforilarlo. También encontramos unas proteínas llamadas CKIs (*Cyclin-dependent Kinase Inhibitor*) las cuales en algunos momentos ayudan a inhibir los complejos CDK-ciclina. Esto es de especial relevancia ya que cuando se necesita dar una respuesta rápida a un estímulo, no se tiene que degradar la ciclina sino que solo basta con inhibir el complejo rápidamente y así se proporciona flexibilidad a la célula para responder a cambios ambientales bruscos.

REGULACIÓN DEL CICLO CELULAR EN *S. cerevisiae*

En *Saccharomyces cerevisiae*, Cdc28 es la CDK que gobierna mayoritariamente todo el ciclo celular. A su paso por START, la célula activa la transcripción de las ciclinas de Cdc28 en G1 y S, Cln1, Cln2, Clb5 y Clb6, pero hasta este punto de G1, Cdc28 está unida a Cln3, con quién llega a START y decide si es viable otra división celular. Cln3 es una ciclina muy inestable y da la especificidad a Cdc28 para fosforilar a la proteína

represora Whi5 (4-6), controlar la proteína dual Stb1 y las histonas deacetilasas (7) facilitando así la aparición de las ciclinas Cln1 y Cln2 y la entrada en START.

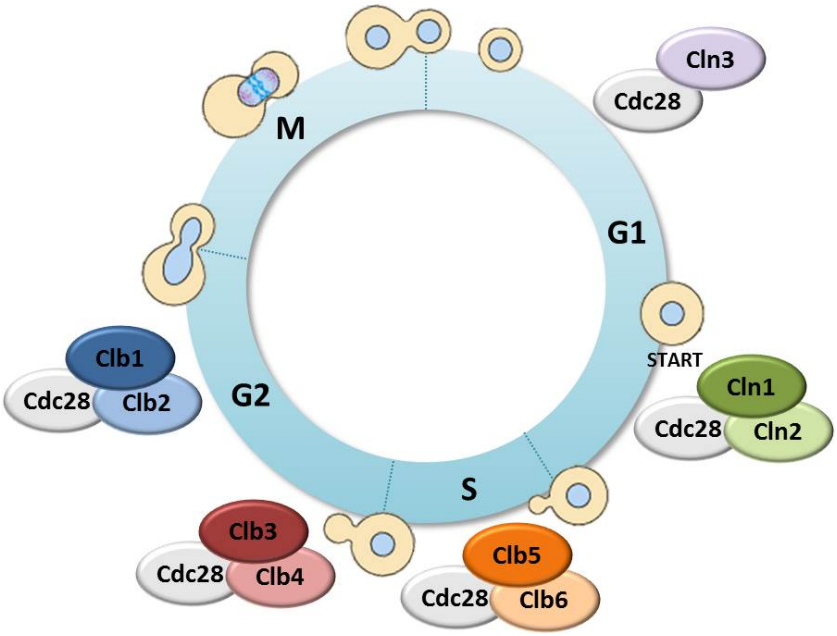

Figura 1. Ciclo celular de *Saccharomyces cerevisiae.* Se representa las 4 fases del ciclo celular con la evolución morfológica de la célula y la CDK esencial, Cdc28, con sus ciclinas asociadas a la regulación de cada fase.

REGULACIÓN DEL CICLO CELULAR POR NUTRIENTES

La célula toma la decisión de ciclar una vez más basada en muchos factores y uno de ellos es la disponibilidad de nutrientes en el medio que la envuelve. La falta de algunos de estos nutrientes, activa una cascada de señalización haciendo que la célula pare y no se comprometa en START. Esto se sabe que es cierto para glucosa, a través de PKA y Snf1 (8), y nitrógeno, mediante la vía de TORC1 (9), pero al inicio de este trabajo no se sabía de qué manera afectaba la falta de fosfato a la parada del ciclo.

Los niveles de fosfato también son controlados por otra vía de señalización, la vía de PHO, la cual tiene otra CDK como elemento principal: Pho85 (10). La ciclina con la que regula la homeostasis del fosfato es Pho80 y Pho81 es su correspondiente CKI, inhibidor de la CDK (11).

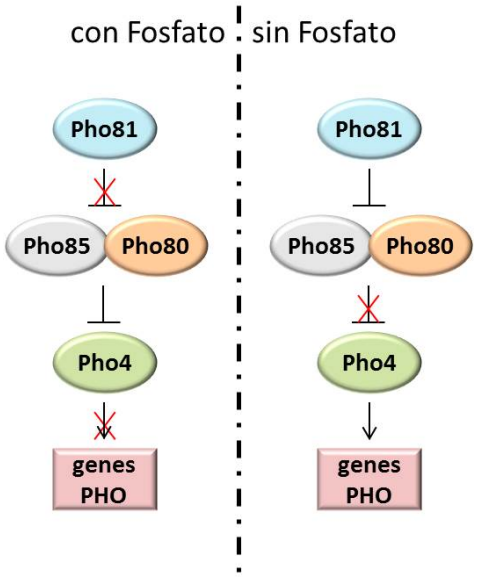

Figura 2. Vía de señalización de Pho85/Pho80 en respuesta a fosfato. Se representa la situación en presencia de fosfato donde el complejo Pho85/Pho80 impide la localización al núcleo de Pho4 mediante fosforilación. En una situación sin fosfato, Pho81 inhibe el complejo Pho85/Pho80 de manera que Pho4 se localiza en el núcleo activando la transcripción de genes de respuesta a la falta de fosfato.

Este complejo formado por Pho85-Pho80-Pho81 es activo cuando el medio es rico en nutrientes (Pho81 no inhibe al complejo Pho85-Pho80) pero cuando las condiciones ambientales varían y la célula se encuentra en un medio sin fosfato, se crean interacciones adicionales entre Pho81 y el complejo Pho85-Pho80 y éste queda inhibido (10, 12). Esto permite que Pho4, un factor de transcripción, deje de ser fosforilado, facilitando su entrada al núcleo para la iniciación de la transcripción de los genes PHO. Éstos derivan en la síntesis de diferentes proteínas para volver a incrementar los niveles de fosfato, cuando Pho81 vuelve a permitir la fosforilación de Pho4 por parte de Pho85-Pho80 (11, 13, 14).

En la regulación del ciclo ante la falta de nutrientes, en este caso el fosfato, Cln3 se mostraba como una buena diana para regular todas estas vías debido a su papel como activador *upstream*. De hecho, se ha demostrado que esta proteína se vuelve más inestable ante la falta de nitrógeno (15).

PHO85 Y SUS CICLINAS

Pho85 dispone de 10 ciclinas con las que se puede asociar para fosforilar diferentes sustratos: Clg1, Pcl1, Pcl2, Pcl9 y Pcl5 en la familia Pcl1/2; y Pcl6, Pcl7, Pcl8, Pcl10 y Pho80 en la familia Pho80 (16, 17). Pcl1 Pcl2 y Pcl9 tienen una expresión cíclica indicando que intervienen controlando el ciclo.

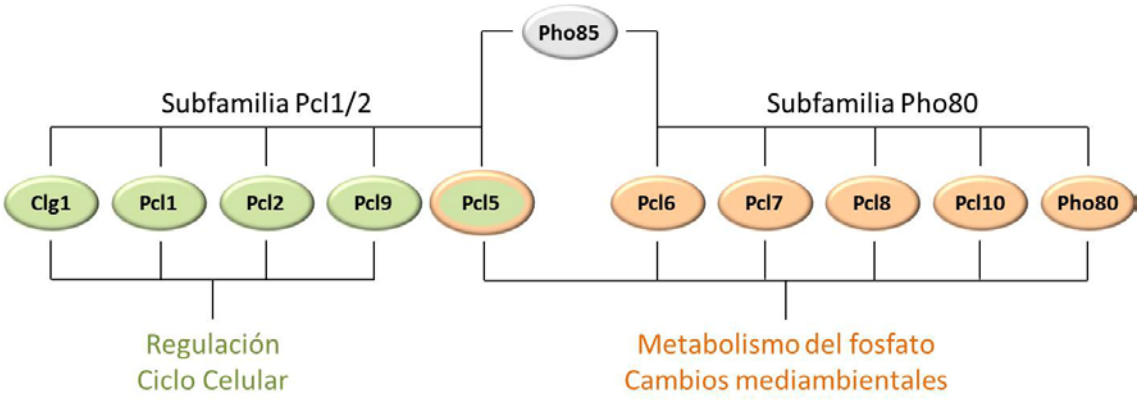

Figura 3. Ciclinas de Pho85. Se observan las 2 subfamilias de ciclinas de Pho85, la subfamilia Pcl1/2, donde hay una presencia mayor de ciclinas oscilatorias durante el ciclo, y la subfamilia Pho80, donde las ciclinas están mayoritariamente implicadas en la regulación del metabolismo, función que también realiza Pcl5 aun siendo de la primera subfamilia.

En G1 encontramos una doble regulación de Cdc28 y Pho85. Sus respectivas ciclinas, Cln1 y Cln2 para Cdc28 y Pcl1 y Pcl2 para Pho85 son transcritas en el mismo momento (START) por los mismos factores de transcripción (2). De hecho, estas ciclinas parecen tener un papel redundante en la regulación de G1-S dado que una cepa KO *cln1*, *cln2*, *pcl1* y *pcl2* no es viable (18). Curiosamente, la mayoría de sustratos de Pho85-Pcl en G1 también lo son de Cdc28-Cln: Sic1, Whi5, Rga2, Ash1, Swi5 y Clb6 (18-23). Al inicio de este trabajo de tesis, no había un significado claro para esta redundancia funcional: ¿Para qué la célula necesita 2 CDKs si regulan los mismos sustratos?

SISTEMAS DE DEGRADACIÓN DE CICLINAS

Los complejos de ubiquitin ligasas E3, el complejo promotor de anafase (APC) y el SCF (Skp1/Cdc53/F-box) controlan las transiciones del ciclo celular. Las ciclinas Clb son ubiquitinadas tras mitosis por el APC (21, 22) y SCF degrada a Cln1, Cln2 y Cln3 tras la

fase G1 (23, 24). Existe gran variedad de SCFs, cada uno de ellos con una F-box diferente; de hecho, el genoma de *Saccharomyces cerevisiae* contiene al menos 17 posibles proteínas F-box, sugiriendo que, en eucariotas, el sistema SCF podría controlar numerosos procesos de regulación. En condiciones estándares de cultivo (YPD a 30ºC), Cln2 es degradado por el complejo SCF-Grr1; el hecho de que Grr1 también controle la homeóstasis de los nutrientes (25) sugiere que podría ser el link entre la detección de los nutrientes y la degradación de Cln2 (26). Aunque se asuma que las Pcls se degradan por el mismo sistema de degradación, lo cierto es que el mecanismo de E3 ligasa involucrado en degradar a estas proteínas nunca se ha descrito.

La especificidad de las interacciones con las proteínas F-box podría depender en señales específicas de destrucción presentes en sus sustratos. De hecho, Cln2 presenta una secuencia descrita recientemente que permite la interacción con Grr1 (27). Además, esta interacción también depende de una modificación previa del sustrato. Por ejemplo, la degradación de ciclinas normalmente depende de la fosforilación por su propia CDK en regiones específicas con un alto contenido en Pro, Glu, Ser y Thr, también llamadas regiones PEST. Además, un Cln2 mutado en los siete posibles sitios de fosforilación por parte de Cdc28 se estabiliza y las células dejan de ser sensibles a la falta de nutrientes y las señales inhibidoras del crecimiento (28). Por lo tanto, es aceptado que la inestabilidad de las ciclinas Cln deriva de la dependencia de la fosforilación por Cdc28, lo que permite la interacción con el complejo SCF-Grr1 en el dominio específico de Cln2 (27, 29, 30). En mamíferos se ha propuesto un modelo similar basado en los descubrimientos hechos en G1 con las ciclinas E y D1 (31-33). Este modelo podría ser también aplicado a otro grupo de ciclinas de G1: las Pcls. Sin embargo, Pcl1 no tiene una clara región PEST, sugiriendo que otra E3 ligasa debería estar involucrada en la degradación de Pcl1 y no los típicos mecanismos SCF.

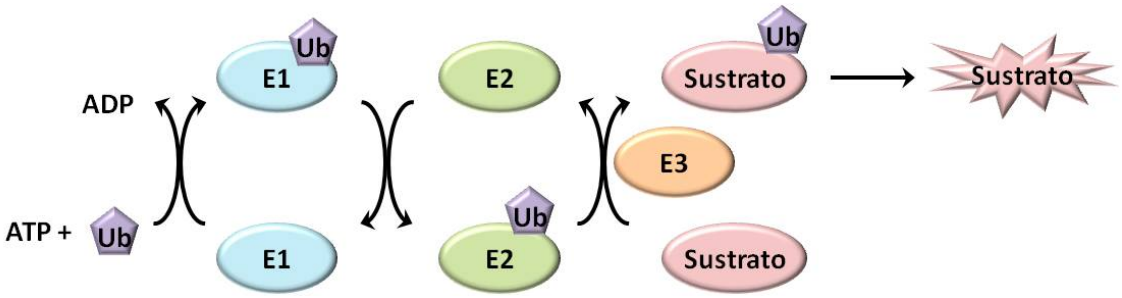

Figura 4. Mecanismo de la vía de ubiquitinación. Para que un sustrato finalice ubiquitinado, es necesaria la presencia de diferentes componentes. La E1, o ubiquitin activadora, es la que capta la ubiquitina libre para comenzar toda la cascada. La E2, o ubiquitin conjugadora, se une a la ubiquitina captada por la E1 y la conjuga de manera que pueda unirse al sustrato. Finalmente, la E3, o ubiquitin ligasa, es la que, en el caso de ser una RING, hace de conexión para que la E2 una directamente la ubiquitina al sustrato, o de ser una HECT, capta la ubiquitina de la E2 y se lo transfiere ella misma al sustrato. Finalmente, éste último es degradado en el proteosoma. Otro tipo de complejos E3 serían los anteriormente mencionados SCF y APC.

Las levaduras contienen un gran número de E3 ligasas, algunas de ellas involucradas en el control del ciclo celular. En estas se incluye a Dma1 y Dma2, que pertenecen a una pequeña clase de proteínas que contienen un dominio FHA (*Fork-Head-Associated*) y un dominio RING (34). Solo hay dos proteínas con estos dominios en humanos: Chfr (*CHeckpoint protein with FHA and RING domains*) (35) y Rnf8 (36).

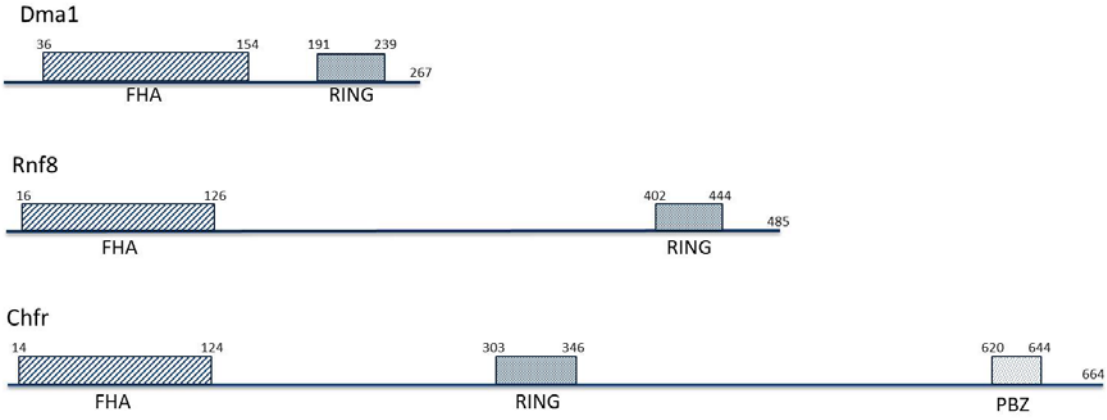

Figura 5. Esquema de E3 ligasas FHA y RING. Dma1 es una proteína de *S. cerevisiae* mientras que Rnf8 y Chfr son proteínas de la misma familia conservadas en mamíferos. Observamos que las 3 proteínas contienen los mismos dominios a pesar de la diferencia evidente de tamaño.

Chfr es una proteína oncosupresora implicada en el *checkpoint* de antefase (35, 37). Durante la fase G2, Dma1 y Dma2 ayudan a controlar el *checkpoint* de posición del huso mitótico (38) y la degradación de Swe1 (39), mientras que en G1, los dos genes controlan la progresión en respuesta a nutrientes mediante un mecanismo desconocido (40). Además, se ha demostrado que Dma1 y Dma2 tienen actividad ubiquitin ligasa *in vitro* (40) aunque al inicio de esta tesis, sus sustratos fisiológicos eran desconocidos.

AUTOFAGIA EN RESPUESTA A NUTRIENTES

Finalmente, otro posible mecanismo de regulación es la autofagia. Este sistema de degradación de proteínas se ha visto implicado también por los niveles de nutrientes mediante PKA y Snf1 para la glucosa, TORC1 para el nitrógeno y Pho85 para el fosfato. Curiosamente, Pho85 regula la autofagia activándola en ausencia de fosfato, pero también la regula de forma cíclica conjuntamente con Cdc28 durante G1 activándola mediante la degradación del represor Sic1. En este caso, la autofagia se postulaba también como un enlace para la degradación de proteínas durante el ciclo y los niveles de nutrientes disponibles durante éste y como posible vía de regulación de Cln3 mediante Pho85 (41).

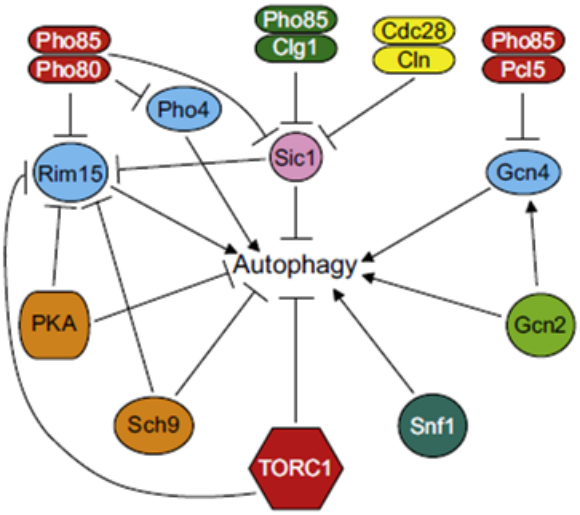

Figura 6. Autofagia en *Saccharomyces cerevisiae.* Se muestran las diferentes vías de activación o inhibición de la autofagia. Pho85 tiene una doble regulación negativa mediante la inhibición de Rim15 y Gcn4 y también positiva mediante la inhibición de Sic1 a través de Pho4 y Clg1. Extraído de Yang Z. *et al*, 2010.

OBJETIVOS

1. Estudiar la autofagia como posible mecanismo de degradación de Cln3

   Artículo 1: *Phosphate-activated cyclin-dependent kinase stabilizes G1 cyclin to trigger cell cycle entry*

2. Encontrar el mecanismo de degradación de la ciclina Pcl1

3. Identificar la región específica de degradación para las ciclinas sin PEST

4. Estudiar el mecanismo de regulación de Pcl1 mediante su CDK Pho85

5. Buscar la relevancia fisiológica para la existencia de 2 sistemas de degradación independientes en G1

   Artículo 2: *Dma1 ubiquitin ligase controls G1 cyclin degradation*

RESULTADOS Y DISCUSIÓN

Phosphate-activated cyclin-dependent kinase stabilizes G1 cyclin to trigger cell cycle entry

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April 2013

Molecular and Cellular Biology

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Mol. Cell. Biol. **2013, 33(7):1273. DOI: 10.1128/MCB.01556-12.**
Published Ahead of Print 22 January 2013.

MCB
Journals.ASM.org

Phosphate-Activated Cyclin-Dependent Kinase Stabilizes G_1 Cyclin To Trigger Cell Cycle Entry

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G_1 cyclins, in association with a cyclin-dependent kinase (CDK), are universal activators of the transcriptional G_1-S machinery during entry into the cell cycle. Regulation of cyclin degradation is crucial for coordinating progression through the cell cycle, but the mechanisms that modulate cyclin stability to control cell cycle entry are still unknown. Here, we show that a lack of phosphate downregulates Cln3 cyclin and leads to G_1 arrest in *Saccharomyces cerevisiae*. The stability of Cln3 protein is diminished in strains with low activity of Pho85, a phosphate-sensing CDK. Cln3 is an *in vitro* substrate of Pho85, and both proteins interact *in vivo*. More interestingly, cells that carry a *CLN3* allele encoding aspartic acid substitutions at the sites of Pho85 phosphorylation maintain high levels of Cln3 independently of Pho85 activity. Moreover, these cells do not properly arrest in G_1 in the absence of phosphate and they die prematurely. Finally, the activity of Pho85 is essential for accumulating Cln3 and for reentering the cell cycle after phosphate refeeding. Taken together, our data indicate that Cln3 is a molecular target of the Pho85 kinase that is required to modulate cell cycle entry in response to environmental changes in nutrient availability.

When environmental conditions change, *Saccharomyces cerevisiae*, like other organisms, takes several coordinated decisions about growth, cell division, and maintaining homeostasis. Nutrient status is among the most important environmental conditions that must be accurately sensed and responded to in order to ensure cell survival. In the absence of nutrients, cells arrest in G_1 phase, and this regulation of cell cycle becomes essential in the adaptation process. However, nothing is known about how nutrients impinge on the cell cycle machinery (1).

In budding yeast, the commitment to a new round of cell division takes place in late G_1, at a point called Start, where transcriptional activation of more than 200 G_1-specific genes occurs (reviewed in reference 2). This includes the transcription of major cell cycle regulators, including the G_1 cyclins Cln1, Cln2, Clb5, and Clb6, as well as numerous genes with functions related to DNA metabolism, budding, spindle pole body duplication, and cell wall synthesis (3, 4). Many of these transcribed genes are targets of the heterodimeric transcription factors SBF and MBF, each of which contains a Swi6 subunit and a distinct DNA-binding subunit: Swi4 and Mbp1, respectively (5). It is interesting to note that, when SBF and MBF are poised at their target promoters during much of G_1 phase, they cannot activate transcription; rather, they repress it (6, 7). To become activators at Start, they need an upstream element: the Cdc28/Cln3 complex.

Cdc28 is a cyclin-dependent kinase (CDK) that governs all of the cell cycle progression, and Cln3 is a highly unstable cyclin that remains fairly constant throughout the cell cycle (8, 9). When Cdc28 is associated with Cln3 cyclin, it unfolds the activation of transcriptional G_1 machinery, chiefly through two mechanisms: phosphorylation of the Whi5 repressor (and presumably of Swi6) at multiple residues (10–12) and control of the Stb1 dual protein and the histone deacetylases (13). In addition to Cln3, other elements involved in G_1 transcription firing have recently been demonstrated: for example, a positive-feedback mechanism involving Cln1 and Cln2 could reinforce SBF/MBF activation (14). At the end of G_1, the rise in Cln/Cdc28 activity results in the phosphorylation and targeting for degradation of Sic1, an inhibitor of the S-phase cyclins, thereby enabling robust entry into S-phase (15).

Nutrients are important trophic factors that control the passage through Start by activating several signaling pathways, including the protein kinase A and Snf1 pathways, which positively regulate cell proliferation in response to glucose availability (16), and the TORC1 pathway, which controls the cell cycle according to nitrogen levels (17). Consequently, inactivation of any of three major pathways results in a cell cycle blockade and, in other typical phenotypes, of the G_0-like growth arrest program, even in the presence of other abundant nutrients. Due to its role as the most upstream activator, Cln3 has emerged as a good target for these pathways. Indeed, it has been demonstrated that this cyclin becomes less stable under nitrogen deprivation, although the mechanism(s) underlying this effect remains obscure (18).

Phosphate is sensed by another signaling pathway, the PHO pathway, which has progressively been uncovered by the O'Shea group (19). The central element of this pathway is another CDK: Pho85, which associates with a family of 10 cyclins, each of which can direct Pho85 to different substrates (for a review, see reference 20). Pho80 is the main cyclin of the PHO pathway, which controls phosphate homeostasis, and Pho81 is the corresponding CDK inhibitor (21). The phosphate sensor that controls this pathway is still unknown: however, a low-P_i signal is known to be transmitted via certain inositol polyphosphate (IP) species (e.g., heptakisphosphate [IP_7]), which are synthesized by Vip1 IP_6 kinase. IP_7 appar-

Received 17 November 2012 Returned for modification 30 December 2012
Accepted 11 January 2013
Published ahead of print 22 January 2013
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doi:10.1128/MCB.01556-12

ently interacts noncovalently with the Pho80/Pho85/Pho81 complex and induces additional interactions between Pho81 and Pho80/Pho85, preventing substrates from accessing the active site of the kinase (19, 22). Thus, the ternary complex Pho80/Pho81/Pho85 is active in rich medium but becomes inactive upon phosphate starvation, leading to the migration of unphosphorylated Pho4 transcription factor into the nucleus and enabling the expression or repression of *PHO* genes (21, 23, 24). This transcriptional response ultimately results in maintenance of the internal phosphate levels.

Besides being constitutively associated with Pho80, Pho85 is also bound to other cyclins (e.g., Pcl1, Pcl2, and Pcl9) (20). In fact, the four Pho85 complexes should be considered different enzymes: they recognize different substrates, they are localized in different subcellular regions, and the respective activities of the Pcl1/Pho85, Pcl2/Pho85, and Pcl9/Pho85 complexes are not regulated by external phosphate. When Pho85 is associated with these Pcl cyclins, it cooperates with Cdc28 in specific morphogenetic events during the G_1-S transition.

It is logical to wonder whether Pho80/Pho85 complexes could also help control the cell cycle. Indeed, there is evidence that Pho80/Pho85 phosphorylates and inhibits Rim15, a PAS kinase that promotes the entry into the G_0 program in stationary cells (25), although whether this mechanism is involved in cell cycle-induced arrest is unknown. In addition, it has been demonstrated that Pho80/Pho85 is essential to restart the cell cycle after G_1 arrest due to DNA damage (26), suggesting that the Pho85 activity is essential when Cdc28 activity is absent (20).

Here, we demonstrate that the lack of phosphate leads to downregulation of Cln3 protein levels and to G_1 arrest. Interestingly, neither effect is observed in cells that overexpress Pho85 or in cells that cannot inhibit it (i.e., *vip1*Δ or *pho81*Δ cells). In accordance, we also demonstrate that Cln3 is less stable in strains with low Pho85/Pho80 activity and that it is phosphorylated *in vitro* by Pho85/Pho80 complexes at S449 and T520. More interestingly, we report that cells carrying a *CLN3* allele encoding aspartic acid substitutions at these sites maintain high levels of Cln3 independently of Pho85 activity. Accordingly, the nonphosphorylatable alanine mutant displays the same low levels as the *pho85*Δ mutant. Finally, we show that when nutrient levels drop, downregulation of Cln3 is essential to establish proper G_1 arrest and that once these levels recuperate, activation of Pho85 is essential to restart the cell cycle from the G_0 state. Together, our findings indicate that phosphate levels regulate the amount of Cln3 by controlling Pho85/Pho80 kinase activity.

MATERIALS AND METHODS

Strains. The strains used are indicated in Table 1. The *pho85-as* strain (YAM67, which carries an analog-sensitive allele of *PHO85*) was provided by Erin O'Shea and was used for inhibition of Pho85 in the presence of the inhibitor 1-Na PP1; (4-amino-1-tert-butyl-3-(1′-naphthylpyrazolo[3,4-D]pyrimidine) kindly provided by K. Shokat) as described previously (27). The Pho85 inhibition was confirmed by following the entry of Pho4-green fluorescent protein (GFP) into the nucleus.

Plasmids. The plasmids used in this work are listed in Table 2. *CLN3-MYC* was expressed from its own promoter in the centromeric plasmid pRS416; this plasmid expresses *CLN3* at a level similar to the expression of the genomic tagged version. To express *MYC*-tagged mutated versions of *CLN3*, we replaced S449 and T520 with alanine or used the substitutions I448D S449D and T519 T520D to mimic the double-negative charge of the phosphate group. *PHO85* and Pho80 were overexpressed under the control of the *GAL1* promoter in the pEG(KG) plasmid. YAM91 carries a plasmid with *CLN3* hemagglutinin (HA)-tagged protein lacking the PEST sequence of *CLN3* (the *cln3-1*-HA allele). The *CLN2* open reading frame (ORF) was placed under the control of the *Schizosaccharomyces pombe adh1* promoter in YCplac22 (28), resulting in plasmid pCM64. Recombinant proteins were expressed from the pGEX6P1 plasmid. Due to its toxicity in *Escherichia coli* cells, full-length Cln3 could not be expressed, and the C-terminal half of Cln3 (from Met 347 to Arg 580) was cloned into the BamHI site of pGEX6P1 (pJC1154).

Growth conditions. Cells were grown in either YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or complete synthetic dextrose (SD) medium (0.67% yeast nitrogen base and 2% glucose) containing amino acids for auxotrophic requirements (15 μg/ml leucine, 5 μg/ml histidine, and 10 μg/ml tryptophan).

Yeast nitrogen base without phosphate was used as recommended by the manufacturer (MP Biomedicals) to prepare SD medium without a phosphate source. Phosphate deprivation experiments were done with cells growing exponentially in SD for 14 to 16 h until they reached an optical density at 600 nm (OD_{600}) 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 prewarmed medium lacking the phosphate source, as previously described (18). The nitrogen deprivation experiment was performed under the same conditions using a yeast nitrogen base without ammonium sulfate (Difco). α-Factor cell synchrony experiments were done as previously described (29).

DNA content, cell volume, cell number, and budding index measurements. Approximately 1×10^7 cells were collected and processed as described previously (30). DNA was stained with SYBR green and then analyzed in a FACSCalibur cytometer (Becton Dickinson). Cell number and volume were quantified using a Scepter cell counter (Millipore). Budding was analyzed microscopically by scoring a minimum of 200 cells.

Immunoblot analysis. The primary anti-Myc or anti-HA monoclonal antibodies (kindly provided by F. Posas) were used at 1:100 and followed by anti-mouse-horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:20,000), using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). Anti-glucose-6-phosphate dehydrogenase (anti-G6PDH; Sigma) was used at 1:500.

Coimmunoprecipitation of PHO85-TAP and CLN3-MYC. Exponential-phase yeast cells were harvested (500 ml at an OD_{600} of 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, phenylmethylsulfonyl fluoride [PMSF], and benzamidine) and phosphatase inhibitors (10 mM sodium orthovanadate and 250 mM β-glycerophosphate). Cells were ruptured by vortexing with glass beads, and the resulting extract was centrifuged at 4°C for 1 h at 12,000 rpm. Amounts of 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.

Recombinant protein purification. For expression of glutathione *S*-transferase (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 β-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 (PBS-T) supplemented with a protease inhibitor mixture (Roche Applied Science), and subjected to mechanical rupture. 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 (1,000 rpm for 1 min at 4°C) and washed with PBS-T three times. The elution was performed by adding 10 mM glutathione.

Kinase assays. Kinase assays were performed essentially as described previously (31). GST-Pho85 and GST-Pho80 were purified from bacteria as described above. A fragment of GST-Cln3 was expressed (plasmid

TABLE 1 Yeast strains used in this study

Strain	Background	Genotype	Source
BY4741	BY4741	*MAT***a** *his3Δ1 leu2Δ200 met15Δ0 ura3Δ0*	Euroscarf
YNR55	BY4741	*CLN3-MYC-KanMX*	This study
YAM78	BY4741	*pho85Δ::LEU2*	This study
YAM78	BY4741	*pho85Δ::LEU2 CLN3-MYC-KanMX*	This study
YAM113	BY4741	pRS416-*CLN3-MYC*	This study
YAM114	BY4741	*pho85Δ::LEU2* pRS416-*CLN3-MYC*	This study
YAM115	BY4741	*pcl1Δ::LEU2* pRS416-*CLN3-MYC*	This study
YAM119	BY4741	*pcl2Δ::KanMX pcl1Δ::LEU2* pRS416-*CLN3-MYC*	This study
YAM116	BY4741	*pcl6Δ::KanMX* pRS416-*CLN3-MYC*	This study
YAM117	BY4741	*pcl7Δ::KanMX* pRS416-*CLN3-MYC*	This study
YAM118	BY4741	*pcl8Δ::KanMX* pRS416-*CLN3-MYC*	This study
YAM99	BY4741	*pcl9Δ::KanMX* pRS416-*CLN3-MYC*	This study
YAM111	BY4741	*pho80Δ::LEU2* pRS416-*CLN3-MYC*	This study
YPC702	BY4741	*SWI6-TAP-KanMX*	This study
YNR11	BY4741	*WHI5-TAP-KanMX*	This study
YAM150	BY4741	*vip1Δ::KanMX* pRS416-*CLN3-MYC*	This study
YNR82	BY4741	*pho81Δ::LEU2 CLN3-MYC-KanMX*	This study
YAM63	BY4741	*CLN3-MYC-KanMX* pEG(KT)-*GAL-PHO85*	This study
YAM91	BY4741	pRS416-*cln3-1-HA*	This study
YAM92	BY4741	*pho85Δ::LEU2* pRS416-*CLN3-1-HA*	This study
YAM151	BY4741	*ubc4Δ::LEU2 CLN3-MYC-KanMX*	This study
YAM142	BY4741	*pho85Δ::URA ubc4Δ::LEU2 CLN3-MYC-KanMX*	This study
YAM146	BY4741	*PHO85-TAP-KanMX* pRS416-*CLN3-MYC*	This study
YNR13	BY4741	*PHO85-TAP-KanMX*	This study
YAM113	BY4741	pRS416-*CLN3-MYC*	This study
YAM163	BY4741	pRS416-*CLN3-*(*T520A S449A-MYC*)	This study
YAM149	BY4741	*pho85Δ::LEU2* pRS416-*CLN3-*(*T519D T520D I448D S449D-MYC*)	This study
YAM103	BY4741	*pho80Δ::LEU2*	This study
YAM45	BY4741	*pho81Δ::LEU2*	This study
YNR46	BY4741	*vip1Δ::KanMX*	This study
YPC631	BY4741	*cln3Δ::KanMX*	This study
YSH10	BY4741	*atg1Δ::LEU2 CLN3-MYC-KanMX*	This study
YSH11	BY4741	*pho85Δ::URA atg1::LEU2 CLN3-MYC-KanMX*	This study
YAM67	K699	*pho85Δ::LEU2 ADE2 PHO4-GFP trp1::PHO85*(*F82G*)*-TRP1*	E. O'Shea
YAN32	W303	*CLN2-HA-URA3 SIC1-MYC-KanMX CLB5-TAP-LEU2*	F. Posas

pJC1154) and purified from bacteria. Cdc28-TAP was purified using IgG Sepharose beads (Sigma). Phosphorylated proteins were detected by using the Pro-Q diamond phosphoprotein gel stain kit (Invitrogen).

Viability assays. The viability experiments were done as described in reference 32. Stationary-phase cells were diluted, plated in YPD, and incubated for 2 days.

RNA isolation and analysis. Cells were harvested and stored at −80°C. Total RNA was isolated by hot phenol extraction and quantified spectrophotometrically. An amount of 2 μg of total RNA was incubated with DNase and reverse transcribed using Quanta qScript cDNA supermix following the manufacturer's instructions. The cDNA was subjected to reverse transcription (RT)-PCR on a C1000 thermal cycler-CFX96 real-time PCR system, and expression was normalized to that of *CDC28*.

RESULTS

The G_1 arrest caused by phosphate depletion may involve active mechanisms to downregulate Cdc28-Cln activity. Nutrients are

TABLE 2 Plasmids used in this study

Name	Relevant characteristics	Reference or source
pCM64	YCplac22-*ADHp-CLN2*	14
pJC1065	pEG(KG)-*PHO85* (cloned in BamHI)	This study
pJC1437	pEG(KG)-*PHO80* (cloned in BamHI)	This study
pJC1280	pRS416-*CLN3-MYC*	This study
pJC1161	pGEX6P1-*PHO80* (cloned in BamHI)	This study
pJC1164	pGEX6P1-*PHO85* (cloned in BamHI)	This study
pJC1154	pGEX6P1-*CLN3* (from nucleotide 1038 to stop codon)	This study
pJC1448	pGEX6P1-*CLN3* (from nucleotide 1038 to stop codon) with the 2 putative Pho85 sites (S449 and T520) mutated to Asp	This study
pJC1456	pGEX6P1-*CLN3* (from nucleotide 1038 to stop codon) with the 2 putative Pho85 sites (S449 and T520) mutated to Ala	This study
pJC1449	pGEX6P1-*CLN3* (from nucleotide 1159 to stop codon) with the 8 putative Cdc28 sites (T420, S455, S462, S466, S466, T478, S514, and T517) mutated to Ala	This study

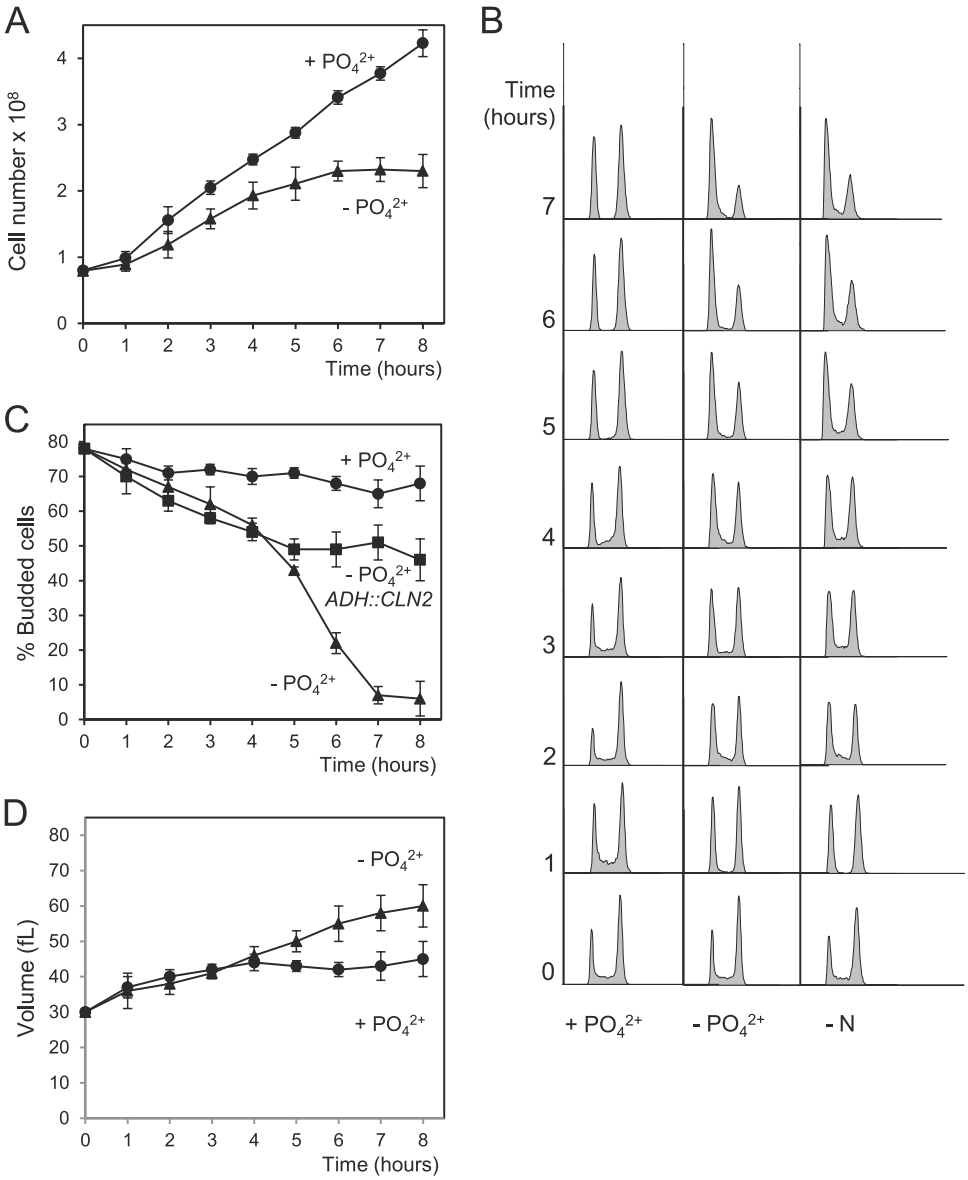

FIG 1 Phosphate deprivation leads to G_1 arrest. Wild-type cells were grown exponentially in synthetic complete medium. At time zero, cells were harvested and incubated either in the same medium ($+PO_4^{2-}$) or in a medium without phosphate ($-PO_4^{2-}$). At the indicated times, samples were collected and then subjected to several analyses: total cell number (A), DNA content (B), percentage of budding (C), and cell volume (D). (B) The DNA contents of wild-type cells incubated in a medium without a nitrogen source (−N) are shown. (C) The percentages of budding of wild-type cells carrying a centromeric plasmid with the *CLN2* gene expressed from an ADH promoter are shown. Data ± standard deviations from three independent experiments are shown.

important trophic factors in all organisms. When deprived of a nitrogen or carbon source, yeast cells use accumulated reserves to complete the current cycle and arrest at the following G_1 phase. We sought to determine whether phosphate starvation also provokes G_1 arrest. To this end, we transferred exponentially growing yeast cells from complete medium to a medium lacking a phosphate source. The rate of division declined slowly over the next 3 h (Fig. 1A) and, finally, the cells arrested in G_1, as indicated by their DNA content and budding index (Fig. 1B and C). Interestingly, the cell cycle arrest was comparable to that observed in the absence of nitrogen (Fig. 1B) and was not merely a consequence of the inability to meet the growth requirements of the G_1/S transition, as suggested by the fact that the cell volume still increases under phosphate deprivation (Fig. 1D).

Considering that cyclins are fundamental to progression through G_1, we asked if the arrest caused by phosphate deprivation requires downregulation of the activity of G_1 cyclins. Cells expressing *CLN2* from a constitutive promoter continue to grow in size (not shown) and, more importantly, cells cross Start despite the absence of phosphate, as deduced from the budding index (Fig. 1C). This indicates that the downregulation of Cln activity may be essential in order to achieve a proper G_1 arrest under phosphate-fasting conditions. Taken together, these results strongly suggest that the G_1 arrest caused by phosphate deprivation is mediated by specific mechanisms, some of which involve the downregulation of Cln proteins, and cannot be explained simply as a passive consequence of a growth arrest that would prevent cells from reaching the critical cell mass necessary to bud and to initiate S phase.

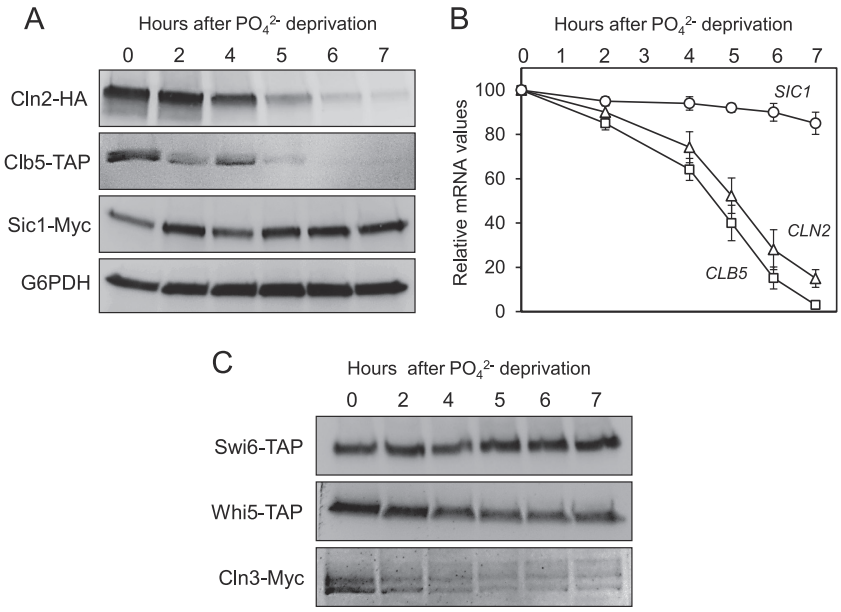

FIG 2 Phosphate deprivation leads to the downregulation of Cln3p. (A) Strain YAN32 (triple tagged: Cln2-HA, Clb5-TAP, and Sic1-Myc) was grown exponentially in synthetic complete medium. At time zero, the cells were harvested and incubated in phosphate (PO_4^{2-})-free medium. At the indicated times, samples were recovered and then analyzed for different proteins by immunoblotting using specific antibodies. (B) The wild-type strain was treated and sampled as described in Materials and Methods. Transcripts were analyzed using RT-PCR with specific primers. Data ± standard deviations from three independent experiments are shown. (C) Strains YPC702 (*SWI6-TAP*), YNR11 (*WHI5-TAP*), and YNR55 (*CLN3-MYC*) were treated, sampled, and analyzed as described for panel A.

External phosphate controls the levels of Cln3 protein. Since constitutive expression of Cln2 could partially reverse the blockade in G_1, we examined whether the absence of phosphate could affect the levels of proteins controlling the G_1/S transition. The results in Figure 2A clearly show that the protein levels of Cln2 and Clb5 (essential for budding and S-phase entry) decrease after 4 h of growth in phosphate-free medium, whereas the levels of the protein Sic1 (essential for arresting the cell cycle before S phase) remain constant. We then analyzed the mRNA levels of all these genes (Fig. 2B), finding that the mRNA of *CLN2* and *CLB5* were also progressively depleted, whereas the transcripts of *SIC1* remained constant. These results suggest that the absence of phosphate may inhibit the transcription of genes required for the G_1/S transition.

The G_1/S transcriptional wave is controlled by the SBF and MBF factors, which may be regulated by phosphate levels. As observed from the results in Figure 2C, the protein levels of Swi6 are not affected during the first 6 h after phosphate deprivation (nor are those of Swi4 or Mbp1 [not shown]), thereby excluding the possibility that SBF and MBF are controlled at the expression level. Whi5 and Cln3 are known to play a key role in the activation of SBF and MBF complexes in late G_1 (10). The results in Figure 2C show that, after 3 h of phosphate depletion, the amount of Cln3 protein decreases rapidly, while the levels of Whi5 repressor remain fairly constant. These results support the idea that phosphate deprivation downregulates SBF and MBF activity as a result of the loss of Cln3. Considering that a triple *cln* mutant cannot undergo the G_1/S transition unless Sic1 is removed (33), we propose here that 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.

Pho85 activity regulates the levels of Cln3 protein. It is known that exogenous phosphate activates Pho85, a CDK that controls the homeostasis of phosphate, and we examined whether this kinase could also be involved in the control of Cln3 cyclin. As reflected in Figure 3A, *PHO85*-depleted cells growing exponentially in rich medium exhibit low levels of Cln3 protein. This decrease is very similar to that observed in wild-type cells grown in phosphate-free medium. *PHO85* gene deletion results in a broad spectrum of defects, and it is possible that our *pho85*Δ laboratory strain bears suppressor mutations that alleviate such defects. To avoid this problem, we took advantage of the *pho85-as* strain, which behaved quite similarly to wild-type cells until the specific inhibitor 1-Na PP1 was added to the culture. As observed by the results in Figure 3B, the addition of 1-Na PP1 to the exponentially growing cells results in a progressive depletion of Cln3 relative to the amount in control cells. The localization of Pho4-GFP is a widely used readout to evaluate Pho85 activity (27), and Cln3 starts to decrease at the same time that Pho4-GFP protein enters the nucleus (not shown). The latter result shows a good correlation between Pho85 inactivation and the reduction of Cln3 protein and suggests that Pho85 activity (rather than the physical presence of the protein itself) is a key factor controlling cellular levels of Cln3.

Upstream elements of the PHO pathway also affect Cln3 protein levels. Pho85 activity is controlled by external phosphate through an upstream signaling transduction pathway that has been progressively elucidated by several research groups (Fig. 4A) (for a review, see reference 34). The *vip1*Δ and *pho81*Δ strains are unable to respond to changes in the external concentration of phosphate and therefore, cannot inhibit Pho85 in a deprivation situation. Thus, we hypothesized that these strains could not downregulate Cln3 protein levels in response to depletion of phosphate, which we confirmed experimentally (Fig. 4B). Conse-

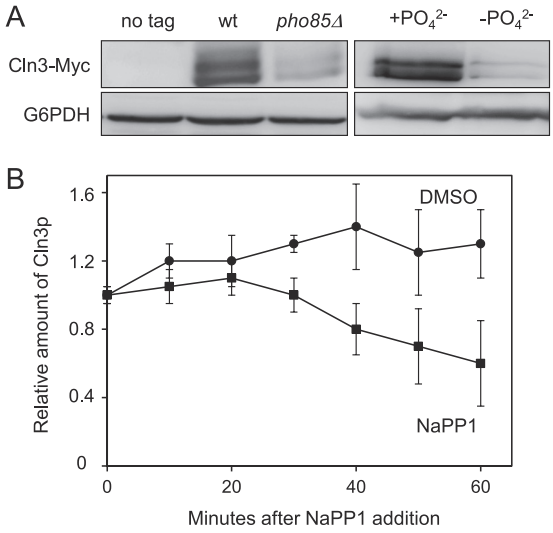

FIG 3 Pho85 inactivation leads to downregulation of Cln3. (A) Wild-type (wt) and *pho85*Δ cells were grown exponentially in YPD and then assessed for levels of Cln3-Myc (left), and wild-type cells were grown in synthetic complete medium with ($+PO_4^{2-}$) or without ($-PO_4^{2-}$) phosphate (right). Samples were taken after 6 h, and levels of Cln3-Myc were monitored. (B) Cells from the YAM67 strain were incubated with either 1-Na PP1 (a specific *pho85-as* inhibitor) or drug vehicle (dimethyl sulfoxide [DMSO]). Samples were taken at the indicated times, and Cln3-Myc was analyzed by immunoblotting. Data ± standard deviations from three independent experiments are shown. Cln3p, phosphorylated Cln3.

quently, the overexpression of *PHO80* has the same effect (Fig. 4C) as the absence of the inhibitors. All these results strongly suggest that the phosphate source controls the quantity of Cln3 cyclin by modulating Pho85 activity.

Loss of a particular Pho85 cyclin can phenocopy some aspects of the phenotype of a *pho85*Δ mutant, and we decided to evaluate the levels of Cln3 protein of the different cyclin mutants. As shown by the results in Figure 4D and E, the *pho80*Δ strain is the only one that shows a reduction in the Cln3 content very similar to that observed in *pho85*Δ cells, suggesting that the effect of the CDK could be mediated by the Pho80 cyclin. This result is in agreement with the fact that the Pho80/Pho85 complexes (together with Pcl7/Pho85) are inhibited when deprived of phosphate (35), providing further evidence that such nutrients control the quantity of Cln3 by modulating the activity of Pho85 kinase.

Pho85 affects Cln3 stability. Pho85/Pho80 complexes control the transcription of many genes, and we reasoned that these complexes could modulate the transcription of *CLN3* in response to changes in phosphate levels. However, there are no differences in the levels of *CLN3* mRNA in wild-type cells cultured in the presence or absence of phosphate (Fig. 5A).

Likewise, we could not detect any changes in the mRNA levels of *CLN3* when comparing wild-type cells, *pho85*Δ cells, and cells that overexpress *PHO85*. Despite the similar levels of mRNA, we again confirmed the differences in Cln3 protein levels under these new experimental conditions (Fig. 5A). These results suggest that Cln3 must be downregulated by posttranscriptional mechanisms.

Next, we tested whether Pho85 activity affects the stability of Cln3 by studying the levels of Cln3 in cycloheximide-treated cells. In wild-type cells, we estimated a Cln3 half-life of 8 min (data not shown), in accordance with values reported by others (18). Unfortunately, we were unable to measure the half-life of Cln3 in the *pho85*Δ strain because of the low levels in these cells. For this reason, we decided to assess the Cln3 stability in the *pho85-as* strain in the presence or absence of the specific inhibitor (Fig. 5B). Under such conditions, the *pho85-as* strain still retains low levels of Pho85 activity but clearly exhibits an apparent reduction in the half-life of Cln3 protein (5.8 min in control cells versus 3.2 min in the presence of the inhibitor). Although this result does not exclude the existence of other regulatory mechanisms (such as the control of the translation of Cln3 mRNA), it strongly suggests that Pho85 activity increases the stability of the cyclin.

Pho85 does not control Cln3 stability through regulation of autophagy. It has been recently described that Pho85 negatively regulates autophagy through diverse mechanisms (36). Autophagy induces the destruction of many proteins under conditions of nutrient deprivation, and we hypothesized that Cln3 could be a likely candidate for destruction. To test this hypothesis, we quantified the Cln3 levels in a *pho85*Δ strain whose autophagy process has been abrogated the (*pho85*Δ *atg1*Δ strain) and found that the Cln3 protein levels still were clearly decreased (Fig. 5C). This result rules out the possibility that Pho85 controls Cln3 stability through the regulation of autophagy.

Pho85 affects Cln3 ubiquitin-dependent degradation. Since Cln3 is constitutively degraded by a ubiquitination-dependent mechanism (9), we decided to test whether Pho85 controls this process. We found 2 pieces of experimental evidence to support this hypothesis: (i) deletion of the PEST region in *CLN3* (the *cln3-1* allele; see the legend to Fig. 6A), which has been shown to prevent ubiquitination, also stabilizes the protein, even in the *pho85*Δ strain (Fig. 5D), and (ii) deletion of the E2 ubiquitin ligase Ubc4, which is important for nutrient homeostasis and is involved in the ubiquitination of Cln3 (9), restores Cln3 levels in the *pho85*Δ strain (Fig. 5E). Therefore, Pho85 may somehow interfere with Cln3 ubiquitination and/or destruction processes. Bearing in mind that Pho85 is a protein kinase, one explanation could be that it phosphorylates Cln3 and thereby interferes with such processes.

Cln3 is an *in vitro* substrate of Pho80/Pho85. To test the latter hypothesis, we performed an *in vitro* phosphorylation experiment, for which we needed to obtain full-length Cln3 protein. We tried to purify Cln3 from wild-type yeast cells, but the cyclin is tightly associated with the endoplasmic reticulum (37) and remains with the particulate fraction of the cell extracts, making purification difficult. We also tried to purify it as a recombinant protein, but the expression of the full-length protein was highly toxic to *E. coli* cells, resulting in very poor expression levels. Finally, we expressed and purified a fragment of the recombinant GST-Cln3 protein (from Met347 to Arg580) containing the PEST region. This fragment of Cln3 was specifically phosphorylated by reconstituted Pho85/Pho80 complexes purified from *E. coli* cells (Fig. 6B). In contrast, we observed that the N-terminal half of the Cln3 protein (from Met1 to Met347) was not phosphorylated by Pho85 (not shown).

Pho85 is a proline-directed kinase that preferentially phosphorylates the consensus sequence S/T-P-X-I/L (17), and Cln3 contains two of these sites (Ser449 and Thr520) located precisely at the ends of the PEST region (Fig. 6A). We replaced both of these sites with either alanine or aspartic acid, rendering Cln3 that was no longer phosphorylated by Pho85 (Fig. 6B) and showing S449 and T520 to be the Pho85/Pho80 targets. A typical substrate of Pho85/Pho80, such as Pho4, was included as a control.

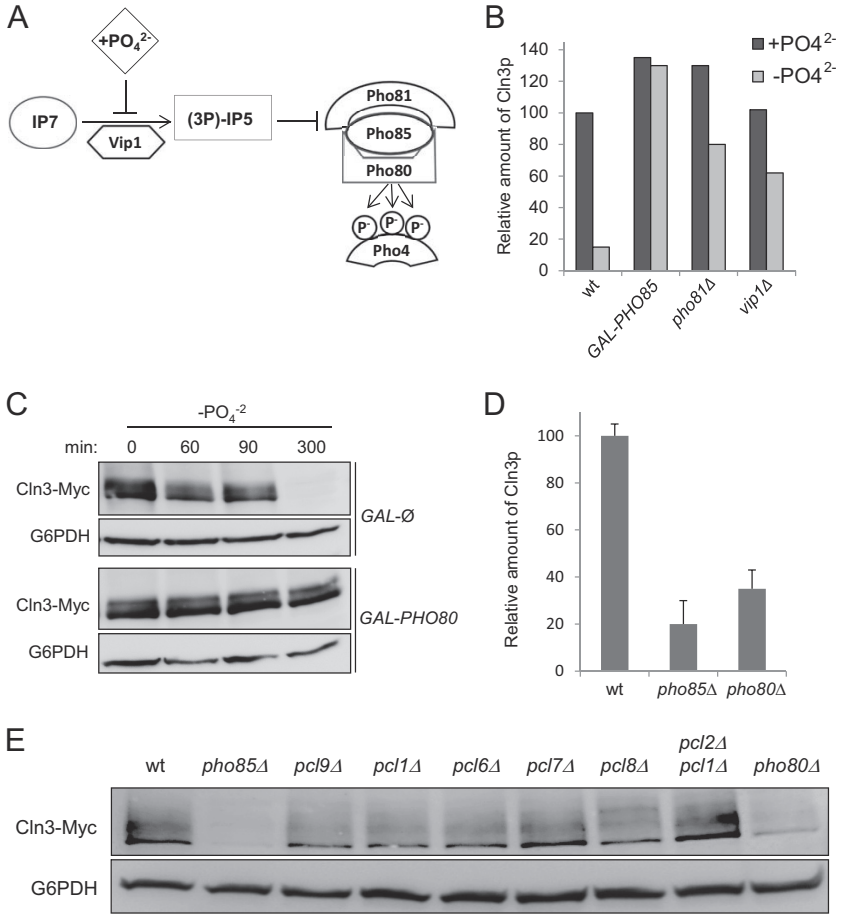

FIG 4 Phosphate controls cellular levels of Cln3 by modulating the PHO pathway. (A) Schematic of the PHO pathway. During phosphate starvation, Vip1 causes an increase in the levels of inositol heptakisphosphate (IP_7), which binds to and changes the conformation state of Pho81, leading to the inactivation of Pho85/Pho80 complexes. (B) Relative amounts of Cln3-Myc in different strains. Wild-type (wt), *pho81*Δ, and *vip1*Δ strains were grown in synthetic complete medium with ($+PO_4^{2-}$) or without ($-PO_4^{2-}$) phosphate. After 5 h, the levels of Cln3-Myc were quantified by immunoblotting using monoclonal antibodies. Cells of the *Gal1-PHO85* strain (a wild-type strain that carries a centromeric plasmid with *PHO85* expressed under the Gal1 promoter) were grown for 5 h in synthetic complete medium with galactose as a carbon source, either with or without phosphate. (C) Pho80 is necessary to maintain high levels of Cln3. As described for panel B, wild-type (GAL-Ø) and *GAL1-PHO80* strains were grown in synthetic complete medium in the presence of galactose without phosphate ($-PO_4^{2-}$). Levels of Cln3-Myc were quantified by immunoblotting using monoclonal antibodies. (D) Pho80 is necessary to maintain high levels of Cln3. Quantification of data in panel E (data ± standard deviations from four independent experiments) is shown. (E) Pho80 is necessary to maintain high levels of Cln3. A plasmid with a Cln3-Myc epitope tag was introduced in strains with the indicated mutations for deficiency of the different Pho85 cyclins. After 3 h of exponential growth in YPD, the levels of Cln3-Myc were analyzed by immunoblotting.

It has been suggested that Cdc28 is involved in the down-regulation of Cln3 levels by acting through the CDK consensus phosphorylation sites in the PEST region of Cln3. Therefore, Cln3 phosphorylation by Cdc28 and Pho85 appears to have the opposite effect. We assayed the same Cln3 fragments against Cdc28-TAP immunopurified from yeast cells. The results in Figure 6C show that Cdc28 phosphorylates Cln3 (to a greater extent than Pho85, probably because it phosphorylates Cln3 at more sites in the PEST region). Interestingly, Cdc28 is still able to phosphorylate the S449A T520A *cln3* mutant to the same extent as the wild type (Fig. 6C), indicating a differential site requirement between Cdc28 and Pho85 on Cln3, at least *in vitro*. A typical substrate of Cdc28, such as Sic1, is included as a control.

Because Cln3 and Pho80 interact *in vivo*, as indicated by the results of coimmunoprecipitation experiments (Fig. 7A), we examined whether Cln3 is also an *in vivo* substrate of Pho85.

***In vivo* phosphorylation of S449 and T520 is essential to maintain Cln3 levels.** We verified the *in vivo* Cln3 phosphorylation status by using mobility shift analysis. However, unphosphorylated Cln3 is highly unstable and becomes nearly undetectable by Western blot analysis, making this experiment very challenging. We attempted to identify the shift using a *pho85-as* strain and adding 5 times more yeast extract to the electrophoresis gel, but the levels of Cln3 remained nearly undetectable (data not shown).

We decided to test the *in vivo* effects of phosphorylation by measuring the levels of different mutant versions of Cln3. We expressed Cln3 from its own promoter in a centromeric plasmid, and when we replaced S449 and T520 with alanines, the levels of Cln3 became nearly undetectable (Fig. 7B and C). We also replaced these 2 sites with aspartic acid, to mimic the effect of phosphorylation, predicting that they would restore Cln3 levels to some degree in a strain without Pho85 activity. The results in Figure 7D and E illustrate how in *pho85*Δ cells (or in wild-type

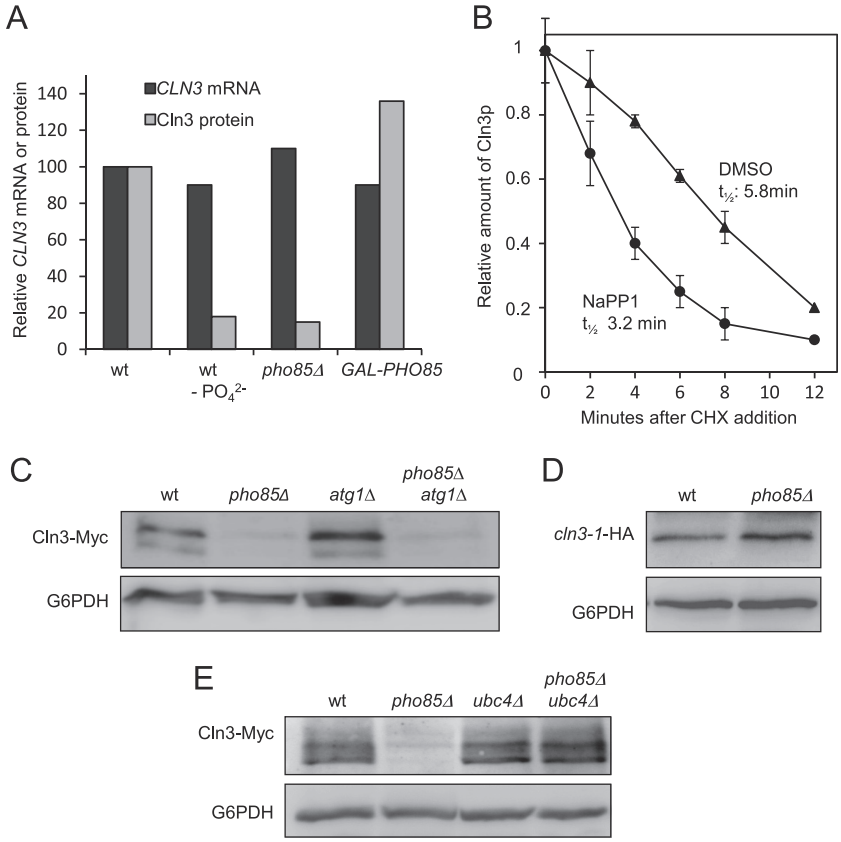

FIG 5 Pho85 activity increases the half-life of Cln3 protein. (A) Different strains with a genomic tagged version of *CLN3* were sampled and then analyzed for *CLN3* mRNA levels (by RT-PCR) or Cln3-Myc levels (by immunoblotting). Wild-type cells were grown exponentially in synthetic complete medium with (wt) or without (wt $-PO_4^{2-}$) phosphate, *pho85*Δ strain cells were grown in the same complete medium with phosphate, and *GAL-PHO85* cells were grown for 5 h in synthetic complete medium with galactose as a carbon source. (B) YAM67 strain cells were incubated with either 1-Na PP1 (a specific *pho85-as* inhibitor) or drug vehicle (DMSO). Forty minutes later (time zero), cycloheximide (CHX) was added to the cultures (final concentration, 10 μg/ml). At the indicated times, samples were taken and analyzed for Cln3-Myc levels by immunoblotting. Data ± standard deviations from four independent experiments are shown. $t_{1/2}$, half-life. (C) Downregulation of autophagy does not restore the diminished Cln3 levels of *pho85*Δ mutants. Strains of the indicated genotypes were grown exponentially in phosphate-rich medium, and Cln3-Myc was analyzed by immunoblot assay using specific antibodies. (D) Absence of the PEST region restores Cln3 levels in *pho85*Δ cells. Wild-type or *pho85*Δ cells carrying a plasmid with a *cln3-1* allele without the PEST region (41) were grown exponentially in YPD. Cln3-HA levels were measured by immunoblotting. (E) Absence of *UBC4* restores Cln3 levels in a *pho85*Δ strain. Strains of the indicated genotypes carrying a genomic Myc-tagged version of Cln3 were grown exponentially in phosphate-rich medium. Cells were harvested, and Cln3 levels were evaluated by immunoblotting.

cells growing in medium without phosphate), the levels of Cln3 carrying the S449 and T520 aspartic acid substitutions are higher than the levels of wild-type Cln3, suggesting that *in vivo* phosphorylation at these sites is essential for maintaining high levels of Cln3. Overall, these results suggest that the proposed phosphorylation sites are involved in the *in vivo* regulation of Cln3 levels.

A possible scenario that emerges from all these results (Fig. 8A) is that in the absence of phosphate, Pho85 becomes inactive and Cln3 is no longer phosphorylated, which in turn would interfere with the ubiquitination and/or the destruction of the cyclin. At this point, we sought to test the physiological validity of our proposed model.

Pho85 inactivation is essential for proper G_1 arrest. Ectopic expression of Cln3 in G_1-arrested cells (due to the presence of rapamycin or to nutrient depletion) leads to accidental entry into S phase and to diminished cell viability (38, 39). To ascertain the relevance of these observations to our work, we arrested cells in phosphate-depleted medium, and we found that, as predicted, cells with a hyperactive form of *CLN3* (the *cln3-1* allele) induced a diminished cell viability which correlated well with an increase in the number of cells that crossed Start, as indicated by the high percentage of budded cells (Fig. 8B). Moreover, as discussed above (Fig. 1), ectopic expression of Cln2 led to a clear increase in the number of budded cells and to a rise in the rate of cell death (not shown). Hence, it seems that downregulation of Cdc28 activity is essential for proper G_1 arrest during phosphate deprivation.

According to our model, overactivation of Pho85 should also be detrimental to cell viability in G_1-arrested cells, and our results demonstrated that this was indeed the case. As shown by the results in Figure 8B, wild-type cells incubated for 7 days in phosphate-free medium exhibited a viability rate of 55%. In contrast, *vip1*Δ or *pho81*Δ cells (which should retain Pho80/Pho85 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%). Even more interesting is the fact that cells carrying the Cln3 with the Asp substitutions that mimic Pho85 phosphorylation also have low viability under phosphate-fasting conditions. Together, these results agree with the

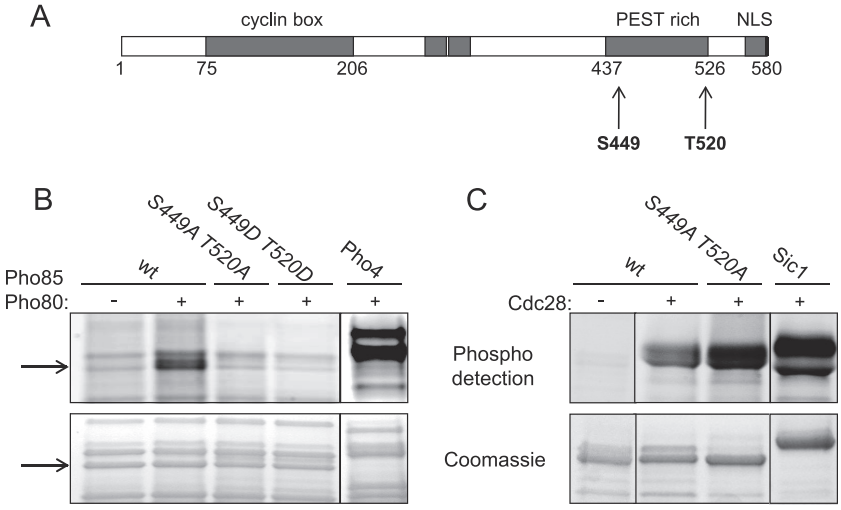

FIG 6 Cln3 is phosphorylated *in vitro* by Pho85/Pho80. (A) Schematic representation of Cln3 with the different functional domains. The PEST region is indicated by shading, and the putative target residues for Pho85 by arrows. NLS, nuclear localization signal. (B) *In vitro* kinase assay of Pho85/Pho80 on Cln3. Recombinant Pho85 and GST-Pho80, purified from bacteria, were incubated with the C-terminal half of Cln3 (also from bacteria) containing the PEST region with the indicated mutations or the wild-type sequence (see Materials and Methods and Table 2). Pho4, a well-known substrate of Pho85/Pho80, was included as a control for the Pho85/Pho80 activity. The arrows indicate Cln3 protein. (C) *In vitro* kinase assay of Cdc28-TAP and the Cln3 mutants with the indicated mutations or the wild-type sequence. IgG-Sepharose beads were used to pull down Cdc28 either from a no-tag strain (−) or the Cdc28-TAP tag strain (+). Sic1 was included as a control for the Cdc28 kinase activity.

notion that, in the absence of phosphate, downregulation of Pho85 activity is essential for decreasing Cln3 levels and for proper G_1 arrest.

Pho85 activation is essential for proper cell cycle reentry. It has been proposed that Pho85 activity is essential in situations with no Cdc28 activity, such as those involving cell cycle restart (20). This hypothesis is consistent with our model, which, if true, predicts that cells with low Pho85 activity should have difficulties reentering the cell cycle from G_0. To test this prediction, we incubated yeast strains in phosphate-free medium and, after 7 h, transferred them to rich medium in order to monitor their growth. The results in Figure 8C show how, after 2 h of incubation in phosphate-rich medium, *pho85*Δ cells present low levels of Cln3 and consequently remain arrested, while wild-type cells already undergo mitosis. It is interesting to note that the differences between strains are only minor when we compare the ability of cell cycle reentry from α-factor arrest (Fig. 8D). This finding suggests that the observed delay in cell cycle reentry is specific rather than a general defect of cell cycle regulation.

In summary, these results reinforce the idea that Pho85 activity plays an indispensable role in exiting from G_0 arrest.

DISCUSSION

Phosphate levels regulate cell cycle progression. So far, there are several lines of evidence implicating glucose and nitrogen as critical elements in cell cycle control and that their absence leads to G_1 arrest. Also, it is known that the absence of phosphate also stops proliferation. At first glance, 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, there is considerable evidence to confirm that the observed arrest is a controlled process, as follows. (i) Under phosphate-fasting conditions, the cells accumulate as mostly unbudded with 1N DNA content; if they had arrested accidentally, we should have found cells in various stages of the cycle. (ii) Metabolomic studies have shown that cells growing under phosphate-limiting conditions maintain a relatively constant free energy of ATP hydrolysis (40), suggesting that in the first moments of phosphate deprivation, cells might have enough energy to initiate an adaptive response, such as finishing one round of division until the next G_1 phase. (iii) The volume of arrested cells continues to increase, indicating that they are metabolically active and not in a collapsed state. (iv) Finally, the arrest caused by lack of phosphate can be reversed with an increase in CDK/Cln2 activity. In conclusion, one of the contributions of the present work is to confirm that phosphate regulates Start through the control of G_1/S transcriptional machinery in *S. cerevisiae*, as suggested some time ago by Johnston et al. (35).

It should be noted that, in our experiments, the cells did not arrest immediately: they gradually accumulated in the G_1 phase, and finally, within approximately 6 h of deprivation, 90% of them had stopped dividing. This gradual response of cultures is probably due to polyphosphate reserves in the cells (41) which serve to buffer the sudden external changes.

Pho85 affects the stability of Cln3. The central idea of this work is that phosphate levels dictate Cln3 stability by regulating the activity of the Pho85/Pho80 complex. It is interesting to note that the amounts of other cyclins (e.g., Cln2) are not affected by Pho85 activity (not shown), underscoring the specificity of the described effect.

Several studies indicate that Cln3 is downregulated by translational repression under conditions of nutrient deprivation (18, 42, 43). Although we cannot rule out the possibility that phosphate limitation has the same effect, our results show that the critical step targeted by phosphate availability on Cln3 is its stability. An analogous destabilization phenomenon has been described in nitrogen-deprived cells, although its mechanism remains unexplained (18). This same study showed that the half-life of Cln3 was again reduced by half. Thus, the destabilization of Cln3, along

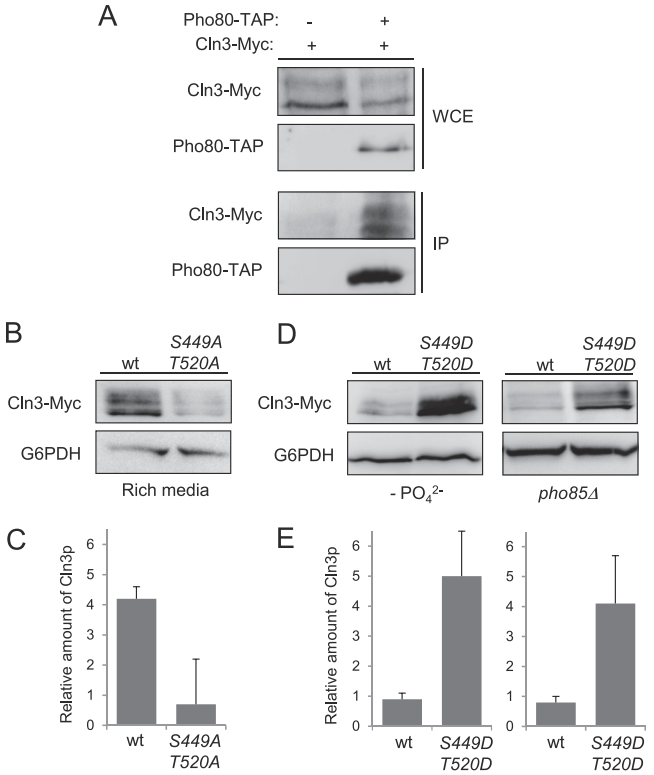

FIG 7 *In vivo* phosphorylation of S449 and T520 is essential to maintain Cln3 levels. (A) Pho80 and Cln3 interact *in vivo*. Yeast extracts (WCE [whole-cell extracts]) containing untagged Pho80 or Pho80-TAP were pulled down with IgG-Sepharose. Tagged Cln3-Myc (from its chromosomal locus) was detected using specific antibodies (IP [immunoprecipitate]). (B) Alanine replacement of S449 and T520 destabilizes Cln3. Wild-type cells were transformed with a centromeric plasmid bearing a Cln3-Myc or a 2-Ala (S449A T520A) mutant version and grown for 4 h in rich medium. The amount of Cln3 was determined by immunoblotting. (C) Quantification of experiment whose results are shown in panel B. Data ± standard deviations of three independent experiments are shown. (D) Aspartic acid replacement of S449 and T520 stabilizes Cln3. Wild-type (in phosphate-deficient medium) or *pho85*Δ (in phosphate-rich medium) cells bearing a plasmid with different versions of *CLN3* were grown exponentially for 4 h. Cln3 levels were analyzed by immunoblotting. To simplify the nomenclature, S449D means a double substitution of Asp into the Ser 449 and the previous residue to mimic the double-negative charge that represents the phosphate group (see Materials and Methods), and the same is true for T520D. (E) Quantification of experiments whose results are shown in panel D. Data ± standard deviations of three independent experiments are shown.

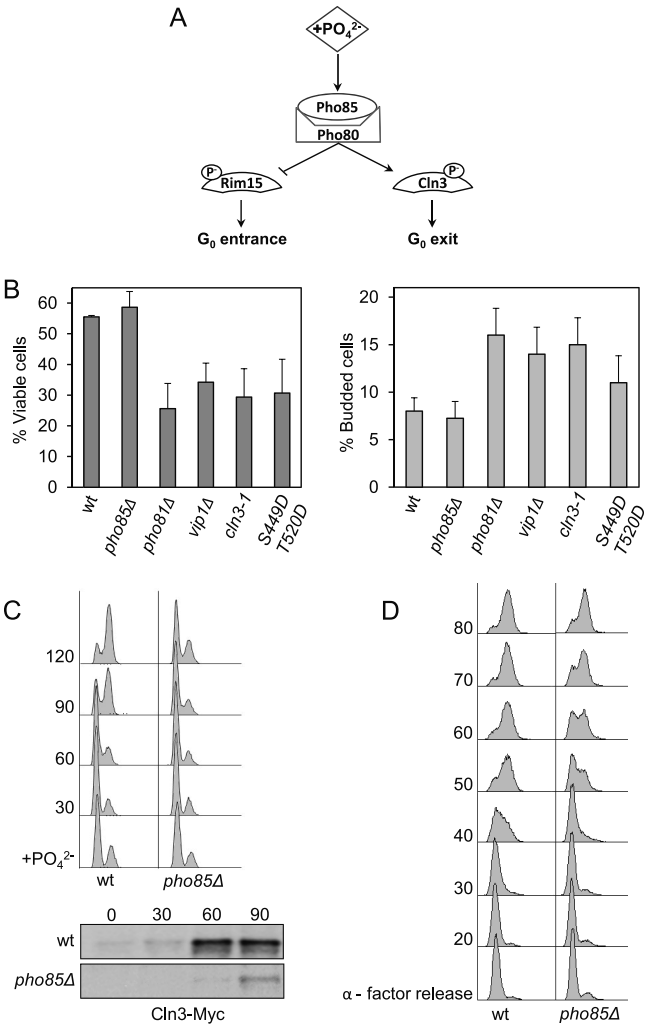

FIG 8 Pho85 activity is necessary for proper G_1 arrest and cell cycle reentry. (A) Proposed model of Pho85 activity. In phosphate-rich medium, Pho85/Pho80 complexes remain highly active. Under such conditions, Pho85 phosphorylates and inactivates Pho4 and Rim15 (25) and, conversely, activates cyclin Cln3. (B) Proper regulation of Pho85 activity is essential for survival under conditions of phosphate deprivation. Cells were incubated in synthetic complete medium for 7 days, at which point cells were collected and assessed for viability by colony counting (left). The percentage of budding was analyzed by counting no less than 200 cells under the microscope (right). Data ± standard deviations from three independent experiments are shown. (C) Pho85 activity is necessary for reentry into the cell cycle after refeeding. Cells transformed with a centromeric plasmid bearing Cln3-Myc were deprived of phosphate for 7 h (time zero) and then refed. At various times, samples were collected and analyzed for DNA content by flow cytometry (top). Times (min) are indicated at left; "$+PO_4^{2-}$" indicates the initiation of refeeding. Cln3 levels were monitored by immunoblotting (bottom) at times (min) indicated above the gel. (D) *pho85*Δ progresses through G_1, with a small delay, after α-factor exit. Wild-type and *pho85*Δ cells were synchronized with α-factor for 3 h and then released into fresh medium at 30°C. At various times, samples were collected. Times (min) are indicated at left; "α-factor release" indicates the time of release following synchronization. Total DNA content was measured as described in Materials and Methods, except that propidium iodide was used instead of SYBR green.

with its possible translational repression, could be a general response of the cell against the limitation of different nutrients. This would limit the activity of the Cln3/Cdc28 complex needed to pass Start in order to slow down the cell cycle and to adapt to the new conditions.

The finding that Cln3 is affected by Pho85/Pho80 activity is relevant for two reasons: on one hand, the Pho85/Pho80 route is shown for the first time to be involved in directly controlling the cell cycle machinery, suggesting coordination between phosphate homeostasis and the cell cycle, and on the other hand, to our knowledge, this is the first reported case of one CDK controlling the activity of another. Interestingly, during the revision process of the manuscript, the group of Kron and collaborators described that Pho85/Pcl2 controlled the amount of Cln3 through the activation of Hsp70 in response to nitrogen, pointing to Pho85 as a general controller of Cln3 in response to variations in nutrient levels (44).

How does Pho85 affect the stability of the cyclin Cln3? Having excluded the possibility of autophagy, we propose here that Cln3

phosphorylation by Pho85 may somehow hinder the normal degradation of Cln3. This raises the possibility that phosphorylation of Cln3 complicates its recruitment by the ubiquitination system, consequently expanding its lifetime. The idea that phosphorylation may prevent interaction with E3 ligases has already been suggested for other cell cycle-regulated proteins (45).

If Cdc28 and Pho85 have opposite effects on Cln3 stability, then it would be important to determine whether these CDKs phosphorylate different residues. The residues of Cln3 phosphorylated by Cdc28 are currently unknown, although some results point to S468 as one of the most likely candidates (9). However, we have demonstrated that a recombinant fragment of Cln3 that presents S449 and T520 mutated to alanine is still phosphorylated by Cdc28 and is no longer phosphorylated by Pho85 (Fig. 6B and C), indicating that the CDKs phosphorylate different residues of Cln3, at least *in vitro*. It is important to note that, although the consensus sequence for the CDK family is SP or TP, Cdc28 and Pho85 have distinct preferences for the sequence adjacent to these sites. Cdc28 prefers a positively charged lysine or arginine at the third position from the phosphoacceptor site, with the consensus site being S/TPXK/R (46, 47), whereas Pho80-Pho85 has the consensus phosphorylation site sequence SPXI/L (24, 48). Therefore, it is formally plausible that both kinases phosphorylate distinct sites, generating distinct effects. In this regard, it is interesting to note that only 2 of 10 SP/TP sites of the PEST region have the preferred sequence for Pho85 (the hydrophobic residue at +3), and these sites are the same two that appear to be phosphorylated by Pho85. There are other examples of proteins that are phosphorylated at SP/TP with opposite effects, such as Sic1, which must exceed a threshold of CDK phosphorylation in several SP/TP sites to be destroyed, but a single mitogen-activated protein kinase (MAPK) phosphorylation in one specific TP makes the protein stable (45).

Physiological significance of Cln3 regulation. The underlying mechanism(s) that enables nutrient sensors to control cell cycle machinery remains 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. We propose that modulation of Cln3 stability may be one of many principal targets in cell cycle regulation, at least in terms of the adaptive response to phosphate scarcity (the proposed model is shown in Fig. 8A). Cln3 cyclin is a good candidate because it is the most upstream control point in the cell cycle and because raising its cellular content by either increased transcription or altered protein turnover profoundly affects its capacity to pass through G_1 (9, 49). Thus, the G_1 arrest caused by phosphate deprivation could be explained by a simple model: due to the reduction in the Cln3 half-life, starved cells cannot reach the Cln3 threshold level required to execute the Start program. However, this mechanism might not be important in rich medium, given that *cln3*Δ cells can still progress through the entire cell cycle, albeit with a longer G_1 phase.

According to our model, Cln3 destabilization is crucial during G_1 arrest due to lack of phosphate. This downregulation could be important, for instance, in maintaining silencing of Swi6-dependent transcription in order to avoid the process of wall remodeling during nutrient deprivation (50). Moreover, Burhan's group has demonstrated that establishing and maintaining proper arrest in G_1 is an important cellular response to nutrient deprivation (38); they report that cells that improperly halt at the S phase suffer replication stress and rapidly lose viability. Furthermore, they clearly demonstrate that ectopic expression of *CLN3* increases the frequency with which nutrient-depleted cells arrest at the beginning of the S phase instead of G_1. Our results confirm that the presence of a hyperstable Cln3 allele (*cln3-1*) also increases the number of S-phase-arrested cells and decreases cell viability. In this context, we propose that in response to the lack of phosphate, Pho85 inactivation is critical for stopping the cycle at G_1, avoiding entry into the S phase with low levels of nucleotides. This assumption is clearly supported by evidence showing that mutants with high Pho85 activity (*pho81*Δ, *vip1*Δ, and Gal-*PHO85*) incubated in medium without phosphate (i) maintain high levels of Cln3, (ii) show large numbers of cells that have entered the cell cycle, and (iii) show a decrease in cell viability. Considering that under the same conditions of deprivation, cells with a *CLN3* allele that encodes aspartic acid substitutions also die prematurely, we propose that such cellular defects chiefly derive from sustained and unscheduled phosphorylation of Cln3.

According to our model, Pho85 could also be important for restarting the cell cycle after refeeding. Our findings support this notion; either the absence of Cln3 or low Pho85 activity greatly hinders reentry into the cell cycle. Interestingly, this role for Pho85 in reentry seems to be specific, as reflected in the almost total lack of differences between wild-type cells and *pho85*Δ cells released from alpha factor arrest. These results are consistent with the hypothesis that Pho85 is essential when Cdc28 activity is lacking (20) and reinforce the idea that Pho85 activity is almost dispensable when yeast grows in nutrient-rich medium (although the cells still undergo a longer G_1 phase), but is essential in other situations (e.g., exiting from nutrient-induced G_0 arrest). Since wild yeast should thrive under diverse nutrient conditions (the availability of phosphate and other nutrients often varies widely), we postulate that Pho85 must be fundamental in controlling the constant cell cycle stalls and reentries that a yeast cell is subjected to under natural conditions.

ACKNOWLEDGMENTS

We gratefully acknowledge F. Posas, W. C. Burhans, J. Jiménez, and N. Casals for stimulating discussions, E. O'Shea for providing strains, K. Shokat for the 1-Na PP1 inhibitor, M. Pérez for technical assistance, and O. Mirallas for his collaboration in some of the experiments.

S. Hernández received a postgraduate Junior Faculty fellowship from the UIC and l'Obra Social la Caixa. This work was supported by grants from Ministerio de Ciencia e Innovación of the Spanish government (BFU 2009-09278).

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Dma1 ubiquitin ligase controls G1 cyclin degradation

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December 2012

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 7, pp. 4704–4714, February 15, 2013

Defective in Mitotic Arrest 1 (Dma1) Ubiquitin Ligase Controls G_1 Cyclin Degradation*[S]

Received for publication, October 10, 2012, and in revised form, December 21, 2012 Published, JBC Papers in Press, December 21, 2012, DOI 10.1074/jbc.M112.426593

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Background: Dma ubiquitin ligases control the cell cycle in diverse organisms. In humans, these enzymes act as tumor suppressors that prevent aberrant mitosis.
Results: Dma1 targets the cyclin Pcl1 for destruction.
Conclusion: Dma1 ubiquitin ligase activity controls stability of G_1 cyclins.
Significance: Pcl1 is the second reported substrate for Dma1 enzymes. Uncovering new Dma1 substrates could help to elucidate cellular functions of these enzymes.

Progression through the G_1 phase of the cell cycle is controlled by diverse cyclin-dependent kinases (CDKs) that might be associated to numerous cyclin isoforms. Given such complexity, regulation of cyclin degradation should be crucial for coordinating progression through the cell cycle. In *Saccharomyces cerevisiae*, SCF is the only E3 ligase known to date to be involved in G_1 cyclin degradation. Here, we report the design of a genetic screening that uncovered Dma1 as another E3 ligase that targets G_1 cyclins in yeast. We show that the cyclin Pcl1 is ubiquitinated *in vitro* and *in vivo* by Dma1, and accordingly, is stabilized in *dma1* mutants. We demonstrate that Pcl1 must be phosphorylated by its own CDK to efficiently interact with Dma1 and undergo degradation. A nonphosphorylatable version of Pcl1 accumulates throughout the cell cycle, demonstrating the physiological relevance of the proposed mechanism. Finally, we present evidence that the levels of Pcl1 and Cln2 are independently controlled in response to nutrient availability. This new previously unknown mechanism for G_1 cyclin degradation that we report here could help elucidate the specific roles of the redundant CDK-cyclin complexes in G_1.

In all eukaryotic organisms, progression through the G_1 phase of cell cycle is controlled by diverse cyclin-dependent kinases (CDKs)[4] associated to numerous isoforms of cyclins. In budding yeast, the passage through G_1 is mediated by two CDKs: Cdc28, associated to the cyclins Cln1 and Cln2, and Pho85 associated to Pcl1 and Pcl2. These two sets of cyclins are transcribed at the same moment (named START), by the same transcription factors, and it is assumed to be destroyed by the same ubiquitin ligase system (1).

Pcls-CDK complexes could perform the same redundant functions as Clns complexes. Indeed, a *cln1 cln2 pcl1 pcl2* strain is unviable: it fails to extend a discernible bud, and has a large amorphous shape (2). Moreover, most of the Pho85 substrates involved in G_1 cell cycle progression are also substrates of Cdc28: there is evidence for phosphorylation of Sic1, Swi5, Ash1, Whi5, Rga2, and Clb6 by both Pcl-Pho85 and Cln-Cdc28 (2–7). The question arises as to why cells need Pho85 when Cdc28 alone appears able to execute essential cell cycle functions. Andrews and collaborators (2) have suggested that phosphorylation by Pho85 regulates proteins as Cdc28 does, but under different conditions. For example, in the context of DNA damage, the Pho85-Pcl1 complex is the only one active, and hence, is essential for restarting the cell cycle in G_1 (4). Although, the mechanisms that enable the alternating presence of these cyclins remain unknown, selective ubiquitination and degradation are believed to be involved.

The E3 ubiquitin ligase complexes, anaphase-promoting complex and SCF (Skp1/Cdc53/F-box protein) control the two main cell cycle transitions. The former ubiquitinates Clb cyclins after mitosis (5, 6), and the latter targets Cln1 and Cln2 after G_1 (7). A variety of SCFs exist, each of which has a different F-box protein; in fact, the *Saccharomyces cerevisiae* genome contains at least 17 putative F-box proteins, suggesting that, in eukaryotes, the SCF system could control numerous regulatory processes. Under standard growth conditions (YPD at 30 °C) Cln2 is targeted by the SCF-Grr1 system; the fact that Grr1 also regulates nutrient homeostasis (8) suggests that Grr1 could be the link between nutrient sensing and Cln2 turnover (9). Although Pcls are assumed to be degraded by the same ubiquitin-ligase system, the E3 ligase involved in Pcl1 destruction is unknown.

The specificity of F-box interactions could depend on specific destruction box signals present in their substrates. In this regard, a motif present in the Cln2 sequence has recently been described that enables interaction with Grr1 (10). Moreover, this interaction also depends on previous substrate modifica-

* This work was supported by Spanish Ministry of Science and Innovation Grant BFU 2009–09278, now known as the Ministry of Economy and Competitiveness.
[S] This article contains supplemental data.
[1] Supported by a post-graduate Junior Faculty fellowship from the Universitat Internacional de Catalunya and l'Obra Social la Caixa.
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[4] The abbreviations used are: CDK, cyclin-dependent kinase; SCF, Skp1/Cdc53/F box protein; Glu-6-PDH, glucose-6-phosphate dehydrogenase.

tion; for example, the destruction of cyclins usually depends on phosphorylation by its own CDK in specific regions with a high content of Pro, Glu, Ser, and Thr, the so-called *PEST* domains. Indeed, Cln2 mutated at the seven potential Cdc28 phosphorylation sites is highly stabilized and render cells insensitive to nutrient and growth inhibitory signals (11). Hence, it is accepted that the instability of Cln cyclins derives from Cdc28-dependent phosphorylation of the cyclin subunit, which allows the interaction of the SCF-Grr1 in a specific domain of Cln2 (10, 12, 13). A similar model has been proposed for mammalian cells, based on findings reported for G_1 cyclins E and D1 (14–16). This model might also be applied to the other group of G_1 cyclins: the Pcls. However, Pcl1 has neither the Grr1 interaction domain proposed in Cln2, nor any PEST sequence, suggesting that an E3 ligase other than SCF might be involved in Pcl1 targeting.

Yeast contains a high number of E3 ligases, some of which are involved in cell-cycle control. These include Dma1 and Dma2, which belong to a small class of proteins that contain a Forkhead-associated (FHA) domain, as well as a RING domain (for review see Ref. 17), of which there are two in humans: Chfr (CHeckpoint protein with FHA and RING domains, (18) and Rnf8 (19). Chfr is a tumor suppressor protein implicated in the antephase checkpoint (18, 20). During the G_2 phase, Dma1 and Dma2 help control the spindle position checkpoint (21) and Swe1 degradation (22), whereas in G_1, the two genes control G_1 progression in response to nutrients by an unknown mechanism (23). Moreover, Dma1 and Dma2 have been described to have ubiquitin ligase activity *in vitro* (23) although their physiological targets are unknown.

Herein we demonstrate that Pcl1 is targeted *in vitro* and *in vivo* by Dma1, and not by Grr1. We uncovered a possible Dma1 docking site sequence required for Pcl1 degradation. Also, we determined that degradation of Pcl1 requires phosphorylation at specific residues of its sequence by its own CDK. Finally, we present evidence that Pcl1 levels change in response to nutrient source through Dma1. The novel mechanism for G_1 cyclin degradation that we report here could help to elucidate the specific roles of the redundant CDK-cyclin complexes in G_1.

EXPERIMENTAL PROCEDURES

Yeast Strains—The strains used in this study are listed in Table 1.

Plasmids—PCL1-TAP and its derivative *pcl1 2A* (with Thr^{39}-Ala and Ser^{43}-Ala substitutions) were expressed from its own promoter in the centromeric plasmid pRS416. The Pcl1, *pcl1 2A*, *pcl1 4A* (with Ser^{14}-Ala, Thr^{70}-Ala, Ser^{117}-Ala, and Ser^{198}-Ala substitutions), *pcl1 DDD** (with Val^{31}-Asn and Pro^{33}-Asn substitutions), and *pcl1 DDD*Δ (with a gap between amino acids Leu^{29} and Asn^{36}) were cloned in pGEX6P1 to be expressed in *Escherichia coli*, and cloned in pRS416 to be expressed in *S. cerevisiae*. *PHO85* was cloned in pEG-KG multicopy plasmid to be overexpressed under control of the *GAL1* promoter and cloned in pGEX6P1 to be expressed in *E. coli*. *PCL9* and *DMA1* genes were cloned in centromeric plasmids (pRS416 and pRS426, respectively) and expressed from their own promoters. *DMA1* was expressed in *E. coli* from pGEX6P1. pGEX6P1::Sic1 construction was previously described in Ref. 24.

TABLE 1
Yeast strains used in this study

Strain	Background	Genotype	Source
	BY4741	*MATa his3Δ1 leu2Δ 200 met15 Δ 0 ura3 Δ 0*	Euroscarf
	W303-1a	*MATa ura3–52; trp1Δ2 leu2–3,112 his3–11 ade2–1 can1–100*	Euroscarf
	BY4741	*Das1::KanMX*	Euroscarf
	BY4741	*Dia2::KanMX*	Euroscarf
	BY4741	*Mfb1::KanMX*	Euroscarf
	BY4741	*Rad9::KanMX*	Euroscarf
	BY4741	*Rcy1::KanMX*	Euroscarf
	BY4741	*Saf1::KanMX*	Euroscarf
	BY4741	*Ubc4::KanMX*	Euroscarf
	BY4741	*Ubc13::KanMX*	Euroscarf
	BY4730	*Ufo1::KanMX*	Euroscarf
	BY4741	*ydr131c::KanMX*	Euroscarf
	BY4741	*ylr224w::KanMX*	Euroscarf
	BY4741	*ymr258c::KanMX*	Euroscarf
	BY4741	*ydr306c::KanMX*	Euroscarf
	BY4741	*ynl311c::KanMX*	Euroscarf
	BY4741	*ylr352w::KanMX*	Euroscarf
YSH75	BY4741	*grr1::KanMX*	This study
YPC639	BY4741	*dma1::KanMX*	This study
YPC651	BY4741	*dma2::KanMX*	This study
YPC748	BY4741	*dma1::LEU2 dma2::KanMX*	This study
YAM42	BY4741	*pho85::URA3*	This study
YAM78	BY4741	*pho85::LEU2*	This study
YSH87	BY4741	*CLN2-TAP::KanMX*	This study
YSH98	BY4741	*CLN2-TAP::KanMX PCL1-TAP::URA3*	This study
YPC708	BY4741	*DMA1-TAP::HIS3*	Euroscarf
YSH82	BY4741	*PCL1-TAP::HIS3*	Euroscarf
YSH83	BY4741	*PCL1-TAP::HIS3 pho85::URA3*	This study
YSH5	BY4741	*PCL9-TAP::KanMX*	This study
YSH12	BY4741	*PCL9-TAP::KanMX pho85::URA3*	This study
YAN31	W303-1a	*SIC1-MYC::TRP1 CLN2-HA::KanMX::NAT*	Posas lab
YSH119	W303-1a	*SIC1-MYC::TRP1 CLN2-HA::KanMX::NAT PCL1-HA::KAN*	This study

Growth Conditions, Cell Synchronization, and Cytometry Analysis—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 (15 mg/ml of leucine, 5 mg/ml of histidine, and 10 mg/ml of tryptophan) supplemented with either 2% dextrose or 2% raffinose (when indicated). Galactose induction was accomplished by initial growth in SC plus raffinose, followed by an addition of 2% galactose. α-Factor cell synchronization experiments were carried out as previously described (25). Flow cytometry analysis was performed on yeast cells stained with SYBR Green.

Genetic Screening—19 strains defective in different ubquitination systems (indicated in the Table 1) were transformed with a centromeric plasmid carrying *PCL1-HA* and the levels of Pcl1 from exponential growing cells were analyzed.

Immunoblot Analysis—Proteins were detected using the following primary antibodies: anti-PAP 1:4,000 (Sigma, P1291), anti-GST 1:1,000 (GE Healthcare, 27-4577-01), anti-HA monoclonal antibody 1:100 (a gift from Posas Lab), anti-ubiquitin 1:1,000 (Cell Signaling, 3936), anti-FLAG M2 1:500 (Sigma, F3165), or anti-Glu-6-PDH 1:500 (Sigma, A9521). The secondary antibodies used were: donkey anti-goat HRP, sheep anti-mouse HRP, and goat anti-rabbit HRP, all at 1:25,000 (all from Jackson laboratories). Immunoblots were developed using Luminata Forte Western HRP Substrate (Millipore) and images were taken with GeneSnap (Syngene) and quantified with GeneTools (Syngene).

RNA Isolation and Analysis—Cells were harvested and stored at −80 °C. Total RNA was isolated by hot phenol extraction and quantified spectrophotometrically. 2 mg of total RNA were incubated with DNase and reverse transcribed using Quanta qScript cDNA SuperMix according to the manufacturer's instructions. The cDNA was subjected to RT-PCR on a C1000 thermal cycler CFX96 RT system, and expression was normalized to *CDC28*.

Inhibition of Proteasome—Inhibition of proteasome was performed as described (26).

Protein Stability Assay—The stability of the proteins was essentially determined as in Ref. 27. Cycloheximide (Sigma, C4859) was used at a final concentration of 10 mg/ml.

Co-immunoprecipitation of GST-Pho85 and Dma1-TAP from Yeast Extracts—Yeast cells were grown exponentially for 4 h in SC with 2% raffinose. Gene expression was then induced for 4 h by adding galactose at a final concentration of 2%. Cells were harvested (500 ml, A_{660} = 1.2), and resuspended in 5 ml of cold extraction buffer A (50 mM Tris, pH 8, 15 mM EDTA, 15 mM EGTA, and 0.1% Triton X-100) containing protease inhibitors (2 mg/ml of pepstatin, 2 mg/ml of leupeptin, 1 mM PMSF, and 1 mM benzamidine), phosphatase inhibitors (10 mM sodium orthovanadate, 25 mM β-glycerophosphate, 1 mM sodium pyrophosphate, and 10 mM sodium fluoride), and 2 mM DTT. Cells were ruptured with glass beads in the FastPrep-24 (Qbiogene, 6 times for 30 s at speed 5) and lysates were clarified by centrifugation at 4 °C for 1 h at 12,000 × *g* and quantified by the Bradford assay (Bio-Rad Laboratories). 3 mg of crude extracts were incubated for 4 h at 4 °C with 200 μl of glutathione-Sepharose beads (GE Healthcare Life Sciences, 17-0756-05). After washing with buffer A, 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.

In Vitro Binding Assay—Recombinant versions of GST-Pcl1 were purified as described above. The glutathione-Sepharose beads were incubated with yeast cell extracts at 4 °C for 1 h. Beads were washed with buffer A and evaluated by immunoblot analysis.

In Vitro Ubiquitination Assay—GST-Dma1 protein ubiquitin (Ub) ligase activity assays were carried out essentially as described (23). The E1 enzyme (Uba1) and the E2 complex (HIS_{6x}-Ubc13, HIS_{6x}-Mms2) used were from Boston Biochem. The E2 enzyme Ubc4 was purified in our laboratory as a recombinant protein. The E3 GST-Dma1 and the substrate GST-Pcl1 were also purified as described above and N terminally FLAG-tagged using Ub (Sigma, U5382). The reaction was incubated for 5 h at 37 °C, and the ubiquitinated species were separated on 4–15% SDS-PAGE gradient gels.

In Vivo Ubiquitination Assays—Pcl1 was tagged at the C terminus with HIS_{3x}-HA-ProtA and overexpressed from the GAL1 promoter. Tagged proteins were purified using the protocol described in Ref. 28, under fully denatured conditions.

In Vitro Kinase Assays—Kinase assays were performed as previously described (29). Phosphorylated proteins were detected using the Pro-Q Diamond phosphoprotein gel stain kit (Invitrogen).

Phos-tag Gels—10 to 20 μg of protein from cell extracts were separated in 7% polyacrylamide/SDS gels with 10 mM Phos-tag (Wako, 304-93521) plus 20 mM $MnCl_2$ according to the manufacturer's instructions.

LC-MS/MS Analysis—*In vitro* ubiquitination reactions were performed as described above and reaction products were separated by SDS-PAGE on a 10% gel. Gel regions corresponding to ubiquitinated proteins were excised from the gel, reduced, alkylated, trypsinized, and analyzed using a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to an EasyLC (Thermo Fisher Scientific (Proxeon), Odense, Denmark). Peptides were loaded directly onto the analytical column at 1.5–2 μl/min using a wash volume of 4 to 5 times injection volume and separated by reversed-phase chromatography using a 12-cm column with an inner diameter of 75 μm, packed with 5-μm C18 particles (Nikkyo Technos Co., Ltd., Japan). Chromatographic gradients started at 97% buffer A and 3% buffer B with a flow rate of 300 nl/min, and gradually increased to 93% buffer A and 7% buffer B in 1 min, and to 65% buffer A/35% buffer B in 60 min. After each analysis, the column was washed for 10 min with 10% buffer A/90% buffer B. Buffer A was 0.1% formic acid in water. Buffer B was 80% acetonitrile + 0.1% formic acid in water.

The mass spectrometer was operated in positive ionization mode with nanospray voltage set at 2.2 kV and source temperature at 250 °C. Ultramark 1621 for the FT mass analyzer was used for external calibration prior the analyses. Moreover, an internal calibration was also performed using the background polysiloxane ion signal at m/z 445.1200. The instrument was operated in DDA mode and full MS scans with 1 microscan at a resolution of 60,000 were used over a mass range of m/z 100–2000 with detection in the Orbitrap. Auto gain control was set to $1E^6$, dynamic exclusion (60 s) and charge state filtering disqualifying singly charged peptides was activated. In each cycle of DDA analysis, following each survey scan the top 20 most intense ions with multiple charged ions above a threshold ion count of 5000 were selected for fragmentation at normalized collision energy of 35%. Fragment ion spectra produced via collision-induced dissociation were acquired in the Ion Trap, auto gain control was set to $5e^4$, isolation window of 2.0 m/z, activation time of 0.1 ms, and maximum injection time of 100 ms was used. All data were acquired with Xcalibur software version 2.2.

Data Analysis—Raw MS/MS spectra were interpreted with the Proteome Discoverer (version 1.3.0.339, Thermo Fisher Scientific) software suite for peptide identification using Sequest (version 1.20) as the search engine. The data were searched against an in-house generated database containing the 6 proteins present in the *in vitro* assay (Pcl1, Dma1, Ubc13/Mms2, Uba1, and GST). A precursor ion mass tolerance of 7 ppm was applied and up to three missed cleavage sites for trypsin were allowed. The fragment ion mass tolerance was set to 0.5 Da. Oxidation of methionine, ubiquitination (Gly-Gly) in lysine, and protein acetylation at the N-terminal were defined as variable modification; carbamidomethylation on cysteine was set as fixed modification. All spectra were manually validated.

Statistical Analysis—Data were expressed as mean ± S.D. Statistical significance was determined by Student's test for the difference between two normal groups. $p > 0.05$ was considered significant.

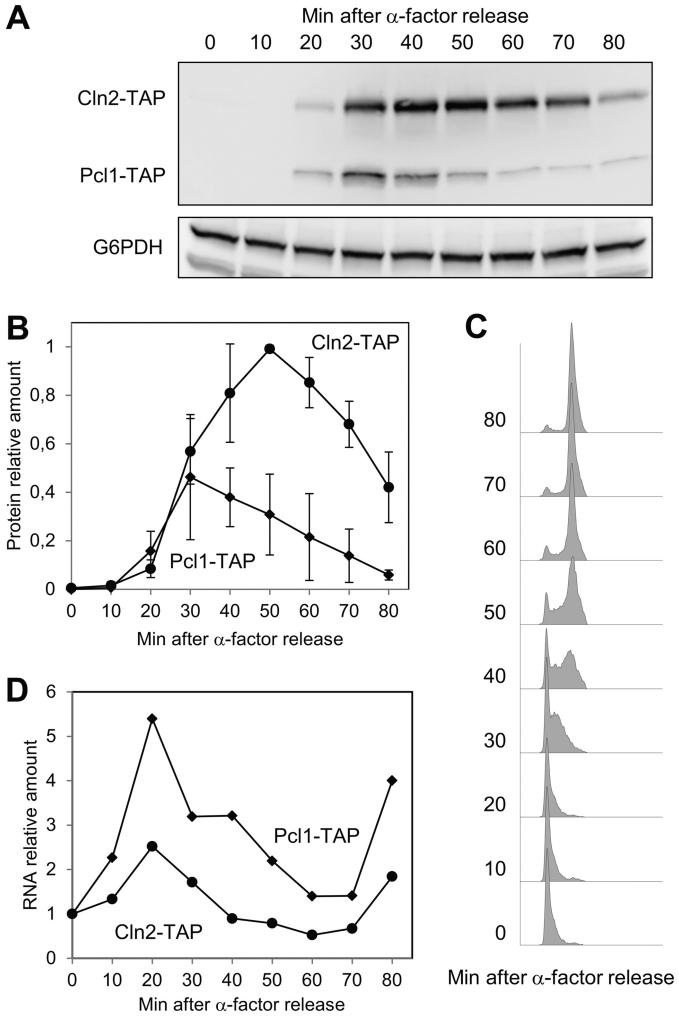

FIGURE 1. **Cln2 and Pcl1 disappear with different kinetics.** Strain YSH98 (double-tagged: *CLN2*-TAP and *PCL1*-TAP) was synchronized at G_1 with α-factor and released in YPD at time 0. Samples were collected at the indicated times and then subjected to several analyses. *A,* Cln2 and Pcl1 protein levels were analyzed by immunoblotting using monoclonal antibodies against TAP-tag; Glu-6-PDH (*G6PDH*) detection was used as a loading control. *B*, quantification of *panel A*. Data ± S.D. from 3 independent experiments are shown. *C,* flow cytometry analysis of DNA content of samples from *panel A*. *D*, RNA levels of Cln2 and Pcl1 of samples from *panel A*. Average from two independent experiments is shown.

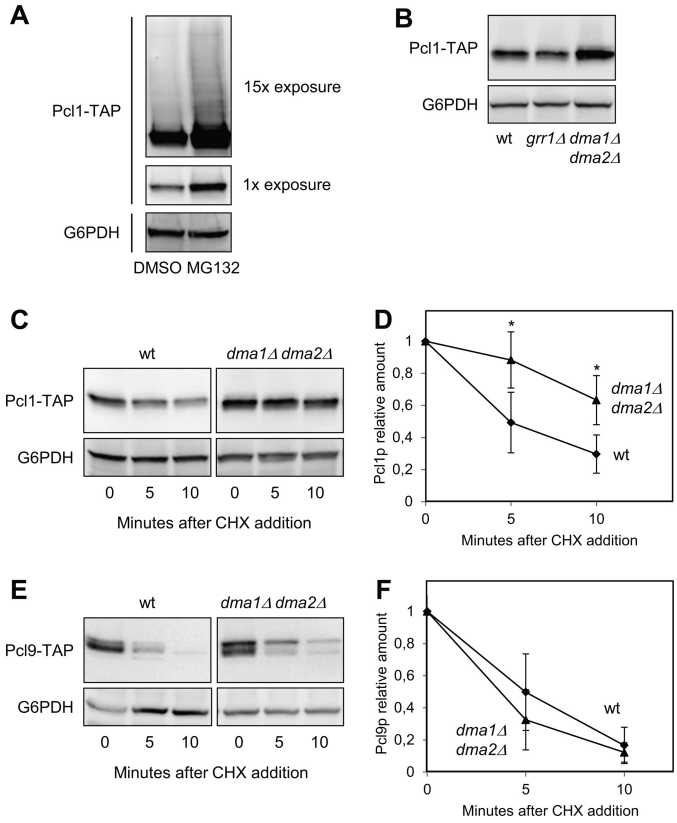

FIGURE 2. **Pcl1 is more stable in the absence of Dma activity.** *A*, Pcl1 levels increased in the presence of proteasomal inhibitors. Cells of strain YSH82 were incubated with either MG132 (a proteasome inhibitor) or a drug vehicle (*DMSO*). 60 min later, samples were taken and Pcl1-TAP protein levels were analyzed by immunoblotting using monoclonal antibodies against TAP-tag. Two different exposures of the immunoblot are shown. As in the rest of the panels, Glu-6-PDH (*G6PDH*) detection was used as a loading control. *B*, relative amounts of Pcl1 in wild type, *grr1*Δ, and *dma1*Δ *dma2*Δ strains. Cells were grown exponentially in rich media, and Pcl1-TAP levels were detected by immunoblotting using monoclonal antibodies. *C,* Pcl1 stability measurements. The indicated strains were grown exponentially in rich media and cycloheximide was added to the medium at time 0. Samples were taken at the indicated times and analyzed for Pcl1-TAP levels by immunoblotting using monoclonal antibodies. *D,* quantification of *panel C*. Data ± S.D. from 3 independent experiments are shown. *, $p > 0.05$ *versus* WT. *E,* Pcl9 stability measurements were carried out as in *panel C*. *F,* quantification of *panel E*. Data ± S.D. from 3 independent experiments are shown.

RESULTS

Turnover of Cln2 and Pcl1 Are Differently Regulated—The G_1 cyclins Cln2 and Pcl1 might act in different physiological situations suggesting that their levels could be differently regulated by uncovered mechanisms. Thus, to elucidate these mechanisms, we examined the levels of the two proteins in a synchronous population of cells as they traversed G_1.

To this end, a double TAP-tagged strain was synchronized in G_1, and then released into the cell cycle. As described, both cyclins appeared simultaneously at 20 min after release (Fig. 1, *A* and *B*), which was 10 min before the cells enter into S phase (Fig. 1*C*). Surprisingly, however, the two cyclins disappeared with different kinetics (Fig. 1*B*), Pcl1 was degraded faster than Cln2. Interestingly, we observed the same result in a strain in which both genes had been tagged with the shorter tag HA, ruling out the possibility that this effect was due to the presence of a specific tag (not shown).

CLN2 and *PCL1* are transcribed simultaneously in G_1 by the same transcription factors and with similar profiles. We confirmed that this was also true under our experimental conditions: both genes show the same expression pattern and peak at 30 min after release (Fig. 1*D*), concluding that differences observed in the levels of the two proteins were probably due to post-transcriptional mechanisms. Thus, our finding suggests that the turnover of each cyclin is regulated differently.

Cln2 and Pcl1 Are Targeted by Different E3 Ligase Systems—Several cyclins are targeted by specific ubiquitination mechanisms and degraded through the proteasome. To test if this is also true with Pcl1, we measured the amount of cyclin in cells treated with the proteasome inhibitor MG132. As shown in Fig. 2*A*, Pcl1 is accumulated in cells whose proteasome had been inhibited. Moreover, under this condition, ubiquitinated proteins are more stable, and normally appear as a "ladder" of slower-moving species on SDS-PAGE. A prominent ladder of this type of Pcl1 species, indicative of ubiquitination, was observed

upon long exposures of Western blots of extracts from cells treated with MG132 (Fig. 2*A*). This observation is consistent with the idea that Pcl1 should be destroyed by ubiquitination targeting and subsequent proteomic degradation. Thus, we decided to search for the ubiquitin ligase involved in such targeting.

Despite the high degree of similarity between Pcl1 and Cln2 at the protein sequence level, Pcl1 lacks the 200 central amino acids of Cln2, which encompass the putative PEST regions as well as a specific domain that promotes ubiquitination by targeting to the E3 ligase Grr1 (10). The absence of this region discards the possibility that Pcl1 was recognized by Grr1. Indeed, whereas the levels of Cln2 are elevated in the *grr1*Δ strain (9), the levels of Pcl1 remain normal (Fig. 2*B*).

To discover the E3-ligase of Pcl1, we transformed a centromeric plasmid carrying *PCL1* to a collection of 18 mutants of different ubiquitination systems, with the aim to find an increase in the amount of cyclin in one of the mutants (see "Experimental Procedures"). This type of screening usually yields small differences, but can help to exclude those strains that do not differ with respect to the amount of Pcl1 in wild type cells. We only observed elevated levels of Pcl1 in two strains: *dia1*Δ and *dma1*Δ *dma2*Δ. Next, we tested the stability of Pcl1 in the *dma1*Δ *dma2*Δ mutant strain by treating cells with cycloheximide. This sort of experiment allows the determination of a protein half-life independently of the initial mRNA levels. In wild type cells, Pcl1 has a half-life of 6.1 ± 0.2 min (Fig. 2*D*), and the same result is observed in the single mutants *dma1*Δ or *dma2*Δ (not shown). However, in the double mutant *dma1*Δ *dma2*Δ, Pcl1 is strongly stabilized (Fig. 2, *C* and *D*).

Finally, we tested whether Dma1 and Dma2 are also involved in control of the stability of Pcl9, a cyclin also present in G_1. As shown in Fig. 2, *E* and *F*, Pcl9 protein has an extremely short half-life (around 4 min) that is not significantly affected by the absence of Dma proteins, suggesting that Dma ubiquitin ligases have a specific effect on Pcl1 stability. Together our findings strongly suggest that Cln2 and Pcl1 are targeted by different E3 ligase systems and that Dma1 and −2 may control G_1 processes by regulating the stability of Pcl1.

Dma1 Interacts with Pcl1 and Targets It for Destruction—We initially tested the possibility that the E3 ligase Dma1 regulates Pcl1 stability through direct targeting by constructing an affinity system based on the expression of Pcl1 in bacteria as a GST fusion protein. Recombinant GST-Pcl1 was then used to bind the Dma1-TAP present in yeast cell extracts. Using this approach, Dma1 was specifically detected via immunoblot analysis of the material retained by the affinity system, indicating that both proteins interact (Fig. 3*A*).

We next checked whether Pcl1-Pho85 complexes interact with Dma1 in yeast cells *in vivo*. As shown in Fig. 3*B*, pull-down of Pho85-GST enables the detection of Dma1, suggesting that Pcl1 and Dma1 proteins also interact *in vivo*.

An *in vitro* autoubiquitination activity of Dma1 and Dma2 has been described (23). We attempted to reconstitute ubiquitination reactions consisting of yeast Uba1, FLAG-tagged Ub, His-tagged E2 enzymes (Msm2 and Ubc13), and full-length GST-Dma1 all purified from *E. coli*. As shown in Fig. 3*C*, this reconstituted system was already able to form long-chain Ub adducts as described (28), indicating that Dma1 was active. Interestingly, when Pcl1 was present, the levels of polyubiquitinated species were far higher, corroborating a Pcl1 ubiquitination that is strictly dependent on the presence of Dma1 associated to Mms2/Ubc13. Ubc4 is another E2 enzyme that promotes Dma1 activity and we wanted to test if such complexes can also ubiquitinate Pcl1. Fig. 3*D* shows that Pcl1 is also ubiquitinated *in vitro* by the Dma1-Ubc4 complexes, although clearly to a lesser extent.

We carried out the same ubiquitination assay, and after 5 h of incubation the reaction mixture was analyzed by mass spectrometry. The spectra clearly showed that some of the ubiquitinated peptides are coming from Pcl1, pointing out residues Lys-82 and Lys-121 as the ones ubiquitinated in our *in vitro* assay (supplemental data). The experiment and posterior mass spectrometry analysis was done in duplicate.

Finally, we decided to check the *in vivo* ubiquitination status of Pcl1. To this end, Pcl1 was double tagged at its endogenous C terminus with a His_6 and TAP epitopes and then purified from denatured lysates using nickel-nitrilotriacetic acid resin (28). Pcl1 ubiquitination levels were determined by immunoblotting for ubiquitin and, to validate that each protein was indeed purified, the TAP epitope also was blotted for analysis. Through this approach, we found that Pcl1 ubiquitination levels were clearly lower in the *dma1*Δ *dma2*Δ strain, indicating that Pcl1 ubiquitination *in vivo* depends on the presence of Dma proteins (Fig. 3*E*). All these results show that Dma1 interacts with Pcl1 and targets it for destruction, a finding that points Pcl1 as the first *bona fide* target of Dma ubiquitin ligases in *S. cerevisiae*.

A Specific Domain Is Necessary for the Dma1-dependent Pcl1 Destabilization—As shown above, Dma1 affects the stability of Pcl1, but not the stability of the very closely related cyclin Pcl9, suggesting that Pcl1 has a region that could facilitate the interaction with Dma1. Alignment of Pcl1 and Pcl9 reveals that the two proteins share a high level of sequence conservation, especially in the N-terminal, except for a short region of 20 amino acids. We wondered whether this region could be a docking site for Dma1. Deletion of amino acids 29 to 36 (a region we called the Dma1 Docking Domain (DDD)) greatly stabilizes Pcl1 *in vivo* (Fig. 4, *A* and *B*), pointing to this region as an important determinant for Dma1 destabilization. Accordingly, the recombinant *pcl1DDD*Δ mutant is less ubiquitinated *in vitro* by Dma1 protein (Fig. 4*C*). It is worth noting that this mutated version of Pcl1 is fully active, as it is still able to allow the phosphorylation of Sic1 (not shown); consequently, we ruled out the possibility that stabilization of the DDD deletion mutant could be due to a general loss of Pcl1 integrity. Also, it is important to stress that the DDD mutants are still phosphorylated by the Pho85 kinase (not shown), excluding the possibility that increased stability comes from the inability to be phosphorylated by the CDK.

High-throughput analyses have revealed proteins associated to Dma1 (30), although the relevance of these interactions remains unknown. Interestingly, several of these proteins contain the DDD motif (including Sid4, a Dma1 substrate in *Schizosaccharomyces pombe* (28)). We performed a ClustalW analysis of the sequence of these proteins, which yielded a consensus sequence for the DDD region: LRVVPS, with the presence of conservative changes in the hydrophobic or charged

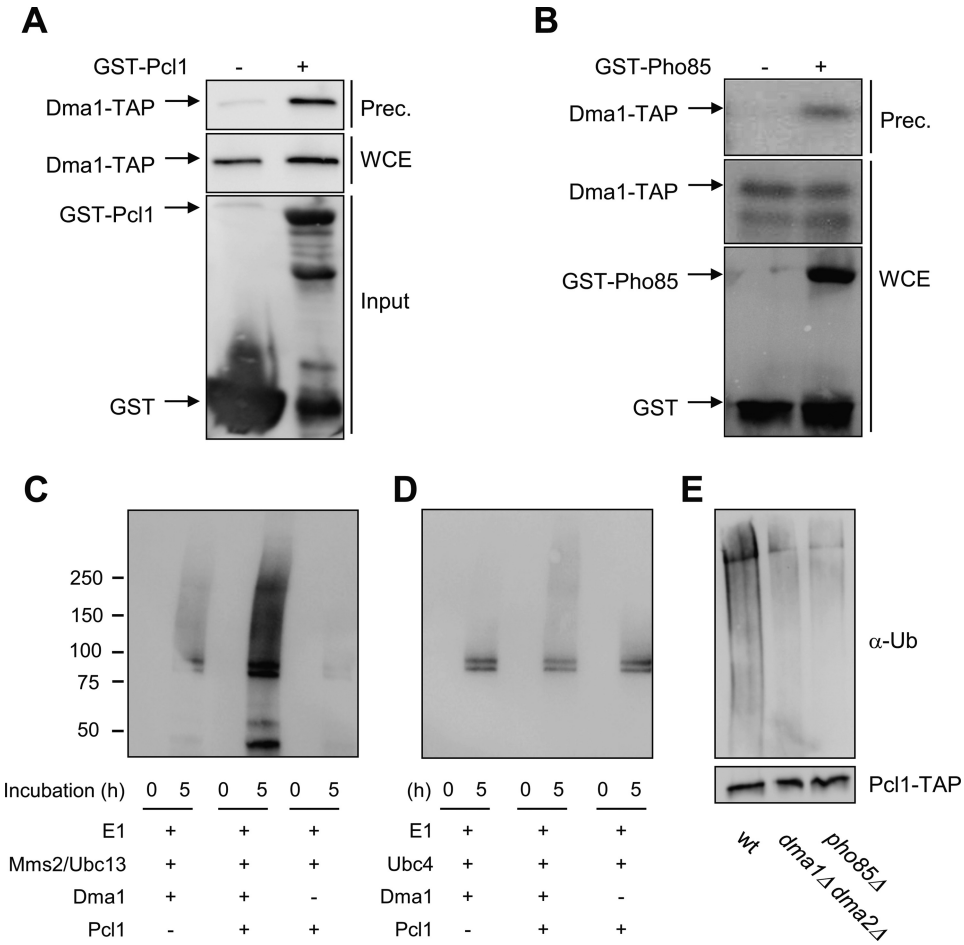

FIGURE 3. **Dma1 interacts and ubiquitinates Pcl1.** *A*, binding *in vitro* assay. Recombinant GST-Pcl1 was purified from *E. coli* and incubated with yeast cell extracts of the strain YPC708 (with a genomic TAP-tagged Dma1) for 45 min, and purified with glutathione-Sepharose beads. Samples were analyzed by immunoblot analysis to detect Dma1-TAP proteins. *WCE,* whole cell extract; *Input*, quantity of Pcl1 used in the trapping. *B*, Pcl1-Pho85 complexes co-immunoprecipitate with Dma1 *in vivo*. Yeast extracts from strains containing untagged *PHO85* or GST-*PHO85* and TAP-tagged *DMA1* (from the chromosomal locus) were precipitated with glutathione-Sepharose beads, and then probed using specific antibodies. *C* and *D,* Pcl1 is ubiquitinated *in vitro* by Dma1. Ubiquitination assays were done by *in vitro* reconstitution of E1-E2-Dma1 complexes (see "Experimental Procedures"). *Panel C* shows Dma1 activity associated to the Mms2-Ubc13 E2 complexes. *Panel D* shows Dma1 activity associated to the Ubc4 E2 enzyme. The reaction was started at time 0 by adding ATP. Samples were taken at 0 and 5 h and analyzed for ubiquitination levels by immunoblotting using α-Ub antibody. *E*, Pcl1 is ubiquitinated *in vivo* in a Dma1-dependent manner. The indicated strains carrying a centromeric plasmid with *PCL1*-His_{6x}-TAP expressed under the *GAL* promoter was grown for 4 h in synthetic complete medium with galactose as a carbon source. The same amount of immunopurified Pcl1-His was separated by SDS-PAGE gels and visualized by immunoblot using an ubiquitin antibody (*top panels*). To validate the levels of Pcl1, the blot was also analyzed using an α-TAP antibody (*bottom panel*).

amino acids (Fig. 4*D*). Interestingly, Cln2 do not have this sequence (Fig. 4*E*), in accordance to the fact that this cyclin is not affected by the presence of Dma1 activity (not shown). To test the importance of such a sequence we changed Pro-5 to Asn, and this substitution strongly stabilized Pcl1 (Fig. 4, *A* and *B*), thereby suggesting that the integrity of DDD is important for the stability of the cyclin. We are currently investigating whether this region could be a true docking domain for Dma1.

Pcl1 Is destabilized by the CDK Pho85—CDK kinases usually phosphorylate their own cyclins, thereby targeting them for destruction, in a negative feedback loop. Hence, we decided to measure Pcl1 protein levels in the absence of Pho85 activity, finding that these levels are six times greater in *pho85*Δ mutants than in wild type cells (Fig. 5*A*). Furthermore, we observed that the half-life of Pcl1 is increased in the absence of Pho85 (Fig. 5, *B* and *C*) to the same extent as Pcl9 (Fig. 5*D*). Accordingly, Pcl1 is less ubiquitinated *in vivo* in the absence of Pho85 activity (Fig. 3*E*). Together these results show that the CDK Pho85 destabilizes Pcl1 probably through a phosphorylation-dependent mechanism.

Pcl1 Is Phosphorylated and Targeted for Destruction by Pho85—We tested this latter possibility by doing *in vitro* phosphorylation experiments using recombinant proteins purified from *E. coli*. Pcl1 has six canonical sites of phosphorylation by Pho85 (Fig. 6*A*) (31). We purified different versions of Pcl1 that carried mutations at these SP/TP sites, and then incubated them with recombinant Pho85 in the presence of ATP. Fig. 6*B* clearly shows that wild type Pcl1 is phosphorylated by Pho85, whereas a mutated version in which Thr^{39} and Ser^{43} are each replaced with an alanine (the *pcl1–2A* version) is markedly less phosphorylated by Pho85. This result does not exclude phosphorylation of other residues, although clearly to a lower extent. Indeed, a Pcl1 that carries a substitution to alanines in the other four SP/TP sites (the *pcl1–4A* version) is phosphorylated to the same extend as wild type Pcl1. This result indicates that Thr^{39} and Ser^{43} of Pcl1 are the major sites phosphorylated by Pho85

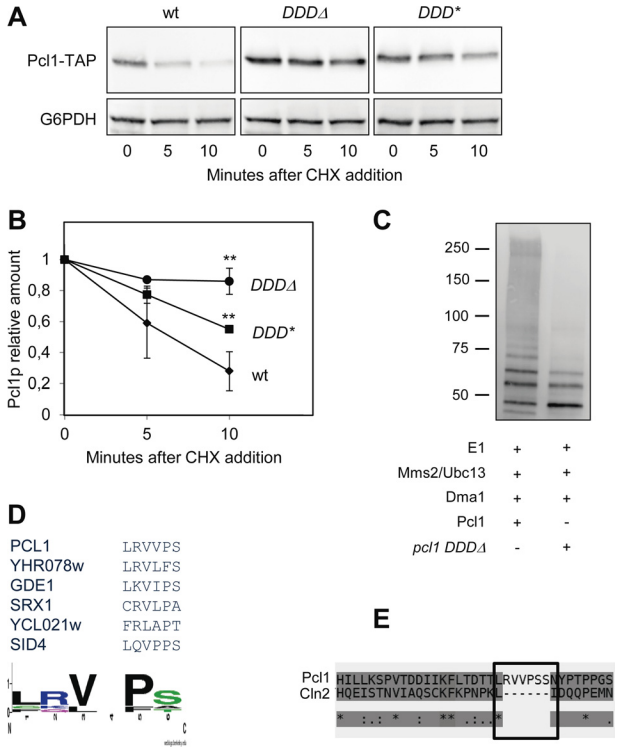

FIGURE 4. **Pcl1 has a putative docking site for Dma1.** *A*, the integrity of the DDD region is essential to maintaining Pcl1 instability *in vivo*. Different alleles of Pcl1 were expressed in wild type cells from a centromeric plasmid under its own promoter: Pcl1 wild type, Pcl1 with a deletion in the DDD region (*DDD*Δ), and Pcl1 with mutations V31N and P33N (*DDD**). All these strains were grown exponentially in rich medium and, at time 0, cycloheximide was added to the medium. Samples were taken at the indicated times and analyzed by immunoblotting using monoclonal antibodies to determine Pcl1-TAP levels. Glu-6-PDH (*G6PDH*) detection was used as a loading control. *B,* quantification of *panel A*. Data ± S.D. from 3 independent experiments are shown. **, $p > 0.01$ *versus* WT. *C, pcl1 DDD*Δ is less ubiquitinated *in vitro* than Pcl1. Ubiquitination assays were done by *in vitro* reconstitution of E1-Mms2-Ubc13-Dma1 complexes (see "Experimental Procedures"). The ubiquitination levels of Pcl1 or *pcl1 DDD*Δ are shown. The reaction was started at time 0 by addition of ATP and finished at 3 h. Samples were taken and analyzed for ubiquitination levels by immunoblotting using α-Ub antibody. *D,* proposed consensus DDD sequence. WebLogo3 analysis (50) of the DDD regions present in several Dma-interacting proteins. *E,* T-Coffee alignment between the N-terminal region of cyclins Pcl1 and Cln2. Cln2 presents a gap that exactly matches the proposed DDD region.

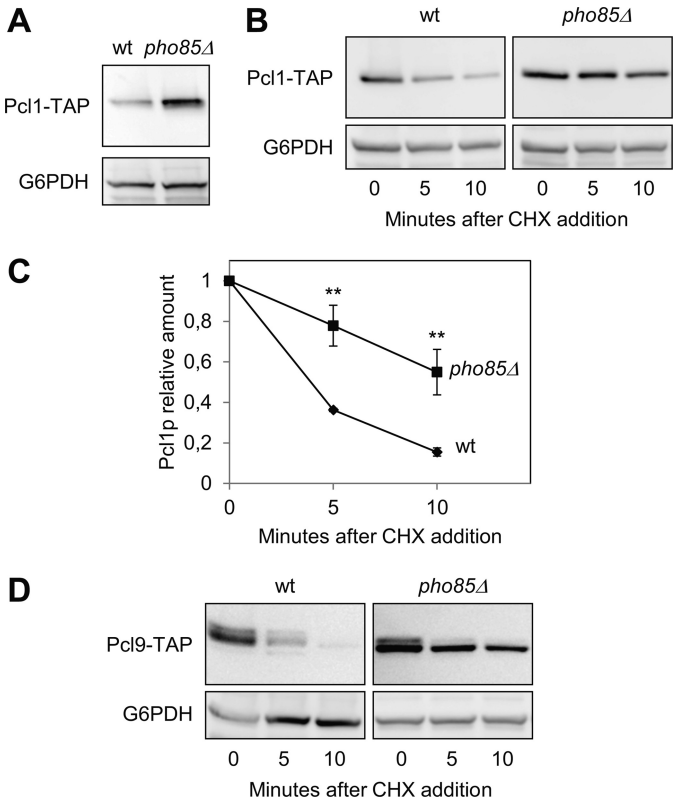

FIGURE 5. **Pcl1 is destabilized by Pho85 activity.** *A*, Pcl1 protein levels are higher in the *pho85*Δ strain. Wild type and *pho85*Δ cells were grown exponentially in YPD, and then Pcl1-TAP levels were determined using immunoblotting with monoclonal antibodies. Glu-6-PDH (*G6PDH*) detection was used as a loading control in each *panel*. *B*, Pcl1 is stabilized in the *pho85*Δ strain. Wild type and *pho85*Δ cells were grown exponentially in rich medium, and cycloheximide was added to the medium at time 0. Samples were taken at the indicated times, and analyzed for Pcl1-TAP levels by immunoblotting using monoclonal antibodies. *C,* quantification of *panel B*. Data ± S.D. from 3 independent experiments are shown. **, $p > 0.01$ *versus* WT. *D,* Pcl9 stability is also controlled by Pho85. Analyses were carried out as in *panel B*.

in vitro. It is interesting to note that these two sites are the nearest to the DDD sequence.

Pcl1 from wild type yeast cells gives a sharp band in standard gel electrophoresis. However, when analyzing extracts in Phos-tag large electrophoresis gels, we distinguished several bands of Pcl1, indicating that such cyclin is phosphorylated *in vivo*. Moreover, the mutant *pcl1–2A* show a clear change in lower mobility bands suggesting that the mutant is phosphorylated to a lesser extent *in vivo* (Fig. 6*C*). More interestingly, the alanine substitution of Thr^{39} and Ser^{43} leads to strong stabilization of Pcl1 (Fig. 6, *D* and *E*), indicating that *in vivo* phosphorylation of Thr^{39} and Ser^{43} is essential for destabilizing the cyclin. As described to other cyclins (10), the substitution of these phosphoacceptor sites by glutamic acid does not mimic the phosphorylation effect and does not decrease the stability of Pcl1 (not shown), suggesting that a negative charge at those two Pcl1 residues is not sufficient for the Dma1 destabilization. Finally, we measured the half-life of the *pcl1–2A* version in a WT and a *dma1*Δ *dma2*Δ strain, and *pcl1–2A* was not further stabilized (Fig. 6*F*), indicating that there was no additive effect between Pho85 and the Ub-ligase.

Pho85 and Dma1 Activities Are Essential for Controlling Pcl1 Levels in the Cell Cycle—At this point, we propose that during G_1, Pcl1 is phosphorylated by Pho85 at the closest residues to the DDD sequence, and that this event increases the ubiquitination of Pcl1 by Dma1 (and probably by Dma2), allowing rapid destruction of the cyclin (see a scheme of the proposed model in Fig. 7*A*). Based on this model, we predicted that cells require both Pho85 and Dma1 activity to down-regulate Pcl1 at the end of G_1. To test this prediction we tracked the levels of Pcl1 from different strains synchronized in G_1 by α factor and subsequently released in rich media. In wild type cells, Pcl1 appears 20 min after release and rapidly disappears, being almost completely depleted at 50 min (Fig. 7*C*). The nonphosphorylatable version of *pcl1–2A* is clearly present for longer periods during the cell cycle (Fig. 7*B*). Similarly, the absence of Dma1/2 proteins also leads to accumulation of Pcl1 beyond the G_1/S transition. The differences observed cannot be explained by differences in the progression through G_1, because the three strains enter into G_2 with the same kinetics (Fig. 7*B*). Thus, we con-

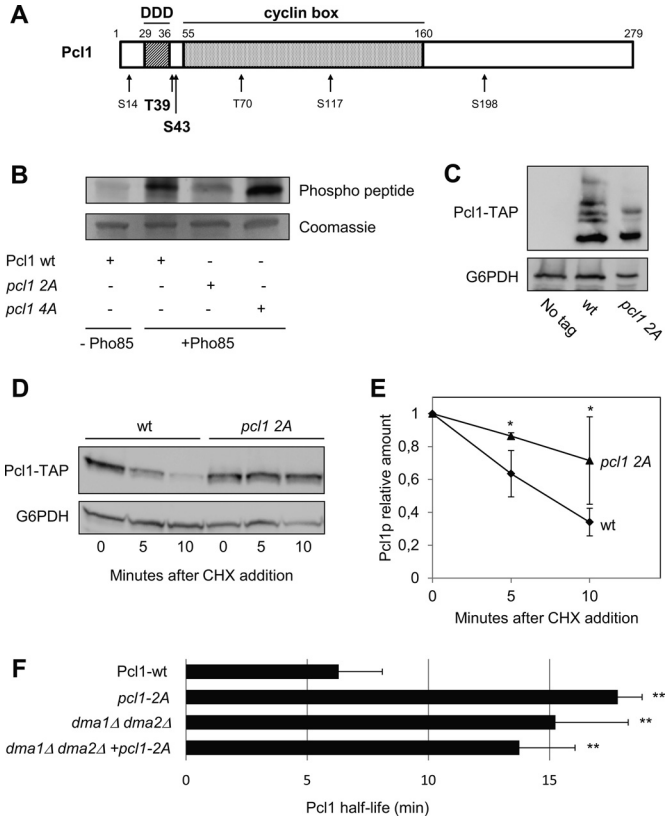

FIGURE 6. **Pcl1 is phosphorylated by Pho85.** *A*, scheme of the Pcl1 protein. *Numbers on top* indicate the amino acid positions. The DDD (Dma1 Docking Domain) region is located between amino acids 29 and 36. *Numbers on bottom* refer to the potential CDK-phosphorylation sites. The proposed Pho85-phosphorylation sites are shown in *bold type* (see below). *B*, Pho85 phosphorylates Pcl1 *in vitro*. Recombinant GST-Pho85 purified from bacteria was incubated with different GST-Pcl1 versions (also purified from bacteria) in the presence of ATP. The 3 Pcl1 versions were: Pcl1 WT, *pcl1 2A* (T39A; S43A), and *pcl1 4A* (S14A; T70A; S117A; S198A). Coomassie staining was used as a loading control. *C*, gel electrophoresis in the presence of 10 mM Phos-tag was carried out to resolve the different phosphorylation populations between Pcl1-TAP and *pcl1 2A*-TAP. Pcl1 levels were analyzed by immunoblotting using monoclonal antibodies. In all panels of the figure, Glu-6-PDH (*G6PDH*) detection was used as a loading control. *D*, nonphosphorylatable versions of Pcl1 are stabilized *in vivo*. Pcl1 WT or *pcl1 2A* were expressed in wild type cells from a centromeric plasmid under its own promoter. Both strains were grown exponentially in rich medium, and cycloheximide was added to the medium at time 0. Samples were taken at the indicated times and analyzed using immunoblotting with monoclonal antibodies to determine Pcl1-TAP levels. *E*, data ± S.D. from 3 independent experiments (performed as in *panel C*) is shown. *, $p > 0.05$ *versus* WT. *F*, Pcl1 half-life is Dma1 and Pho85 dependent. Pcl1-TAP or *pcl1 2A-TAP* were analyzed by Western blot in the noted strains after the addition of cycloheximide (*CHX*). Data ± S.D. from 3 independent experiments are shown. **, $p > 0.01$ *versus* WT.

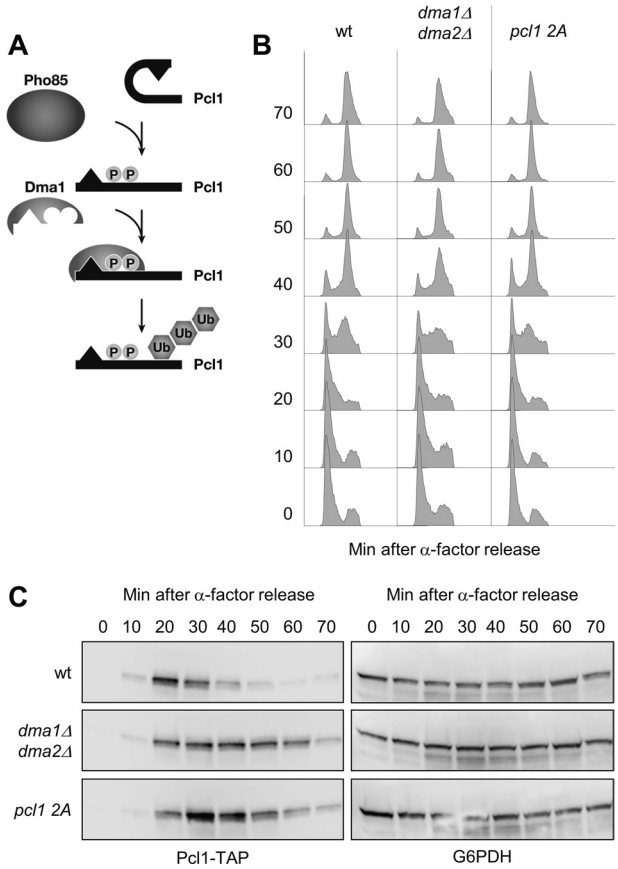

FIGURE 7. **Pho85 and Dma1 activities are essential for controlling Pcl1 levels in the cell cycle.** *A*, proposed model of Pcl1 targeting. Pho85 phosphorylates Pcl1 allowing the recognition by Dma1 that polyubiquitinates and targets Pcl1 for destruction. *B*, the DNA content from cells collected in the experiment shown in *C* was analyzed by flow cytometry at the indicated time points. *C*, Pcl1 requires Dma1 activity to be destabilized under physiological conditions. Wild type cells carrying a Pcl1 or *pcl1 2A* version expressed from a centromeric plasmid and *dma1*Δ *dma2*Δ cells expressing Pcl1, were synchronized with α-factor, and released in rich medium. Samples were collected at the indicated times and then subjected to several analyses. Glu-6-PDH levels were used as a loading control.

cluded that Pho85 and Dma1 are necessary to control Pcl1 levels during the cell cycle.

G_1 Cyclin Levels Are Controlled in Response to Nutrients—An important question arising in this work is: what is the advantage of controlling G_1 cyclins by two different ubiquitination systems? We reasoned that this double control could enable better response to diverse environmental situations. In this regard, Grr1 has been implicated in response to variations in nutrient levels, a finding that links cell cycle control to nutrient availability (9). Dma1 has also been related to nutrient response and G_1 control (32), although the underlying molecular mechanism remains unknown. In this context, we sought to ascertain the behavior of the two cyclins under different nutrient situations.

To this end, we synchronized yeast cells in G_1, released them either in rich media (YPD) or in a synthetic complete media (SD), and then followed the levels of Pcl1 and Cln2. In YPD, Cln2 was more abundant than Pcl1; pointing to the relevance of Cln2 in this growing condition (Fig. 8*A*). However, when the cells are released in SD media, the cyclins seem to have inverted profiles: Pcl1 is more abundant than Cln2. Considering that the two cyclins are transcribed at the same levels under both conditions (not shown), we reasoned that the stability of Cln2 and Pcl1 proteins is differentially affected according to the nutrient status.

It has been predicted that Dma1 levels might be modulated by nutrient conditions (32). Thus, we asked whether the high levels of Pcl1 in SD media could be a consequence of the down-regulation of Dma1 activity. This was indeed the case: Dma1 levels in cells grown in SD were clearly lower than those of cells grown in YPD (Fig. 8*B*). This result indicates that nutrient status regulates levels of Dma ubiquitin ligases, and suggests that physiological control of Pcl1 depends on Dma1 activity. In summary, we propose here that cyclins Pcl1 and Cln2 are degraded by different mechanisms, and that this differential control

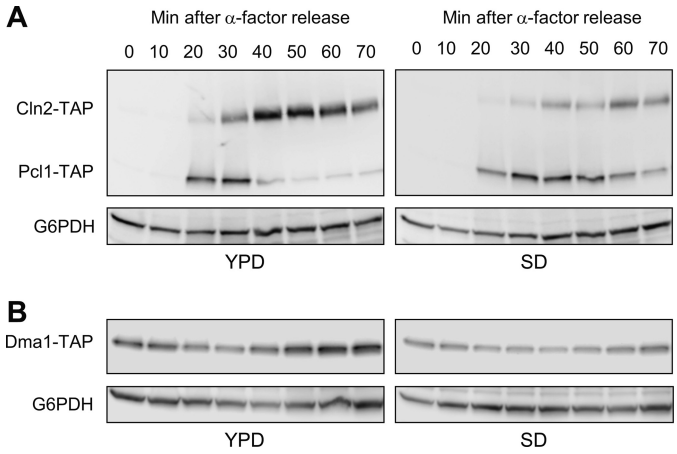

FIGURE 8. **G_1 cyclin levels are controlled in response to nutrients.** *A,* Pcl1 levels are controlled in response to nutrients. The strain YSH98 (double tagged: *CLN2*-TAP and *PCL1*-TAP) was synchronized at G_1 with α-factor. At time 0 the cells were released in either YPD or SD media. Samples were collected at the indicated times, and Cln2 and Pcl1 levels were analyzed by immunoblotting using monoclonal antibodies against TAP-tag. Glu-6-PDH (*G6PDH*) levels were used as a loading control. *B,* Dma1 levels are controlled in response to nutrients. Strain YPC708 was synchronized, sampled, and analyzed as described in *panel A*.

could be essential to ensure correct progression through G_1 in distinct situations of nutrient availability.

DISCUSSION

There are high number of cyclins that control the G_1 phase of eukaryotic cell cycle (33). Such multiplicity is thought to facilitate cellular adaptation to different physiological situations; accordingly, the levels of different cyclins should be differentially regulated. Here we provide substantial evidence that in *S. cerevisiae*, two redundant cyclins expressed at START by the same transcription factors are post-translational regulated by two different E3 ligase systems.

Our motivation for this work came from our earlier observation that Pcl1 is destroyed at the G_1/S boundary, unlike Cln2, which persists until the end of the S phase. Based on this result, we suspected that in *S. cerevisiae* different ubiquitination systems might be involved in destroying distinct G_1 cyclins.

A Novel Function for Dma1: G_1 Cyclin Targeting—The screening designed in this work enabled us to determine the E3 ligases Dia2, and Dma1/2 as controllers of the Pcl1 levels. Interestingly, absence of Dia2 (the name comes from Dig into agar) leads to an invasive growth phenotype (34). It is tempting to speculate that the control of Pcl1, a cyclin involved in morphogenetic events, could be one of the roles of Dia2. Regardless, cycloheximide experiments show that Dia2 activity does not affect Pcl1 stability (not shown), and we propose that the observed effect could be transcriptional. In contrast, we have demonstrated that Dma1 activity does affect Pcl1 stability and that Pcl1 is actually a Dma1 substrate, both *in vitro* and *in vivo*. We think that this result is important for various reasons.

First, the Dma ubiquitin ligases are well conserved across evolution and have been demonstrated to control the cell cycle in diverse organisms: in humans *CHFR* regulates the antephase checkpoint (18); in *S. pombe*, Dma1 controls the SIN, at the end of mitosis (35); and in *S. cerevisiae*, it controls G_2 progression (23). Thus, considering the new G_1 role that we describe here, the Dma ubiquitin ligases can be considered as general cell-cycle regulators.

Second, although Dma enzymes were discovered 10 years ago, Pcl1 is the first *bona fide* substrate of Dma1 described in *S. cerevisiae*. Dma1 also controls Swe1 and Elm1 localization but the mechanism underlying such regulation is unknown and could be an indirect effect (27, 36). Interestingly, Dma1 affects the functionality of septins (21), Swe1, Elm1, and Pcl1, all of these proteins are located at the bud neck, suggesting that the action of the Dma1 is focused mainly at the bud neck.

Different E3 Ligases Control G_1 Cyclin Turnover—The novelty suggested from this work is that the multiplicity of G_1 cyclins is controlled by different E3 ligase systems. To date, SCF was the only E3 involved in the targeting for destruction of several G_1 elements. Indeed, SCF_{Cdc4} is required for degradation of CDK inhibitors Sic1, Far1, Cdc6, and Gcn4 (36–38). Furthermore, SCF_{Grr1} targets cyclins Cln1 and Cln2 (and probably Cln3) for degradation (7, 39). However, our present findings indicate that at least another E3 ubiquitin ligase helps orchestrate G_1 destruction.

The *in vitro* ubiquitination systems reconstituted in our laboratory have shown that Pcl1 is ubiquitinated by Dma1 to a greater extent when it is associated with the Msm2/Ubc13 dimer than when it is associated to Ubc4. The nature of ubiquitin conjugation by E2-E3 complexes is critical because the outcome of ubiquitination is usually determined by the topology of the conjugate. Ubc13-Msm2 is a heterodimeric E2 ligase that forms Lys^{63} chains of polyubiquitin, whereas Ubc4 shows no specificity in ubiquitin lysines and the linkage is usually driven by the ubiquitin ligase (40, 41). Even though Lys^{48}-linked polyubiquitination is the usual signal for directing a protein to proteasomal destruction, Lys^{63}-linked ubiquitin chains have been reported to be involved in some cases of proteasome-dependent degradation (42, 43). Thus, we postulate here that targeting of Pcl1 for degradation by Dma1 may occur via Ubc4 or Msm2/Ubc13.

Specificity of Dma1-Pcl1 Interactions—The specificity of E3 ligase interactions with their substrates probably depends on specific destruction box signals in the latter. In this regard, a short sequence in Cln2 has recently been described as a possible docking site for Grr1 (10). Here we provide preliminary evidence that Dma ubiquitin ligases also might recognize their substrates through a specific sequence, which we have named the DDD. The DDD sequence found in Pcl1 is LRVVPS. However, we must emphasize the fact that, although some amino acids are represented in a greater proportions (see logo of Fig. 4*D*), the DDD region allows for conservative changes in sequence. In the absence of crystallographic data to confirm our hypothesis, we propose here the sequence L(O)V(J)PS(*X*)N as a general DDD sequence (where O represents any charged amino acid, J represents any hydrophobic amino acid, and *X* any amino acid). Interestingly, we searched for this sequence in the yeast proteome and found it in several proteins that control the cell cycle. We are currently investigating if such proteins might be undescribed substrates of Dma1.

Pcl1 Is Phosphorylated and Targeted for Destruction by Its Own CDK—Degradation of Pcl1 after self-catalyzed phosphorylation ensures self-limitation of the activity of the Pho85-Pcl1

complex *in vivo*. This is not a surprising result, given that the cyclins Pcl5 and Pcl9 are destabilized by Pho85 activity (Refs. 45 and 46, and this work), and that Cln1 and Cln2 are also targeted by their own CDK, Cdc28. Thus, our results reinforce the idea that phosphorylation is a general mechanism conserved among the CDKs to control the amount of their own cyclins.

We show that Pcl1 is phosphorylated at Thr^{39} and Ser^{43}. Without excluding other possible residues, we propose that these two residues are the most physiologically relevant to controlling stability of the protein. Indeed, the substitution to alanines of these two residues renders Pcl1 highly stable and, interestingly, the same two residues are found to be the uniquely phosphorylated *in vivo* in a high throughput analysis (44).

The fact that Thr^{39} and Ser^{43} are the two nearest residues to the DDD suggests that this phosphorylation could somehow facilitate interaction between Pcl1 and Dma1. One possibility is that the DDD is usually hidden in Pcl1, but becomes exposed upon phosphorylation of these residues by Pho85, thereby facilitating the interaction between Pcl1 and the E3 ligase. A similar situation has been described in Cln2 that needs a specific region plus the neighboring phosphorylation to be degraded by the Grr1 (10).

Multiplicity in Control, Flexibility in Decisions—The double system of cyclin degradation proposed in this work is probably widespread. For instance, Clb5 and Clb6, which are essential for proper management of S phase, are degraded by anaphase-promoting complex and SCF, respectively (27). Clb5 and Clb6 perform different functions, and accumulation of Clb6 outside of S phase is toxic to cells. However, the G_1 cyclins in *S. cerevisiae* seem to be functionally redundant, which begs the question: are the complexes Cln2-Cdc28 and Pcl1-Pho85 differentially regulated?

Although we cannot definitively answer this question, we have ruminated upon a suggestion that both CDK complexes regulate proteins in the same way but under different conditions (45). This idea is supported by the finding that the G_1 profile of Cln2 and Pcl1 levels depends on nutrient availability. It is striking that Grr1 and Dma1 are also related to the response to changes in nutrient availability (9, 32), and we show here for the first time that Dma1 levels are regulated by nutrient status. Albeit we do not know the mechanism underlying the down-regulation of Dma1, we propose that this process is essential to increase the levels of cyclin Pcl1. Overall, our work supports the notion that the multilayered control of different G_1 CDK-cyclin complexes furnishes cells with the flexibility to adapt to new situations.

A New Conserved Role for Dma1?—Protein sequence alignments between Pcl1 from *S. cerevisiae* and its orthologs from other *Saccharomyces* species indicate that the two putative CDK phosphorylation sites and the DDD sequence are extremely conserved. Moreover, the Pcl1 of *Candida* and other, less related yeasts also contain the DDD region. More interestingly still, is the fact that DDD and the neighboring sequence are conserved in the uncharacterized human cyclin I, which is most similar to Pcl1, and also lacks PEST regions (46). These observations suggest that the destruction of Pcl1 at the G_1/S transition may be conserved through the evolution.

In humans, Dma proteins (named Chfr) play a specific role in the antephase checkpoint. The significance of *CHFR* function in mitosis is reflected by the finding that this E3 ligase is absent or nonfunctional in several transformed cell lines and tumors (47). The study of *CHFR*-null mice presents the *CHFR* as a tumor suppressor that normally acts to prevent aberrant mitosis (48). Although the final target of Chfr is unknown, several lines of evidence support the idea that Cyclin A may be the initial target of the antephase checkpoint (49) thus pointing to the possibility that, in mammals, Chfr would also control cyclin stability. Our laboratory is currently seeking to determine which aspects of the mechanism that we present here are conserved in mammals.

Acknowledgments—We gratefully acknowledge F. Posas for advice and valuable mentoring of J. Clotet all these years. We also gratefully acknowledge S. Menoyo, B. Andrews, and M. Aldea for stimulating discussions, F. Posas for providing yeast strains, M. Pérez for technical assistance, and O. Mirallas for collaboration in some of the experiments. We also thank Armando Terrero for the execution of the scheme shown in Fig. 7A.

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DISCUSIÓN GLOBAL

Pho85 controla la transición a lo largo de G1 mediante un doble mecanismo que involucra la regulación de su ciclina Pcl1 y la fosforilación de la ciclina Cln3 como sustrato.

En el caso de Cln3, ciclina de Cdc28, se sabía que es una ciclina que normalmente está controlada en condiciones de pocos nutrientes en el medio mediante una bajada en las tasas de transcripción y traducción. Esta ciclina es muy inestable, con una vida media muy corta, pero a pesar de ello observamos que la cantidad de esta ciclina es estable a lo largo del ciclo. Nuestros resultados demuestran que Pho85/Pho80 afectan a la estabilidad de la ciclina cuando la disponibilidad de fosfato en el medio queda comprometida. Esta triple manera de regular Cln3 a nivel de transcripción, traducción y estabilidad sería importante para limitar su disponibilidad y proporcionar a la célula una respuesta rápida ante la falta de disponibilidad en el medio de nutrientes.

Además, es la primera vez que la ruta de Pho85/Pho80 es involucrada directamente en la regulación de la maquinaria del ciclo celular, sugiriendo la coordinación entre la homeóstasis del fosfato y el ciclo celular. También es de especial relevancia destacar que es el primer caso descrito donde una CDK regula la ciclina de otra. De hecho, durante la realización de este trabajo doctoral, otro grupo ha descrito una función similar donde Pho85/Pcl2 regula la cantidad de Cln3 a través de Hsp70 en respuesta a nitrógeno (15), destacando a Pho85 como un controlador general de Cln3 en respuesta a la variación de niveles de nutrientes.

Dado que Pho85 está involucrada en autofagia (41), planteamos la posibilidad de que esta pudiese ser el mecanismo de destrucción de Cln3. Comprobamos que Cln3 se acumulaba en una cepa incapaz de realizar autofagia (*atg1Δ)*, indicando que este podría ser el mecanismo de degradación. Pero, a continuación, observamos que, en el doble mutante sin autofagia ni la actividad de Pho85, Cln3 era degradado igualmente descartando esta hipótesis.

Actualmente nuestro grupo está buscando la E3 ligasa que podría estar ubiquitinando a Cln3 bajo estas condiciones. Para ello, hemos realizado un *screening* como el descrito en el presente trabajo para Pcl1, en diferentes cepas sin las ubiquitin ligasas, y no hemos encontrado ninguna ligasa concreta (resultados no publicados). Esto es acorde con la reciente publicación del grupo de Benanti (24), en el que se describió que Cdc4 y Grr1 actúan conjuntamente en la degradación de Cln3. Por otra parte, el análisis estructural de esta Cln3 permite detectar la presencia del DDD descrito para la ubiquitinación por parte de Dma1, indicando que esta podría ser otra E3 ligasa que quizá actuaría ante la falta de fosfato. Esta múltiple regulación de la degradación de Cln3 podría permitir la especulación de la múltiple degradación de Cln3 en diferentes sub-localizaciones celulares, donde Cdc4 se encargaría de degradar la ciclina Cln3 del núcleo, Grr1 la del citoplasma y Dma la del cuello o el extremo de la célula hija.

Con respecto a Pcl1, al inicio de este trabajo era asumido que el complejo encargado de la degradación de todas las ciclinas de G1 era el SCF. De hecho, las dos ciclinas Cln1 y Cln2 son degradadas por el SCF-Grr1 (23, 42) pero en este trabajo hemos demostrado que hay al menos una nueva E3 ligasa que ayuda destruir otras ciclinas de G1: como mínimo a Pcl1.

Gracias al *screening* que hemos realizado, pudimos demostrar que la actividad de Dma1 era necesaria para la estabilidad de Pcl1. De nuevo, la estabilidad emerge como factor clave de la regulación de las ciclinas. Curiosamente, Dma1 es una E3 ligasa conocida generalmente por su actividad durante la mitosis del ciclo regulando sustratos como las septinas (38). Por ello, resultó curioso observar que también regulaba una proteína de G1, mostrándose así como un regulador general del ciclo celular.

Hemos podido demostrar mediante ensayos de ubiquitinación *in vitro* que Pcl1 es preferentemente ubiquitinado por Dma1 cuando está asociado a las E2 conjugadoras Mms2/Ubc13 y lo es menos cuando está asociada a Ubc4. Esto es importante dado que la naturaleza de la proteína E2 conjugadora es la que determina la tipología de la ubiquitinación. En este caso, Mms2/Ubc13 es una E2 heterodimérica que forma cadenas de poliubiquitina en las Lys63 (43) y éstas están involucradas en el marcaje de las proteínas para su destrucción (44, 45).

Han sido varias las proteínas de *S. cerevisiae* sospechosas de ser substratos de Dma1 (todas ellas se sitúan en el cuello de la levadura, indicando que su función la lleva generalmente a cabo en esta localización), pero este trabajo es relevante también por el hecho de haber permitido el descubrimiento del primer substrato *bona fide* de Dma1.

El complejo SCF tiene un degrón conocido: las secuencias PEST. Obviamente, al no conocerse ningún substrato, al inicio de este trabajo se desconocía cual podía ser el degrón de Dma1. En esta tesis doctoral se propone una corta secuencia a la que he denominado DDD (*Dma1 Docking Domain*) que podría ser la región que reconoce Dma1 en sus sustratos para poder actuar sobre ellos. Esta región está conservada en prácticamente todas las proteínas que mediante aproximaciones proteómicas se sabe que interactúan con Dma1 y Dma2 y, en cambio, no está presente en otras ciclinas de G1 como Cln2 que no son marcadas para degradación mediante esta E3 ligasa.

Cabe remarcar que aunque la secuencia del DDD presente en Pcl1 es LRVVPS, ésta admite algunos cambios conservativos cuando es buscada en otras proteínas. Por ello, se propone la secuencia L(O)V(J)PS(X)N como secuencia general del DDD (donde O significa un aminoácido polar, J representa un aminoácido hidrofóbico y X es cualquier aminoácido).

Además de la actividad Dma sobre Pcl1 descrita, también hemos observado que es necesaria la actividad de su CDK, Pho85, para que Pcl1 sea degradada correctamente. En ausencia de Pho85, Pcl1 se estabiliza *in vivo*, lo que refuerza la idea de que la fosforilación por parte de la CDK es un mecanismo general conservado para controlar la cantidad de sus propias ciclinas, como también es el caso de Cdc28 la cual fosforila a Cln1 y Cln2 para que puedan ser degradadas.

El hecho de que la Thr39 y la Ser43 sean los dos residuos de fosforilación más cercanos al DDD, sugiere que de alguna manera, facilitan la interacción entre Pcl1 y Dma1. Una posibilidad es que la secuencia DDD se encuentre normalmente escondida en Pcl1, pero quede expuesta cuando ésta es fosforilada por Pho85 en estos residuos. Una situación similar ha sido descrita para Cln2 la cual necesita una región específica y la fosforilación para ser degradada por Grr1 (27).

Este trabajo sugiere que la degradación diferencial de las ciclinas Cln y Pcl permite comprender el viejo dilema de la redundancia: es cierto que los Clns y Pcls fosforilan los mismos substratos pero deben hacerlo en condiciones ambientales diversas (16). Efectivamente en esta tesis se ha demostrado que Cln2 y Pcl1 tienen perfiles de expresión diferentes dependiendo de la disponibilidad de nutrientes en el medio. Esto es consistente con la idea de que Grr1 y Dma1 también están relacionados con la respuesta a los cambios en la disponibilidad de los nutrientes (26, 46), pero es la primera vez que los niveles de Dma1 aparecen regulados también por esta condición.

Todos estos resultados, apoyan la idea de que la regulación de las CDK/ciclina mediante diferentes mecanismos proporciona a la célula flexibilidad para adaptarse a nuevas situaciones.

Finalmente, cabe preguntarse el grado de conservación evolutivo que tienen los mecanismos descritos en esta tesis. Al alinear la región DDD con bancos de datos de proteínas de otras especies se observa que dicha región se encuentra conservada en algunas proteínas. La región DDD con posibles sitios de fosforilación contiguos, es encontrada en *Candida* y también en una serie de ciclinas poco conocidas humanas. De hecho, la primera proteína que aparece como resultado de un análisis de tipo BLAST es una ciclina: la ciclina I1, aunque también puede encontrarse ligeramente modificada en , Ciclina I2, CNTD1 y CNTD2. Estas ciclinas son de menor tamaño que las ciclinas canónicas de mamífero, contienen una única cada Cyclin Box, tiene conservado el Asp136 (necesario para la activación en ausencia de Cak1) y no tienen secuencias PEST claras (47). Todo ello recuerda a las Pcls de Pho85 y por ello, a estas nuevas 4 ciclinas, nuestro grupo las ha denominado como HPC (*Human Pickle Cyclins*).

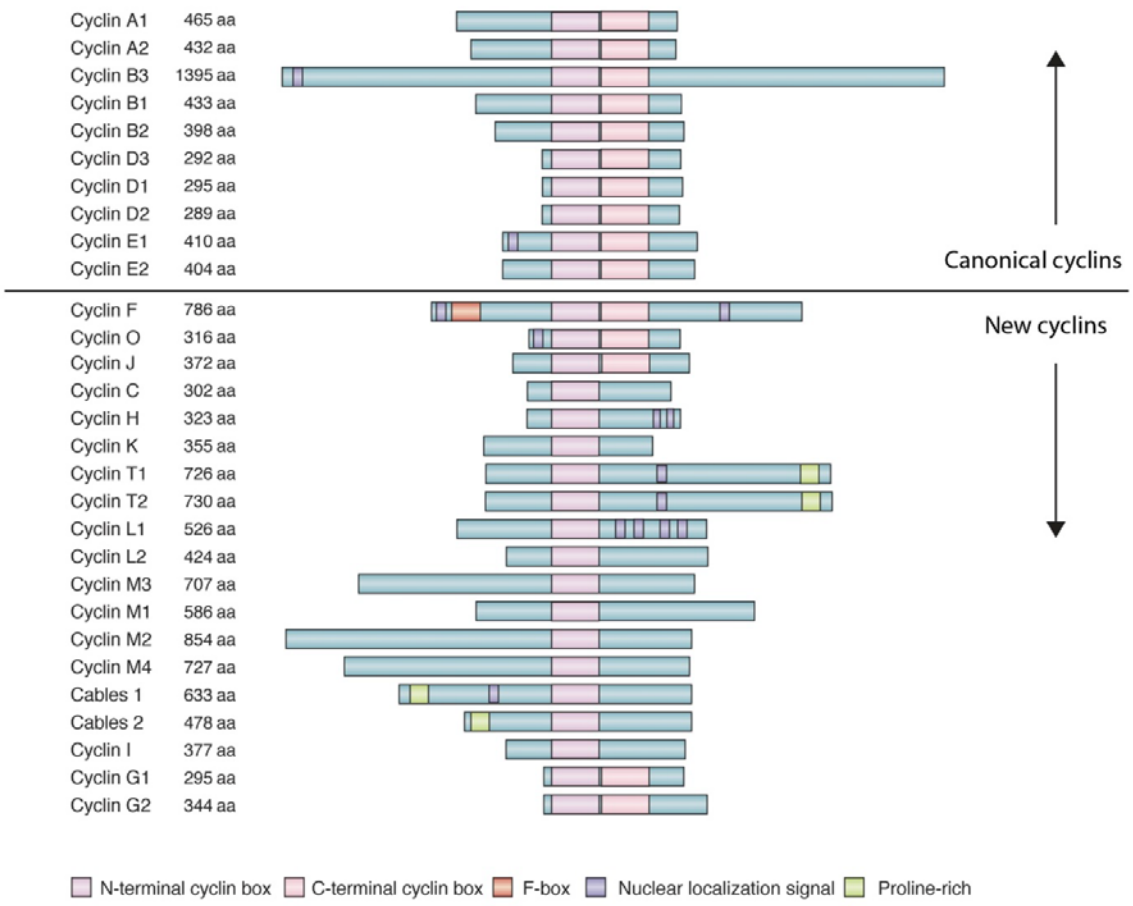

Figura 7. Esquema de ciclinas en mamíferos. Se muestran en la parte superior las ciclinas canónicas conocidas hasta ahora y en la parte inferior las nuevas ciclinas descubiertas a partir del proyecto de secuenciación del genoma humano. Adaptado de Malumbres y Barbacid, 2005.

Sorprendentemente, hasta el momento, se desconoce su papel en la fisiología de las células normales y mucho menos en células tumorales. De la única que se conoce algo más acerca de su involucración en la regulación del ciclo celular es de ciclina I, a la que muy recientemente se ha implicado en la regulación del ciclo de células HeLa (47). Nuestro grupo se encuentra actualmente profundizando en el conocimiento de estas proteínas (a qué CDK se asocian, qué sustratos regulan y en qué tejidos se expresan).

Del mismo modo, sería también interesante conocer los mecanismos de degradación de estas HPC. En este sentido, la región DDD se encuentra bien conservada en la ciclina I, sugiriendo que las HPC podrían ser degradadas a través de las E3 ligasas del tipo Dma. De hecho, disponemos de resultados preliminares que parecen indicar que ciclina I es ubiquitinada *in vitro* por Rnf8, uno de los homólogos humanos de Dma1. Hasta el momento, se desconocen cuáles son los sustratos de Chfr como oncosupresor pero hay evidencias de que podría ser ciclina A (48), apoyando la hipótesis de que, en mamíferos, estas proteínas, también podrían estar controlando la estabilidad de las ciclinas. Cabría hipotetizar que la caída de actividad de estas E3 ligasas podría inducir la acumulación de ciclinas HPC en células tumorales.

CONCLUSIONES

1. Cln3 no es degradado por autofagia, es degradado por ubiquitinación mediante la vía de la E2 conjugadora Ubc4 cuando su actividad es controlada por Pho85.

2. Pcl1 es degradado por el proteosoma tras ser poliubiquitinado por Dma1

3. Dma1 usa la región DDD para reconocer sus sustratos para ubiquitinarlos. Esta secuencia está específicamente presente en las ciclinas de G1 que no contienen las secuencias PEST canónicas de destrucción.

4. La fosforilación de Pcl1, en la Thr39 y la Ser43, mediante Pho85 es necesaria para la degradación de la primera.

5. Dma1 es menos activo en un medio pobre en nutrientes, permitiendo la acumulación de Pcl1 en esta situación y siendo esta la ciclina principal encargada del correcto proceso de la fase G1 en detrimento de Cln2.

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