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Degradation of *Saccharomyces cerevisiae* Transcription Factor Gcn4 Requires a C-Terminal Nuclear Localization Signal in the Cyclin Pcl5▽‡

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Pcl5 is a *Saccharomyces cerevisiae* cyclin that directs the phosphorylation of the general amino acid control transcriptional activator Gcn4 by the cyclin-dependent kinase (CDK) Pho85. Phosphorylation of Gcn4 by Pho85/Pcl5 initiates its degradation via the ubiquitin/proteasome system and is regulated by the availability of amino acids. In this study, we show that Pcl5 is a nuclear protein and that artificial dislocation of Pcl5 into the cytoplasm prevents the degradation of Gcn4. Nuclear localization of Pcl5 depends on the β-importin Kap95 and does not require Pho85, Gcn4, or the CDK inhibitor Pho81. Pcl5 nuclear import is independent on the availability of amino acids and is mediated by sequences in its C-terminal domain. The nuclear localization signal is distinct from other functional domains of Pcl5. This is corroborated by a C-terminally truncated Pcl5 variant, which carries the N-terminal nuclear domain of Pho80. This hybrid is still able to fulfill Pcl5 function, whereas Pho80, which is another Pho85 interacting cyclin, does not mediate Gcn4 degradation.

Cyclin-dependent kinases (CDKs) are protein kinases (7) that are activated by binding of an auxiliary subunit, the cyclin (7, 20). Cyclins play an important role in targeting the kinase to specific substrates (15, 49, 56; reviewed in reference 35). CDKs were originally identified due to their role in regulating the eukaryotic cell division cycle (29) but are also involved in several additional cellular processes, including the control of gene transcription (35). Cyclins are defined as a group of structurally related proteins, which bind and activate CDKs (36). They harbor a common region of moderate sequence conservation, the cyclin box (CB) which assumes a typical five-helix structure (4, 20). The region of the cyclin responsible for substrate recognition depends on the protein and can be different. It includes the N terminus of human cyclin D1 for the interaction with Rb (9) and the C terminus of human cyclin E with Rb interaction (13). In the case of cyclin A, the cyclin box itself is indispensable for substrate recognition (5, 47, 49).

Most CDKs are able to interact with more than one cyclin, resulting in different substrate specificities for the kinase. In budding yeast, six different CDKs are known, of which Pho85 is related to mammalian Cdk5, a kinase involved in neurogenesis that was suggested to be involved in the pathology of Alzheimer's disease (16). Pho85, which has been implicated in cell cycle control, gene expression, and metabolic regulation, interacts with 10 different cyclin partners (14, 28, 32). The Pho85/Pho80 complex specifically phosphorylates the transcription factor Pho4 in a phosphate-rich environment (41), resulting in reduced activity. In response to phosphate starvation, the Pho4 phosphorylation activity is inhibited by the presence of the CDK inhibitor Pho81 (22, 28). Recent data suggest that the two terminal loops of the N and C termini, as well as the D-loop within the cyclin box of Pho80, are involved in targeting the substrate Pho4 (17). Pcl8 and Pcl10 play a role in glycogen metabolism by targeting the glycogen synthase Gsy2 (15, 56). Pcl5 targets Pho85 to the JUN-like transcriptional activator Gcn4 (33, 52). The amount of Gcn4 within the cell is controlled at the translational level in the cytoplasm, where starvation for amino acids results in phosphorylation of the eukaryotic translation initiation factor 2α (eIF-2α) by the ribosome-attached kinase Gcn2 favoring *GCN4* mRNA translation (8, 12). Amino acid starvation also mediates increased protein stability of the otherwise highly unstable Gcn4 in the nucleus and therefore provides an additional level of Gcn4 activity control (26).

The cyclin Pcl5 and its substrate Gcn4 have coevolved between *Saccharomyces cerevisiae* and the distantly related pathogenic yeast *Candida albicans*, so that phosphorylation of Gcn4 by Pho85 depends on the species-specific Pcl5 (11). Phosphorylation of Gcn4 initiates the degradation pathway and marks the protein for ubiquitination by the E3 SCF^{CDC4} ubiquitin ligase, followed by degradation by the 26S proteasome (18, 26, 33).

Under amino acid starvation conditions, the activity of Pho85/Pcl5 is reduced, resulting in stabilization of Gcn4 (33, 52). This reduced activity may be due to dissociation of Pcl5 from Pho85 under amino acid starvation conditions (3) combined with autophosphorylation-induced instability of Pcl5 (2, 52).

The kinase Pho85, as well as the substrate Gcn4, is predominantly localized in the nucleus (45). Gcn4 contains two nuclear localization signals (NLSs), of which NLS2 requires the activity of the karyopherins Srp1 and Kap95 (44). In *S. cerevisiae* 14 different karyopherins are known to mediate the regulated transport in and out of the nucleus. Srp1 is the only importin

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TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
RH2701	*MAT*α *mtr10*::*HIS3 ade2 leu2 trp1 his3 ura3*	51
RH2702	*MAT***a** *kap104*::*HIS3 ura3 his3 trp1 leu2 lys2*	1
RH2703	*MAT***a** *pse1-1 ura3 trp1 leu2*	50
RH2704	*MAT*α *rsl1-4*(*kap95ts*) *ura3 trp1 leu2 ade2*	25
RH2706	*MAT***a** *pse1-1 kap123*::*HIS3 ura3 trp1 leu2*	50
RH2707	*MAT*α *kap123*::*HIS3 ura3 trp1 leu2*	50
RH2708	*MAT*α *nmd5*::*HIS3 ura3 his3 leu2 ade2 ade8*	10
RH2709	*MAT***a** *pdr6*::*HIS3 ura3 his3 leu2 trp1*	27
RH2710	*MAT***a** *sxm1*::*HIS3 ura3 his3 trp1 leu2*	50
RH3058	*MAT***a** *kap114*::*HIS3 ura3 his3 trp1 leu2 lys2*	43
RH3237	*MAT***a** *ura3-1 can1-100 GAL*$^+$ *leu2-3 trp1-1*	3
RH3238	*MAT***a** *ura3-1 can1-100 GAL*$^+$ *leu2-3 trp1-1 pcl5*::*hisG*	3
RH1168	*MAT***a** *leu2-3 ura3-52 gal2*	This study
RH3239	*MAT***a** *leu2-3 ura3-52 gal2*; *PCL5-yEGFP-kanMX4*	This study
RH3241	*MAT***a** *ade2 trp1 leu2-3 his3 ura3-52 pho81*::*HIS3*	40
EY0140	*MAT***a** *ade2 trp1 can1-100 leu2-3,112 his3-11,15 ura3 pho85*::*LEU2*	41
RH3242	*MAT***a** *ade2 trp1 can1-100 leu2-3,112 ura3 pho85*::*LEU2*	This study
RH1408	*MAT***a**; *ura3-52*; *gal2*; *gcn4-103*	45

α besides at least 13 different β importins, including four export and nine import receptors (21). Since the amino acid-dependent Gcn4 stability regulation occurs in the yeast nucleus (45), Pcl5 has presumably to be transported into the nucleus to fulfill its function.

Subcellular localization and substrate specificity are two essential features that determine the cellular activity of cyclins. Here, we analyzed Pcl5-green fluorescent protein (GFP) hybrids to test whether nuclear localization represents a distinct domain of this cyclin and compared them to the localization of Pcl5-Pho80-GFP chimera (2).

MATERIALS AND METHODS

***S. cerevisiae* strains and growth conditions.** All yeast strains used in the present study are either congenic to the *S. cerevisiae* S288c (RH3239, RH1168, and RH1408) or W303 (RH3237, RH3238, RH3241, EY0140, RH3242, RH2701, RH2702, RH2703, RH2704, RH2706, RH2707, RH2708, RH2709, RH2710, and RH3058) genetic background. Details of the strains are given in Table 1. RH3242 was obtained by replacing the mutant *his3-11* allele of yeast strain EY0140 by a wild-type *HIS3* allele using BamHI linearized plasmid B1683 (Table 2). Strain RH3239 expressing GFP epitope-tagged version of *PCL5* at endogenous levels was obtained by PCR-mediated gene tagging (24). Briefly, primers were designed specific for amplification of the *yEGFP-kanMX4* module with homologous sequences to the *PCL5* 3′ end using plasmid pYM12[6] as a template. The PCR product was transformed directly into yeast strain RH1168 and plated onto rich medium with 200 μg of G418 (Geneticin; Gibco)/ml. Transformants were replica plated onto the same medium. The GFP-tagged *PCL5* version was confirmed by Southern hybridization. Standard methods for genetic crosses and transformation were used (19).

The strains were grown in standard yeast extract-peptone-dextrose (1% yeast extract, 2% peptone, 2% dextrose) and minimal yeast nitrogen base (YNB) medium (1.5 g of yeast nitrogen base lacking amino acids and ammonium sulfate/liter, with 5 g of ammonium sulfate/liter and 2% dextrose, galactose, or raffinose and supplemented with the appropriate amino acids).

Plasmid constructions. The plasmids used in the present study are listed in Table 2, and details of important primers are given in Table S1 in the supplemental material. Plasmid pME2844 expressing *PCL5-GFP* was obtained by amplifying the *PCL5*-open reading frame (ORF) with *Pfu* polymerase and introducing it via SmaI/ClaI into the low-copy-number GFP-C-Fus vector (pME2843) (38). pME2846 expressing *PCL5-GFP* was constructed by amplifying the *PCL5*-ORF with *Pfu* polymerase and cloning it as a SmaI/HindIII fragment into p426MET25 (37). Afterward, a 750-bp BglII-fragment encoding the *GFPuv* variant of GFP that was amplified from plasmid pBAD-GFP (Clontech, Heidelberg, Germany) was inserted behind the *PCL5*-ORF.

The construction of plasmids pME2849, pME2850, pME2851, pME2853, pME2854, pME2855, pME2856, pME2857, pME2858, pME2859, pME3370, pME3371, and pME3574 expressing *GFP* fused to the 3′ end of different *PCL5* fragments driven from the *MET25* promoter was started by amplifying the *GFP*-ORF as described before and introducing it via SmaI/ClaI into p426MET25. *PCL5* fragments were fused via SpeI/SmaI to the 5′ end of *GFP*. pME2950 was constructed by amplifying $PCL5_{bp610\text{-}654}$-*GFP* (encoding $Pcl5_{aa204\text{-}218}$-GFP) from plasmid pBAD-GFP by using primers KB57 and KB16 (see Table S1 in the supplemental material) and cloning them via SmaI/ClaI into p426MET25 (37).

Plasmid pME3573 encoding $Pcl5_{aa1\text{-}180}$-GFP was constructed via amplification of the *PCL5-GFP* fragment from pME2853 and introducing it as a SmaI/ClaI fragment into p416MET25 (37).

The plasmid pME2951 was obtained by amplifying *GFP-ARO7-*$PCL5_{bp619\text{-}645}$ (encoding GFP-Aro7-$Pcl5_{aa207\text{-}215}$) with *Pfu* polymerase using the primers RP14 and KB55 (see Table S1 in the supplemental material) and introducing it via SmaI/EcoRI into p426MET25 (37).

The plasmid pME2860 was constructed by amplifying the hybrid ORF $PHO80_{bp1\text{-}219}$-$PCL5_{bp235\text{-}534}$-$PHO80_{bp508\text{-}882}$ (encoding $Pho80_{aa1\text{-}73}$-$Pcl5_{aa79\text{-}178}$-$Pho80_{aa170\text{-}294}$) with *Pfu* polymerase from plasmid KB1360 and fused it via SpeI/SmaI to the 5′ end of *GFP* on plasmid pME2849. To obtain pME2948, the hybrid $PHO80_{bp1\text{-}219}$-$PCL5_{bp235\text{-}534}$ (encoding $Pho80_{aa1\text{-}73}$-$Pcl5_{aa79\text{-}178}$) was amplified via *Pfu* polymerase from plasmid pME2860 and ligated as SpeI/SmaI fragments in front of the *GFP*-ORF of pME2849. The plasmid pME3576 was constructed in two steps. First, the *PCL5* promoter (coordinates −890 to −3) was amplified with *Phusion* polymerase as SacI/XbaI fragment using the primers oFLS95 and oFLS96 (see Table S1 in the supplemental material) and ligated in SacI/XbaI-digested pRS416MET25, leading to pME3575. Next, the hybrid ORF $PHO80_{bp1\text{-}219}$-$PCL5_{bp235\text{-}534}$-*GFP* was amplified with the primers oFLS97 and oFLS98 (see Table S1 in the supplemental material) from plasmid pME2948 and inserted as SpeI/ClaI fragment into XbaI/ClaI-digested pME3575.

Construction of plasmids pME3370, pME3577, and pME3578 was started by site-directed mutagenesis of the *PCL5*-ORF via *KOD* polymerase using the primers oFLS35 and oFLS36 (see Table S1 in the supplemental material) resulting in the amino acid substitutions K209A, R210A, and R212A. The mutated *PCL5****-ORF and the mutated fragments $Pcl5_{aa61\text{-}229***}$ and $Pcl5_{aa181\text{-}229***}$ were introduced as a SpeI/SmaI fragment in front of the *GFP*-ORF of plasmid pME2849.

To obtain pME3371, the $Pcl5_{aa61\text{-}180}$-encoding fragment was amplified with *Pfu* polymerase using the primers KB35 and KB100 (see Table S1 in the supplemental material) and ligated as an SpeI/SmaI fragment in front of the *GFP*-ORF of pME2849.

In the *PCL5-GFP-NES* fusion (pME2861), the nuclear export sequence (NES) from PKI (inhibitor of the cAMP-dependent protein kinase) (54) was engineered at the 3′ end of *PCL5-GFP* in two steps. The *PCL5*-ORF was amplified with *Pfu* polymerase and inserted into p426MET25 as a SmaI/HindIII fragment as the first step. Second, the *GFP-NES* cassette was amplified using the primers KB5 and KB40 (encoding GFP-NESaa GMDELYKNELALKLAGLDINKTKLTLA) (see Table S1 in the supplemental material) and introduced as a BglII fragment at the 3′ end of *PCL5*.

To construct a fusion of glutathione *S*-transferase (GST) with full-length Kap95, the *KAP95* coding region was amplified by PCR using *Pfu* polymerase and the primers Kap95-BamHI-START and Kap95-STOP-XbaI (see Table S1 in the supplemental material) and inserted as a BamHI/XbaI fragment in-frame downstream of the *GST*-ORF into pYGEX-2T to yield pME3372. To obtain plasmid pME3447, the *KAP104*-ORF was amplified by PCR using the primers oFLS74BamHI-KAP104 and oFLS75KAP104-SpeI (see Table S1 in the supplemental material) and inserted as a BamHI/SpeI fragment in-frame downstream of the GST-ORF into pYGEX-2T (46). Plasmid pME3448 expressing *GST-PSE1* was similarly constructed using the oligonucleotides oFLS76BamHI-PSE1 and oFLS77PSE1-SpeI (see Table S1 in the supplemental material). To receive plasmid pME3579 expressing a ninefold myc epitope-tagged fusion of the *NLS*-mutated *PCL5****-ORF (containing the amino acid substitutions K209A, R210A, and R212A), the mutated *PCL5****-ORF was amplified with *Pfu* polymerase and inserted as a SmaI/HindIII fragment into p425GAL1. A 360-bp BglII fragment carrying myc^9 was introduced into a BglII site in front of the third amino acid of Pcl5***. Similarly, plasmids pME3580 encoding myc^9-$Pcl5_{aa181\text{-}229}$ and pME3581 encoding myc^9-$Pcl5_{aa181\text{-}229***}$ with mutated NLS motif were

TABLE 2. Plasmids used in this study

Plasmid	Description[a]	Source or reference
pBKSII	2.96-kb vector, Amp^r (*bla*), *lacZ ori*	Stratagene (La Jolla, CA)
pRS414	*TRP1 CEN*, Amp^r (*bla*), *ori*	53
pRS415	*LEU2 CEN*, Amp^r (*bla*), *ori*	53
pRS416	*URA3 CEN*, Amp^r (*bla*), *ori*	53
pRS425	*LEU2*, 2μm, Amp^r (*bla*), *ori*	53
pRS426	*URA3*, 2μm, Amp^r (*bla*), *ori*	53
p414GAL1	pRS414 containing *GAL1* promoter, *CYC1* terminator	37
p415GAL1	pRS415 containing *GAL1* promoter, *CYC1* terminator	37
p425GAL1	pRS416 containing *GAL1* promoter, *CYC1* terminator	37
p416MET25	pRS416 containing *MET25* promoter, *CYC1* terminator	37
p425MET25	pRS425 containing *MET25* promoter, *CYC1* terminator	37
p426MET25	pRS426 containing *MET25* promoter, *CYC1* terminator	37
pYGEX-2T	*GAL1-10prom-GST*, *URA3*, 2μm	48
pME2843	pGFP-C-FUS	38
pME2844	*MET25prom-PCL5-GFP* fusion in pGFP-C-FUS	This study
pME2846	*MET25prom-PCL5-GFP* fusion in p426MET25	This study
pME2848	*GAL1prom-myc^3-GCN4* fusion in p415GAL1	This study
pME2849	*MET25prom-GFP* fusion in p426MET25	This study
pME2850	*MET25prom-PCL5$_{aa1-95}$-GFP* fusion in p426MET25	This study
pME2851	*MET25prom-PCL5$_{aa1-127}$-GFP* fusion in p426MET25	This study
pME2853	*MET25prom-PCL5$_{aa1-180}$-GFP* fusion in p426MET25	This study
pME2854	*MET25prom-PCL5$_{aa61-229}$-GFP* fusion in p426MET25	This study
pME2855	*MET25prom-PCL5$_{aa111-180}$-GFP* fusion in p426MET25	This study
pME2856	*MET25prom-PCL5$_{aa111-229}$-GFP* fusion in p426MET25	This study
pME2857	*MET25prom-PCL5$_{aa153-229}$-GFP* fusion in p426MET25	This study
pME2858	*MET25prom-PCL5$_{aa61-180}$-GFP* fusion in p426MET25	This study
pME2859	*MET25prom-PCL5$_{aa181-229}$-GFP* fusion in p426MET25	This study
pME2865	*GAL1-10prom-myc^9-PCL5* fusion in p425GAL1	3
pME2866	*GAL1-10prom-GST-PHO85* fusion in pYGEX-2T	3
pME2950	*MET25prom-PCL5$_{aa204-218}$-GFP* fusion in p426MET25	This study
pME2951	*MET25prom-GFP-ARO7-PCL5$_{aa207-215}$* fusion in p426MET25	This study
pME2860	*MET25prom-PHO80$_{aa1-73}$-PCL5$_{aa79-178}$-PHO80$_{aa170-294}$-GFP* fusion in p426MET25	This study
pME2948	*MET25prom-PHO80$_{aa1-73}$-PCL5$_{aa79-178}$-GFP* fusion in p426MET25	This study
pME2861	*MET25prom-PCL5-GFP-NES* fusion in p426MET25	This study
pME3370	*MET25prom-PCL5$_{aa61-229***}$-GFP* fusion containing three Pcl5 amino acid substitutions K209A, R210A, and R212A in p426MET25	This study
pME3371	*MET25prom-PCL5$_{aa61-180}$-NLSSV40-GFP* fusion in p426MET25	This study
pME3372	*GAL1-10prom-GST-KAP95* fusion in pYGEX-2T	This study
pME3447	*GAL1-10prom-GST-KAP104* fusion in pYGEX-2T	This study
pME3448	*GAL1-10prom-GST-PSE1* fusion in pYGEX-2T	This study
pME3572	*GAL1prom-myc^3-GCN4* fusion in p414GAL1	This study
pME3573	*MET25prom-PCL5$_{aa1-180}$-GFP* fusion in p416MET25	This study
pME3574	*MET25prom-PCL5$_{aa79-178}$-GFP* fusion in p426MET25	This study
pME3575	*PCL5prom-CYC1term* in pRS416	This study
pME3576	*PCL5prom-PHO80$_{aa1-73}$-PCL5$_{aa79-178}$-GFP* fusion in pME3575	This study
pME3577	*MET25prom-PCL5$_{aa1-229***}$-GFP* containing three Pcl5 amino acid substitutions K209A, R210A, and R212A in p426MET25	This study
pME3578	*MET25prom-PCL5$_{aa181-229***}$-GFP* containing three Pcl5 amino acid substitutions K209A, R210A, and R212A in p426MET25	This study
pME3579	*GAL1-10prom-myc^9-PCL5*** fusion containing three Pcl5 amino acid substitutions K209A, R210A, and R212A in p425GAL1	This study
pME3580	*GAL1-10prom-myc^9-PCL5$_{aa181-229}$* fusion in p425GAL1	This study
pME3581	*GAL1-10prom-myc^9-PCL5$_{aa181-229***}$* fusion containing three Pcl5 amino acid substitutions K209A, R210A, and R212A in p425GAL1	This study
pME3583	*MET25-KAP95* in p425MET25	This study
B1683	1,720-bp *HIS* gene in pBKSII	J. E. Hill
pYM12[6]	*yEGFP-kan*MX4-module	24
KB1360	*GAL1prom-PHO80$_{aa1-73}$-PCL5$_{aa79-178}$-PHO80$_{aa170-294}$* fusion in p416GAL1	2

[a] Amp^r, ampicillin resistance.

constructed. After amplification of the corresponding fragment with *Pfu* polymerase and insertion as a SmaI/HindIII fragment into p425GAL1 a 360-bp BglII fragment encoding myc^9 was introduced after the first amino acid of the respective Pcl5 fragment.

To express *KAP95*, the ORF was amplified as a BamHI/SalI fragment and introduced into BamHI/SalI-digested p425MET25 (37) to yield pME3583.

Plasmid pME2848, expressing a triple myc epitope-tagged version of *GCN4* under the control of the *GAL1* promoter, was obtained by amplifying *GCN4* with

Pfu polymerase and subsequent insertion in p415GAL1 (37) as blunt/HindIII fragment. A 120-bp BamHI fragment carrying the triple myc epitope was inserted into a BglII restriction site after the fifth amino acid of Gcn4. To construct plasmid pME3572, the triple myc epitope-tagged version of *GCN4* was amplified as a SpeI/ClaI fragment from pME2848 using *Pfu* polymerase and inserted into p414GAL1 (37).

Protein analysis. (i) Shutoff-Western procedure. Yeast cells were pregrown in selective minimal medium with glucose as carbon source. Cells were collected by centrifugation and incubated in minimal medium containing 2% galactose to express *myc*^{3}*-GCN4* from the *GAL1* promoter. After 3 h of induction, 2% glucose was added to shut off the *GAL1* promoter. Samples were analyzed at the indicated time points after promoter shutoff (0-min time point).

(ii) Purification of GST fusions. Yeast strains expressing *GST*, *GST-PHO85*, *GST-KAP95*, *GST-KAP104*, or *GST-PSE1*, together with a myc-tagged version of *PCL5*, were pregrown in selective minimal medium containing raffinose as the carbon source. Two percent galactose was added to induce the expression of the *GAL1*-driven fusions. After 3 h of induction, cells were collected by centrifugation, and protein extracts were prepared and incubated with glutathione-Sepharose overnight at 4°C. The beads were repeatedly washed and collected to purify GST fusions and any associated proteins (3). Samples were denatured in sodium dodecyl sulfate (SDS) loading dye (65°C, 15 min) and analyzed by Western hybridization.

(iii) Whole-cell extracts of *S. cerevisiae*. Extracts were prepared from yeast cultures grown to exponential phase. Cells were washed in ice-cold buffer B (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 50 mM dithiothreitol), lysed with glass beads in 200 μl of buffer B plus PIM (phenylmethylsulfonyl fluoride, tosyl-L-lysine-chloromethylketone, tosyl-L-phenylalanine-chloromethylketone, *p*-aminobenzamidine–HCl, and *o*-phenanthroline [1 mM each]) plus 3% Triton X-100 plus 0.8% SDS at 4°C, and spun at 3,500 rpm for 15 min to remove glass beads and large cell debris. Extracts (10 μl) were removed to determine the total protein concentration using a protein assay kit from Bio-Rad (Munich, Germany). Proteins were denatured in SDS loading dye by heating at 65°C for 15 min. Proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. GFP and myc fusion proteins, Cdc28, and eIF-2 were detected using enhanced chemiluminescence technology (Amersham, United Kingdom). For the first incubation, monoclonal mouse anti-GFP (Clontech), monoclonal mouse anti-myc (9E10), polyclonal rabbit anti-GST (Santa Cruz Biotechnologies, Santa Cruz, CA), polyclonal rabbit anti-Cdc28, or polyclonal rabbit anti-eIF-2 antibodies were used. Peroxidase-coupled goat anti-rabbit or goat anti-mouse immunoglobulin G was used as a secondary antibody (Dianova, Hamburg, Germany).

GFP fluorescence microscopy. Yeast strains harboring plasmids encoding Pcl5-GFP fusion proteins were grown to early log phase and analyzed under sated and starved conditions. Amino acid starvation was induced by transferring these *leu2*-deficient yeast cells from minimal medium containing leucine to minimal medium lacking leucine for 1 h. Cells from 1-ml portions of the cultures were harvested by centrifugation and immediately viewed in vivo on a Zeiss Axiovert microscope by either differential interference contrast microscopy (DIC) or fluorescence microscopy using a GFP filter set (AHF Analysentechnik AG, Tübingen, Germany) or, in the case of DAPI (4′,6′-diamidino-2-phenylindole) staining, a standard DAPI filter set. DAPI staining was used for visualization of nuclei. Cells were photographed using a Hamamatsu-Orca-ER digital camera and Improvision Openlab software (Improvision, Coventry, United Kingdom).

Growth tests. For spot dilution assays, yeast strains were precultured to the same optical densities (optical density at 600 nm [OD_{600}] = 1) and spotted onto appropriate YNB media, as indicated. Tenfold dilutions, starting with 3×10^4 cells per 20 μl, were spotted onto the plates, incubated for 3 days at 30°C, and photographed under white light. For assaying growth in liquid culture, overnight cultures of the yeast strains were pregrown in YNB medium with raffinose as the carbon source, gently harvested, washed with sterile water, and diluted in 100 ml of the appropriate selective medium with either glucose or galactose as the carbon source to a final OD_{600} of 0.1. Cells were incubated with shaking at 30°C. The OD_{600} was measured every hour. A graph was plotted by using OD_{600} values, and the doubling times during logarithmic growth were calculated.

RESULTS

Pcl5 is a nuclear protein. The CDK Pho85 and the Pcl5 target Gcn4 are localized in the nucleus (45). Therefore, we reasoned that the cyclin Pcl5 has to be transported in the nucleus at least in sated cells, when Gcn4 is rapidly degraded.

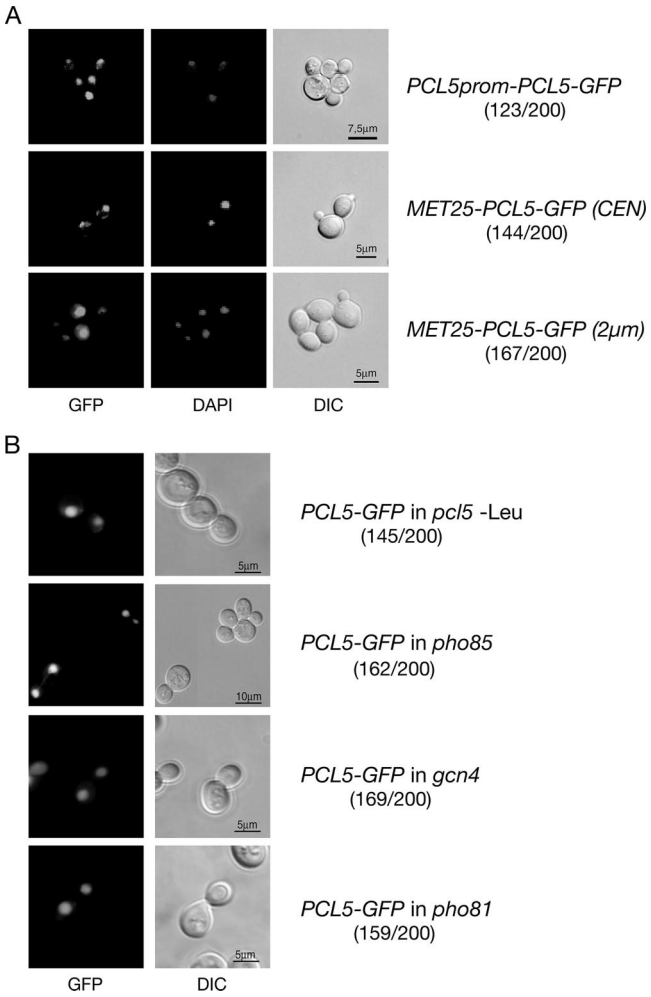


FIG. 1. Pcl5-GFP is transported into the *S. cerevisiae* nucleus. (A) Nuclear localization of high and low amounts of the functional cyclin Pcl5-GFP. Localization was analyzed in *S. cerevisiae* cells (RH3239) expressing Pcl5-GFP fusion protein derived from its endogenous *PCL5* promoter at the chromosomal locus or *pcl5* mutant cells (RH3238) expressing Pcl5-GFP fusion protein from the *MET25* promoter on a low-copy (*CEN*) plasmid (pME2844) or on a high-copy (2μm) plasmid (pME2846). Cells were grown to early log phase at 30°C and analyzed by DIC microscopy, DAPI staining, and fluorescence microscopy (GFP). "X/200" in parentheses means "X" number of 200 cells expressing *PCL5-GFP* detectable in the nucleus. (B) Nuclear localization of Pcl5-GFP is independent of the availability of amino acids or the proteins Gcn4, Pho85, or Pho81. Nuclear localization of Pcl5-GFP expressed from the *MET25* promoter on a high-copy plasmid (pME2846) in *pcl5* (RH3238) under amino acid starvation conditions (−Leu), *pho85* (RH3242), *gcn4* (RH1408), and *pho81* (RH3241) mutant strains. Yeast cells were grown and analyzed as described above. Leucine starvation (−Leu) was induced in leucine-auxotrophic yeast cells by shifting to synthetic medium lacking leucine.

Since Pcl5 is itself an unstable protein with a half-life of <3 min (52), monitoring of the subcellular localization of the protein is a challenge. Overexpression of Pcl5 carrying an epitope tag at the C terminus stabilizes the protein in contrast to a more physiological unstable Pcl5 with an N-terminal tag (2). When *PCL5* is expressed from its endogenous promoter, no stabilization was observed using a C-terminal epitope tag (2). We analyzed Pcl5 fusions to GFP at low and high levels of

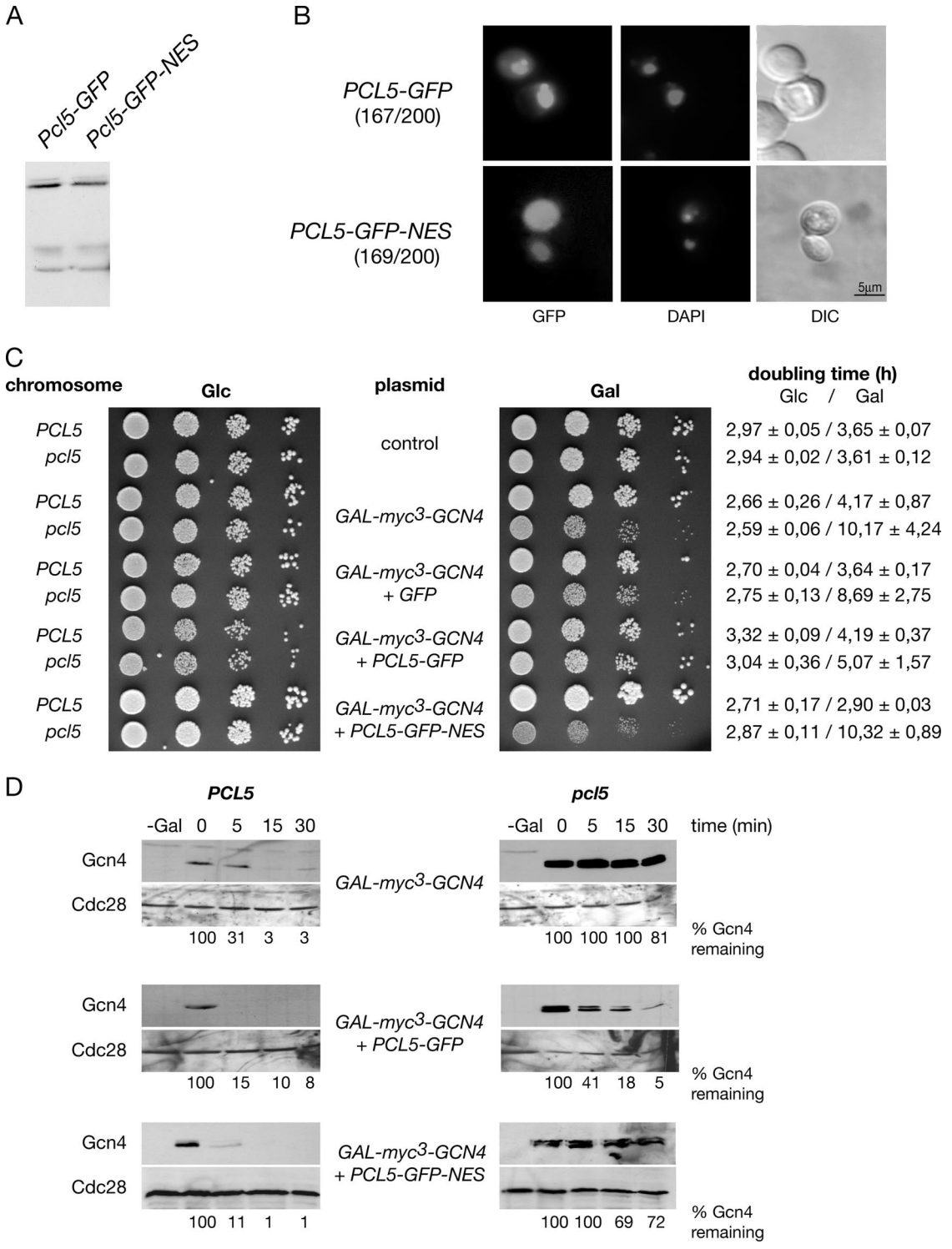


FIG. 2. Nuclear localization of yeast Pcl5 is required for Gcn4 degradation. (A) *PCL5-GFP-NES* is correctly expressed as 54-kDa equivalent to *PCL5-GFP*. Yeast *pcl5* cells (RH3238) were transformed to express either *PCL5-GFP* (pME2846) or *PCL5-GFP-NES* (pME2861) from the high-copy plasmids under the control of the *MET25* promoter. Cells were grown to early log phase, and the expression of the GFP fusion proteins was analyzed by Western blotting with monoclonal anti-GFP antibodies. (B) Pcl5-GFP-NES is transported out of the nucleus into the cytoplasm. Localization of the fusion proteins Pcl5-GFP (pME2846) and Pcl5-GFP-NES (pME2861) expressed under the control of the *MET25* promoter on high-copy (2µm) plasmid was analyzed in a *pcl5* mutant strain (RH3238) by fluorescence microscopy (GFP), DAPI staining, and DIC microscopy. The numbers in parentheses represent the total amount of cells investigated ($n = 200$) and the respective amount showing the displayed localization. (C) Pcl5-GFP-NES is incapable of suppressing the toxicity of overexpressed *GCN4* in the absence of a functional *PCL5* gene. Wild-type cells (RH3237) and *pcl5* mutant cells (RH3238) expressing *GAL1*-driven *myc*^{3}*-GCN4* from the low-copy plasmid pME2848 alone or together with *GFP* (pME2849), *PCL5-GFP* (pME2846), or *PCL5-GFP-NES* (pME2861) under the control of the *MET25* promoter on a high-copy

expression by cloning C- or N-terminally GFP-tagged versions of Pcl5 on both high- and low-copy vectors under the repressible *MET25* promoter to increase expression levels and to study Pcl5 localization independently of its expression. The effect of native levels of expression and regulation of *PCL5* was tested after chromosomal integration of the ORF for *GFP* at the 3′ terminus of the *PCL5*-ORF.

Expression of the different Pcl5-GFP fusion proteins was verified by Western hybridization of *S. cerevisiae* cell extracts using monoclonal anti-GFP antibodies (data not shown). The functionality of all Pcl5-GFP hybrids was tested by their ability to suppress Gcn4 overexpression toxicity. High overexpression of *GCN4* inhibits cellular growth, possibly by the interference of Gcn4 with other transcriptional activation pathways (55). A *pcl5*-deficient yeast strain is hypersensitive to even moderately overexpressed *GCN4* fused to the *GAL1* promoter (52). Whereas GFP by itself is unable to suppress overexpression toxicity of Gcn4 on solid medium, as well as in liquid culture, all Pcl5 fusions tested carrying either C-terminal (see Fig. 2B) or N-terminal GFP (data not shown) complemented the *pcl5* mutant phenotype, indicating that the addition of GFP does not influence the localization of Pcl5 or interfere with kinase activation or substrate recognition. A GFP fusion of *PCL5*, integrated in a single copy at the original locus, is already sufficient to suppress Gcn4 toxicity (data not shown).

Localization of Pcl5 was monitored by fluorescence microscopy. Pcl5-GFP expressed from the endogenous *PCL5* locus, from the *MET25* promoter on a centromeric (*CEN*) plasmid or from the *MET25* promoter on a high-copy (2μm) plasmid were all predominantly localized within the nucleus, which was confirmed by DAPI staining (Fig. 1A). Similar results were obtained with N-terminal GFP-fusions of Pcl5 (data not shown). A quantitative analysis of transformed cells ($n = 200$) revealed that 60 to 80% of the cells expressed Pcl5-GFP were visible in the nucleus depending on promoter strength and copy number. The occurrence of cells where Pcl5-GFP is not detectable decreases when *PCL5-GFP* is expressed from the *MET25* promoter on a high-copy (2μm) plasmid, but overexpression does not alter the localization.

Constitutive nuclear localization of Pcl5 is independent of amino acid starvation and the known interacting proteins Gcn4, Pho85, and Pho81. Amino acid starvation results in Gcn4 stabilization. We analyzed whether Gcn4 stability regulation may be caused by an altered localization of Pcl5 dependent on the availability of amino acids. Pcl5-GFP localization was analyzed in leucine-auxotrophic yeast cells briefly shifted to synthetic medium lacking leucine, conditions that cause the stabilization of Gcn4 in *PCL5* wild-type cells (26). Promoter shutoff experiments with a *pcl5* strain expressing *GAL1*-driven *myc*^{3}*-GCN4* from the low-copy plasmid pME3572, together with *PCL5-GFP* under the control of the *MET25* promoter from the high-copy plasmid pME2846, revealed that under leucine starvation conditions Gcn4, if at all, is only slightly stabilized (data not shown). Nevertheless, Fig. 1B demonstrates that Pcl5 is constitutively located in the nucleus in leucine-starved cells.

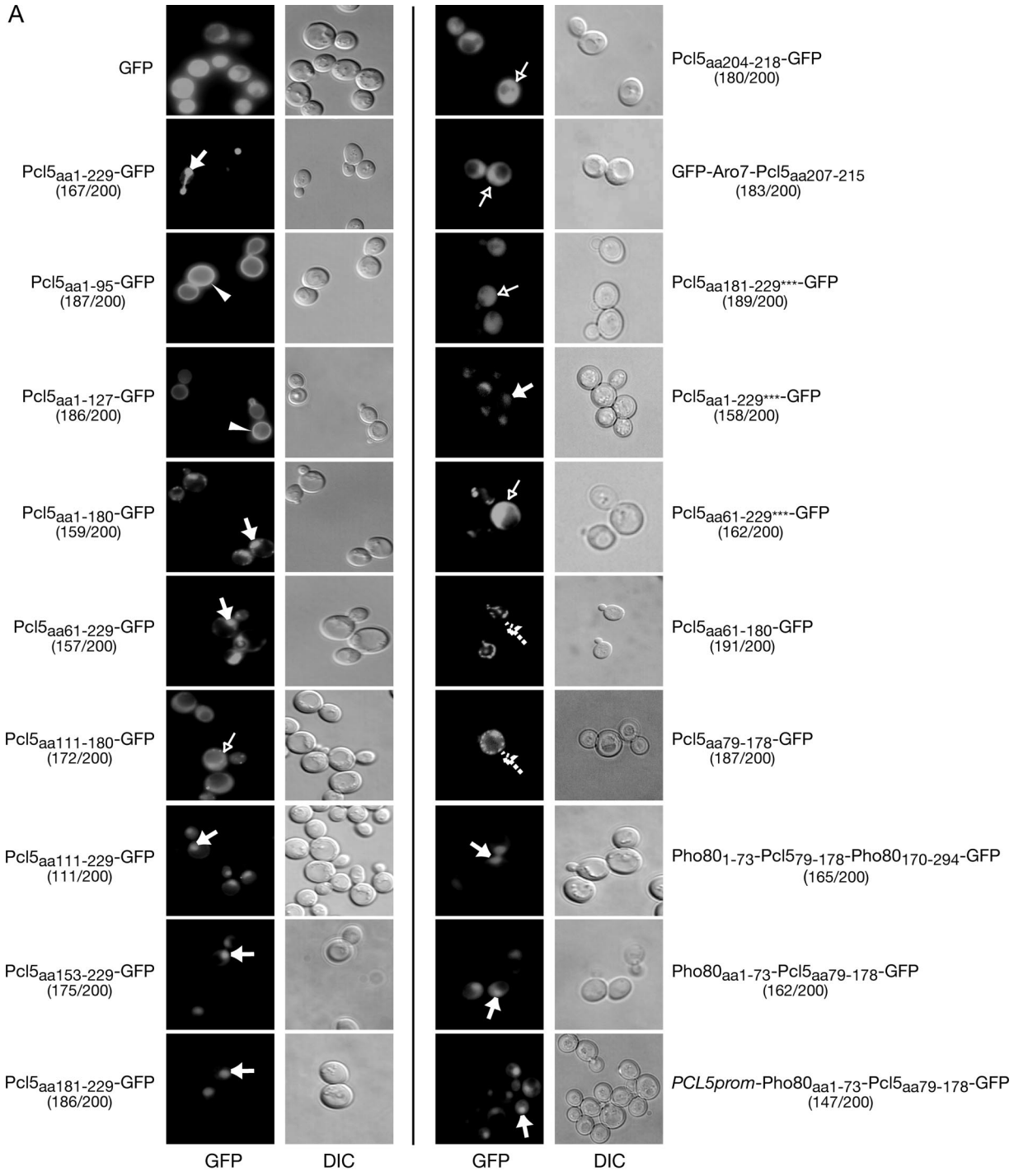
The amino acid sequence of Pcl5 contains neither a monopartite basic NLS as in the simian virus 40 (SV40) large tumor antigen (23) nor the bipartite motif of nucleoplasmin (46). Therefore, we examined whether Pcl5 is indirectly imported into the nucleus by interaction with another nuclear protein. The CKI inhibitor Pho81 is involved in amino acid-dependent stabilization of Gcn4, and both Pho85 and Pho81 are able to interact with the cyclin Pcl5 (3). Gcn4, Pho85, and Pho81 are exclusively nuclear proteins independently of the availability of amino acids (45). We tested whether the substrate Gcn4, the kinase Pho85, or the inhibitor Pho81 are required for Pcl5 nuclear import by analyzing the localization of Pcl5-GFP in the corresponding *S. cerevisiae* mutant strains. The cyclin was detected predominantly in the nucleus in all tested mutant strains (Fig. 1B) without any significant differences in frequency compared to the *GCN4 PHO85 PHO81* wild-type strain shown in Fig. 1A. This indicates that nuclear import of Pcl5 does not require these Pcl5 interacting partners and suggests that Pcl5 possesses an autonomous NLS for direct interaction with karyopherins.

Nuclear localization of yeast Pcl5 is required for Gcn4 degradation. Previous experiments have suggested that phosphorylation and ubiquitination, which are the first two steps of the Gcn4 degradation pathway, are restricted to the nucleus (45). Since Pcl5 is a predominantly nuclear protein (see Fig. 1, above), we wanted to verify that nuclear localization of the Gcn4-specific cyclin Pcl5 is a prerequisite for efficient Gcn4 degradation in sated cells.

The localization of Pcl5-GFP was manipulated by fusing an NES to the C terminus of the Pcl5-GFP hybrid. The used NES sequence derived from the polypeptide inhibitor (PKI) of the cyclic AMP-dependent protein kinase. It represents a short and hydrophobic motif with high leucine content (**L**A**L**K**L**A**G** **L**D**I**) (39). Western analysis confirmed that this hybrid, expressed under the *MET25* promoter, migrated at the expected size of 54 kDa (Fig. 2A). Quantitative fluorescence microscopy revealed that, similar to *PCL5-GFP* 80% of the transformed cells expressed *PCL5-GFP-NES* but in contrast to the nuclear localization of the Pcl5-GFP fusion, the Pcl5-GFP-NES was predominantly localized in the cytoplasm (Fig. 2B). Expression of *PCL5-GFP-NES* from the stronger *GAL1* promoter also resulted in a clear cytoplasmic localization (data not shown).

The activity of the Pcl5-GFP-NES construct was first tested by assaying the ability of this protein to suppress Gcn4 over-

plasmid were spotted in 10-fold dilutions on glucose (repressing conditions) and galactose (inducing conditions) to induce expression of *GCN4* driven by the *GAL1* promoter. As controls, wild-type and *pcl5* mutant cells transformed with the empty vector were used (control). The given doubling times are the average of at least three independent growth tests in liquid culture with glucose (repressing conditions) or galactose (inducing condition) as a carbon source. (D) Pcl5-GFP-NES is unable to induce Gcn4 degradation. The same wild-type and *pcl5* yeast strains as in panel C were transformed to express *myc*^{3}*-GCN4* from plasmid pME2848 alone or together with *PCL5-GFP* (pME2846) or *PCL5-GFP-NES* (pME2861). Protein levels of myc^3-Gcn4 or Cdc28 were determined at the indicated time points after the shift to glucose medium. The numbers given below each lane indicate the remaining Gcn4-percentage compared to Cdc28 as an internal standard.


A
GFP
Pcl5aa1-229-GFP (167/200)
Pcl5aa1-95-GFP (187/200)
Pcl5aa1-127-GFP (186/200)
Pcl5aa1-180-GFP (159/200)
Pcl5aa61-229-GFP (157/200)
Pcl5aa111-180-GFP (172/200)
Pcl5aa111-229-GFP (111/200)
Pcl5aa153-229-GFP (175/200)
Pcl5aa181-229-GFP (186/200)
Pcl5aa204-218-GFP (180/200)
GFP-Aro7-Pcl5aa207-215 (183/200)
Pcl5aa181-229***-GFP (189/200)
Pcl5aa1-229***-GFP (158/200)
Pcl5aa61-229****-GFP (162/200)
Pcl5aa61-180-GFP (191/200)
Pcl5aa79-178-GFP (187/200)
Pho801-73-Pcl579-178-Pho80170-294-GFP (165/200)
Pho80aa1-73-Pcl5aa79-178-GFP (162/200)
PCL5prom-Pho80aa1-73-Pcl5aa79-178-GFP (147/200)
GFP
DIC
GFP
DIC
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pME2846
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pME2950
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pME3578
pME3577
pME3370
pME2858
pME3574
pME2860
pME2948

expression toxicity as described previously. The Gcn4 toxicity assay revealed that, in contrast to nuclear Pcl5-GFP, the activity of cytoplasmic Pcl5-GFP-NES is decreased in the suppression of Gcn4-mediated growth inhibition (Fig. 2C). The activity of Pcl5-GFP-NES was further tested by assaying its ability to promote Gcn4 degradation. A *pcl5* strain was transformed with a *GAL-myc*^{3}*-GCN4* plasmid and either Pcl5-GFP or Pcl5-GFP-NES. A promoter shutoff experiment of the *GAL-myc*^{3}*-GCN4* construct revealed that Pcl5-GFP efficiently complemented the Gcn4 degradation defect of the *pcl5* mutant. In contrast, expression of the cytoplasmic Pcl5-GFP-NES was unable to complement the *pcl5* mutant, indicating that Pcl5-GFP-NES is unable to degrade Gcn4 (Fig. 2D). This suggests that nuclear localization of Pcl5 is required for efficient Gcn4 degradation.

The C-terminal part of Pcl5 directs nuclear localization. To determine which part of Pcl5 is responsible for its nuclear localization, we constructed a set of Pcl5-GFP fusions. The first 60 N-terminal amino acids of Pcl5 are followed by the central part of the protein (amino acids [aa] 61 to 180) containing the predicted cyclin box domain (see Fig. 6). This central domain, which is conserved between the different Pho85 cyclins (32), is followed by an additional 50 aa at the C terminus. The subcellular localization of a set of truncated Pcl5-GFP hybrids was analyzed to identify the Pcl5 *cis*-acting sequences responsible for nuclear import (Fig. 3A and 6). First, all fusion constructs were driven from the repressible *MET25* promoter on a 2μm plasmid to facilitate visualization of truncated Pcl5-GFP hybrids. Expression of Pcl5-GFP derivatives was verified by Western analysis of *pcl5* mutant cell extracts using monoclonal anti-GFP antibodies (Fig. 3B).

Localization of GFP and the different Pcl5-GFP fusion proteins was examined by fluorescence microscopy in sated *pcl5* cells. The native GFP protein by itself was found throughout the cell in both the nucleus and the cytoplasm, whereas Pcl5-GFP displayed a distinctly nuclear localization, as shown above (Fig. 1 and 3A). In contrast, N-terminal sequences of Pcl5 extending between positions 1 and 95 or between positions 1 and 127 showed a very different staining pattern, displaying a pericellular staining distinctive of plasma membrane localization (Fig. 3A). This indicates that the N-terminal region of Pcl5, extending from residues 1 to 95, is associated with the plasma membrane. Pcl5-GFP constructs lacking the N- or C-terminal domain ($Pcl5_{aa1\text{-}180}$ and $Pcl5_{aa61\text{-}229}$) showed predominantly nuclear staining, but cytoplasmic and membrane dot-like staining was also observed (Fig. 3). Expression of the Pcl5 aa 1 to 180 from a low-copy (*CEN*) plasmid did not change its localization (data not shown). The conserved central part of Pcl5 (aa 61 to 180) and the contained cyclin box (aa 79 to 178) are visualized via GFP only in an aggregate-like form spread over the whole cell (Fig. 3A) even when a classical SV40 large tumor antigen NLS is fused to the C terminus (data not shown). The C-terminal domain of the protein, either by itself ($Pcl5_{aa181\text{-}229}$) or with part of the cyclin box ($Pcl5_{aa111\text{-}229}$ and $Pcl5_{aa153\text{-}229}$) efficiently targeted the fusion protein to the nucleus (Fig. 3A). These data suggest that the carboxy-terminal 49 aa residues of Pcl5 include an NLS.

The C terminus of Pcl5 contains a PVKRPRESD motif between positions 207 and 215. The structure of this motif resembles the c-myc NLS (PAAKRVKLD), where the proline and aspartic acid residues flanking the basic cluster play an important role in nuclear import (31). We tested the function of this putative NLS motif by mutating the basic cluster to alanine (PVAAPAESD). $Pcl5_{aa1\text{-}229^{***}}$-GFP containing the mutated motif shows nuclear but also cytoplasmic staining, with dot-like aggregates similar to $Pcl5_{aa1\text{-}180}$-GFP and $Pcl5_{aa61\text{-}229}$-GFP. Pcl5 aa 61 to 229 lacking the N terminus are sufficient for nuclear localization. Therefore, we analyzed the effect of the mutated putative NLS motif of this Pcl5 fragment on localization. Figure 3A shows that this $Pcl5_{aa61\text{-}229^{***}}$-GFP fusion displayed a predominant cytoplasmic staining with additional membrane dot-like structures, suggesting that the putative NLS motif plays an active role for nuclear localization. Furthermore, we analyzed the localization of the C-terminal domain containing the mutated cluster fused to GFP. This $Pcl5_{aa181\text{-}229^{***}}$-GFP is, in contrast to the nonmutated C-terminal domain, found in the cytoplasm, as well as in the nucleus, similar to GFP alone (Fig. 3A), corroborating the importance of the putative NLS motif. To test whether this putative NLS motif is sufficient to direct nuclear import, we constructed fusions encompassing this motif N and C terminally to two different reporters. The sequence including aa 204 to 218 of Pcl5 was fused to the N terminus of GFP (pME2950), and the $Pcl5_{aa207\text{-}215}$ sequence was fused to C terminus of GFP-Aro7 (pME2951), an exclusively cytoplasmic protein (45). Neither of the constructs was found in the nucleus (Fig. 3A). Together, these data indicate that aa 207 to 215 of Pcl5 are necessary but not sufficient for nuclear Pcl5 localization.

Pcl5 nuclear transport requires the importin Kap95. We analyzed the subcellular localization of Pcl5-GFP in a set of *S.*

FIG. 3. The C-terminal part of Pcl5 directs nuclear localization. (A) Yeast *pcl5* mutant strain RH3238 was transformed to express either GFP alone (pME2849) or in N-terminal fusion with $Pcl5_{aa1\text{-}229}$ (pME2846), $Pcl5_{aa1\text{-}95}$ (pME2850), $Pcl5_{aa1\text{-}127}$ (pME2851), $Pcl5_{aa1\text{-}180}$ (pME2853), $Pcl5_{aa61\text{-}229}$ (pME2854), $Pcl5_{aa111\text{-}180}$ (pME2855), $Pcl5_{aa111\text{-}229}$ (pME2856), $Pcl5_{aa153\text{-}229}$ (pME2857), $Pcl5_{aa181\text{-}229}$ (pME2859), $Pcl5_{aa204\text{-}218}$ (pME2950), Aro7-$Pcl5_{aa207\text{-}215}$ (pME2951), $Pcl5_{aa181\text{-}229^{***}}$ (with the mutated NLS motif marked by asterisks) (pME3578), $Pcl5_{aa1\text{-}229^{***}}$ (pME3577), $Pcl5_{aa61\text{-}229^{***}}$ (pME3370), $Pcl5_{aa61\text{-}180}$ (pME2858), $Pcl5_{aa79\text{-}178}$ (pME3574), $Pho80_{aa1\text{-}73}$-$Pcl5_{aa79\text{-}178}$-$Pho80_{aa170\text{-}294}$ (pME2860), or $Pho80_{aa1\text{-}73}$-$Pcl5_{aa79\text{-}178}$ (pME2948) from the *MET25* promoter on a high-copy plasmid. In addition, $Pho80_{aa1\text{-}73}$-$Pcl5_{aa79\text{-}178}$ was expressed from a low-copy plasmid under the control of the *PCL5* promoter (pME3576). GFP signals were analyzed by fluorescence microscopy (GFP) or DIC microscopy. Nuclear localization is indicated by white colored arrows. An arrowhead only marks plasma membrane localization. Accumulation in the cytoplasm is highlighted by a rendered blank arrow. A dashed arrow is used in the case of cytoplasmic aggregates. The differences in sizes of the cells shown are due to different enlargement. "X/200" in parentheses represents the fraction "X" of 200 cells displaying the marked staining pattern. (B) Yeast *pcl5* cells transformed with the same high-copy plasmids as in panel A beside pME2849 encoding GFP alone and the low-copy plasmid pME3576 expressing $Pho80_{aa1\text{-}73}$-$Pcl5_{aa79\text{-}178}$ were grown to early log phase, and the expression of the GFP fusion proteins was analyzed by immunoblotting with monoclonal anti-GFP antibodies. Protein extracts of the cells are blotted in the same order as in panel A (left panel, top to bottom; right panel, top to bottom).

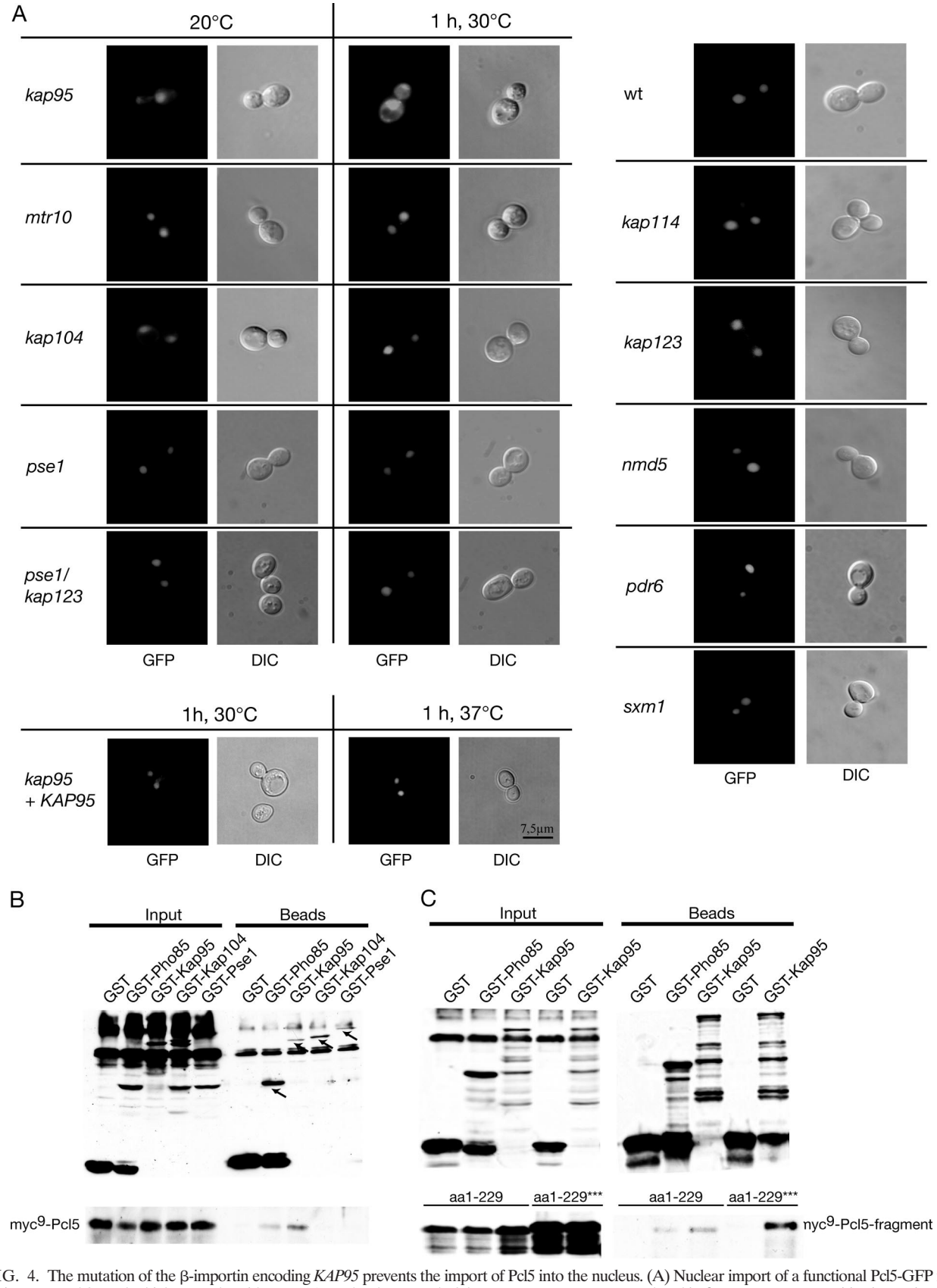


FIG. 4. The mutation of the β-importin encoding *KAP95* prevents the import of Pcl5 into the nucleus. (A) Nuclear import of a functional Pcl5-GFP fusion protein expressed from the high-copy plasmid pME2846 under the control of the *MET25* promoter was analyzed in five temperature-sensitive importin mutant strains by fluorescence microscopy (GFP) and DIC microscopy. Pcl5 translocation is not affected in the mutant strains *mtr10* (RH2701), *kap104* (RH2702), *pse1*

cerevisiae mutant strains that are defective in particular β-importins. Yeast strains deleted for the nonessential importin genes *kap114*, *kap123*, *nmd5*, *pdr6*, and *sxm1* were cultured and analyzed at a temperature of 30°C. Strains carrying the temperature-sensitive mutations *kap95*, *mtr10*, *kap104*, *pse1*, or *pse1*/*kap123* were analyzed at the permissive temperature of 20°C and the restrictive temperature of 30°C. Localization studies showed that Pcl5-GFP was nuclear in the *kap114*, *kap123*, *nmd5*, *pdr6*, *sxm1*, *mtr10*, *kap104*, *pse1*, or *pse1*/*kap123* mutant strains. A cytoplasmic accumulation of Pcl5-GFP was observed in the *kap95* mutant cells at their restrictive temperature of 30°C, suggesting that Kap95 is necessary for nuclear localization of Pcl5 (Fig. 4A). Transformation of *kap95* mutant cells with *KAP95* under the control of the *MET25* promoter on a 2μm plasmid restored nuclear localization of Pcl5-GFP at the restrictive temperatures of 30 and 37°C (Fig. 4A). The high expression of *KAP95* results also in a 30% subpopulation of yeast cells, which showed Pcl5-GFP aggregate formation similar to *kap95* cells.

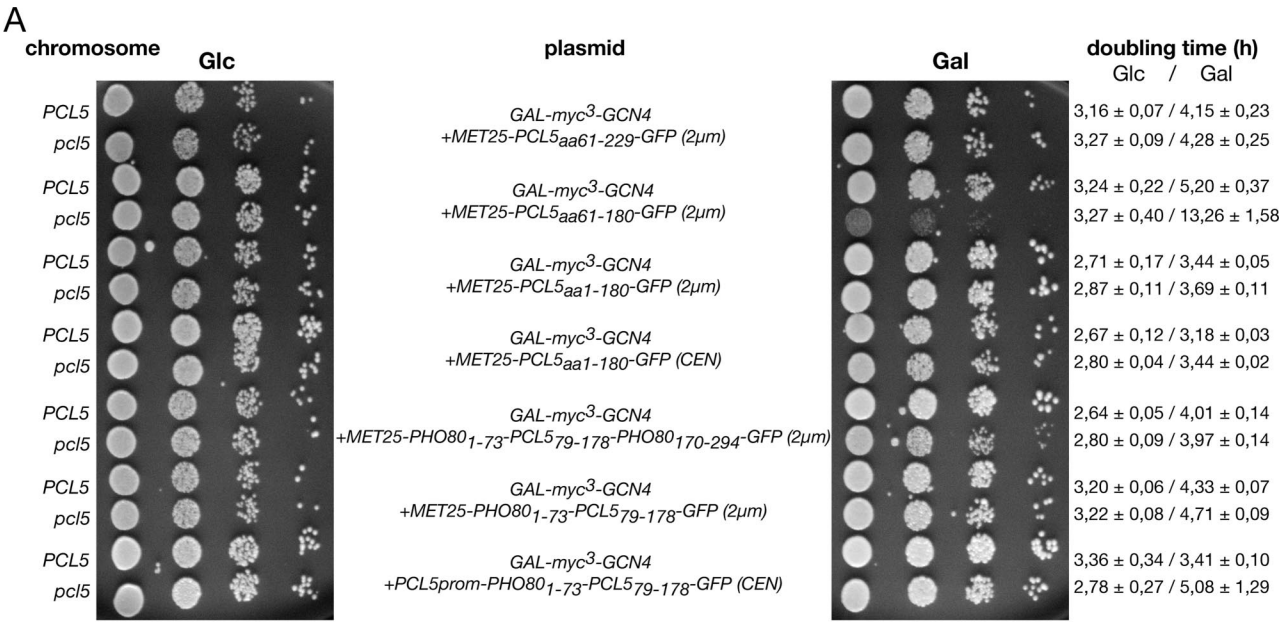
Next, we wanted to know whether there is a direct interaction between Kap95 and Pcl5. Kap95/Pcl5 interaction was investigated by an in vivo coprecipitation assay under conditions when cellular Gcn4 is unstable. *GST-KAP95* and *myc*^{9}*-PCL5* were expressed from the *GAL1* promoter. The protein fusions were induced and purified with glutathione beads to isolate the GST fusion and its associated proteins. Figure 4B shows that myc^9-Pcl5 copurifies with GST-Kap95 under the tested condition. To exclude that the physical interaction of Pcl5 and Kap95 is an overexpression effect, we tested the two other importin fusions, *GST-PSE1* and *GST-KAP104*, concerning their interaction with the cyclin Pcl5. Neither GST-Pse1 nor GST-Kap104 copurified myc^9-Pcl5 in comparison to GST-Kap95 (Fig. 4B). We also analyzed the interaction of GST-Kap95 and Pcl5*** fragments with an altered C-terminal NLS motif. myc^9-Pcl5$_{aa1\text{-}229***}$ copurifies with GST-Kap95 like the nonmutated myc^9-Pcl5 (Fig. 4C), whereas neither myc^9-Pcl5$_{aa181\text{-}229}$ nor myc^9-Pcl5$_{aa181\text{-}229***}$ interacted with GST-Kap95 under the tested conditions (data not shown). This suggests that a tight interaction between Kap95 and Pcl5 requires more than the C-terminal domain of Pcl5. The N-terminal myc^9-tag might impair binding of the C-terminal domain alone.

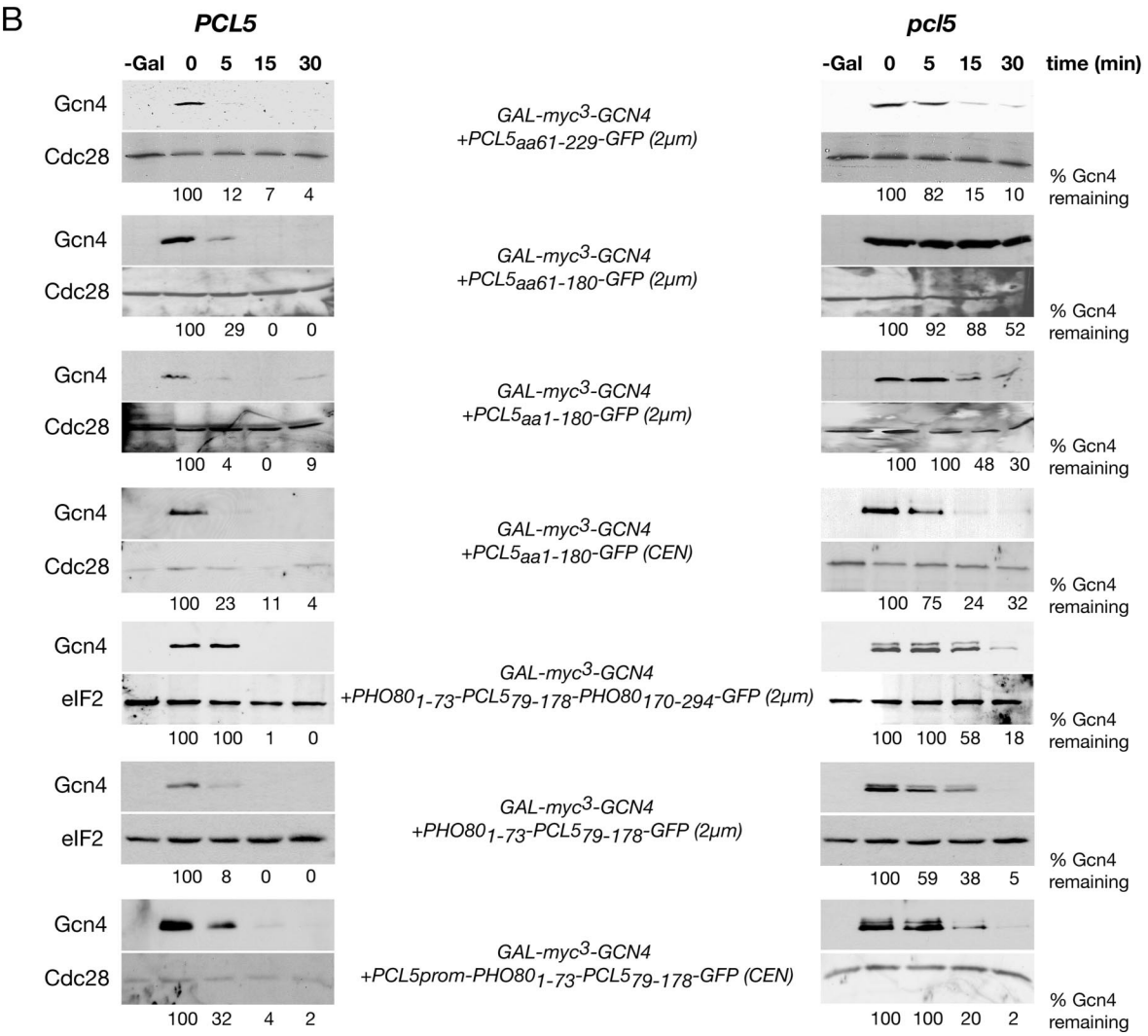
In summary, we have shown that Kap95 is required for accurate nuclear import of the yeast cyclin Pcl5 by a physical interaction.

Analysis of the activity of the Pcl5-GFP hybrids. We used a set of different truncated Pcl5-GFP versions described above to define the specificity domain for the Pho85/Pcl5 complex, which promotes Gcn4 phosphorylation and subsequent degradation. The function of all Pcl5-GFP hybrids was first tested in the Gcn4 overexpression toxicity assay on solid medium as well as in liquid culture. We found that in the *pcl5* background the toxicity of *GCN4* moderately overexpressed from the *GAL1* promoter can only be suppressed by Pcl5$_{aa61\text{-}229}$ or Pcl5$_{aa1\text{-}180}$ (Fig. 5A), but not by any of the other truncated Pcl5-GFP hybrids. For a more detailed analysis, i.e., to determine whether the truncated Pcl5 versions are able to promote Gcn4 degradation, we tested the effect of the truncated *PCL5-GFP* hybrids on Gcn4 degradation. Wild-type *PCL5* and *pcl5* mutant cells were transformed to express *GAL-myc*^{3}*-GCN4*, as well as the truncated Pcl5-GFP fusions. Promoter shutoff experiments of myc^3-Gcn4 revealed a rapid degradation of this protein when Pcl5 aa 61 to 229 or aa 1 to 180 are expressed (Fig. 5B). The N-terminal domain with the central part (aa 1 to 180) expressed from a low-copy (*CEN*) plasmid is still able to suppress *GCN4* overexpression toxicity and degrades efficiently Gcn4 (Fig. 5B). The central domain of Pcl5 (aa 61 to 180) containing the cyclin box domain (aa 79 to 178) (shown schematically in Fig. 6) is unable to direct nuclear import on its own and showed no Pcl5 function in the Gcn4 toxicity assay (Fig. 3A and 5A). In agreement, Gcn4 is highly stable when *PCL5* encoding aa 61 to 180 is present by simultaneous expression (Fig. 5B). Similar results were observed for Pcl5$_{aa79\text{-}178}$-GFP (Fig. 3A and data not shown). A fusion of nuclear localized Pcl5 aa 61 to 180 to SV40 large tumor antigen NLS motif (pME3371) was not sufficient to restore nuclear localization and subsequently Gcn4 degradation (data not shown).

The C-terminal NLS of Pcl5 can be replaced by the N-terminal NLS of Pho80. We analyzed whether the Pcl5 NLS can be replaced by the NLS of another Pho85 cyclin without

(RH2703), or *pse1*/*kap123* (RH2706), whereas the *kap95* (RH2704) mutation impairs the uptake of Pcl5-GFP at the restrictive temperature of 30°C. Expression of *KAP95* under the control of the *MET25* promoter on a high-copy plasmid (pME3583) in *kap95* mutant cells (RH2704) restores the uptake of Pcl5-GFP to the nucleus (~ 40%) at 30 and 37°C. Furthermore, five mutant strains with the nonconditional importin mutations *kap114* (RH3058), *kap123* (RH2707), *nmd5* (RH2708), *pdr6* (RH2709), and *sxm1* (RH2710) were examined for subcellular localization of Pcl5-GFP by fluorescence microscopy. Nuclear import of Pcl5 was unaffected in all five mutant strains and was indistinguishable from that in the wild-type (wt) control (RH3237). (B) Protein-protein interaction of Pcl5 with Kap95. Yeast *pcl5* mutant strain RH3238 was transformed to express either *myc*^{9}*-PCL5* (pME2865) with GST (*GST* on pYGEX-2T), *GST-KAP104* (pME3447), or *GST-PSE1* (pME3448) as negative controls or *myc*^{9}*-PCL5* (pME2865), together with *GST-PHO85* (pME2866), as a positive control. In addition, yeast strain RH3238 was transformed to express *myc*^{9}*-PCL5* (pME2865), together with *GST-KAP95* (pME3372). Protein levels of the fusion proteins were determined by Western blotting with rabbit anti-GST and mouse anti-myc antibodies before and after incubation with glutathione-Sepharose. The left part (Input) represents the GST, GST-Kap95, GST-Kap104, GST-Pse1, and myc^9-Pcl5 before glutathione-Sepharose incubation to ensure that the initial protein extracts contain similar amounts of the fusion proteins. On the right (Beads) the elutions of the glutathione beads are shown. The GST fusion proteins are marked with an arrow in the respective lane of the elutions. (C) Mutation of the C-terminal NLS motif in Pcl5 does not impede the interaction to Kap95. This is the same experiment as shown in panel B. Yeast *pcl5* mutant strain RH3238 was transformed to express either *myc*^{9}*-PCL5* (pME2865) together with GST (*GST* on pYGEX-2T), *GST-PHO85* (pME2866), or *GST-KAP95* (pME3372). In addition, *pcl5* mutant cells (RH3238) were transformed to express *myc*^{9}*-PCL5**** with a mutated NLS motif (pME3577), together with GST (*GST* on pYGEX-2T) or *GST-KAP95* (pME3372). Input (left part) represents the initial protein extracts before glutathione-Sepharose incubation. On the right side (Beads) the elutions of the beads are shown.


A
chromosome
Glc
plasmid
Gal
doubling time (h)
Glc / Gal
PCL5
pcl5
GAL-myc3-GCN4
+MET25-PCL5aa61-229-GFP (2µm)
3,16 ± 0,07 / 4,15 ± 0,23
3,27 ± 0,09 / 4,28 ± 0,25
+MET25-PCL5aa61-180-GFP (2µm)
3,24 ± 0,22 / 5,20 ± 0,37
3,27 ± 0,40 / 13,26 ± 1,58
+MET25-PCL5aa1-180-GFP (2µm)
2,71 ± 0,17 / 3,44 ± 0,05
2,87 ± 0,11 / 3,69 ± 0,11
+MET25-PCL5aa1-180-GFP (CEN)
2,67 ± 0,12 / 3,18 ± 0,03
2,80 ± 0,04 / 3,44 ± 0,02
+MET25-PHO801-73-PCL579-178-PHO80170-294-GFP (2µm)
2,64 ± 0,05 / 4,01 ± 0,14
2,80 ± 0,09 / 3,97 ± 0,14
+MET25-PHO801-73-PCL579-178-GFP (2µm)
3,20 ± 0,06 / 4,33 ± 0,07
3,22 ± 0,08 / 4,71 ± 0,09
+PCL5prom-PHO801-73-PCL579-178-GFP (CEN)
3,36 ± 0,34 / 3,41 ± 0,10
2,78 ± 0,27 / 5,08 ± 1,29


B
PCL5
pcl5
-Gal 0 5 15 30
time (min)
Gcn4
Cdc28
eIF2
GAL-myc3-GCN4
+PCL5aa61-229-GFP (2µm)
100 12 7 4
100 82 15 10
+PCL5aa61-180-GFP (2µm)
100 29 0 0
100 92 88 52
+PCL5aa1-180-GFP (2µm)
100 4 0 9
100 100 48 30
+PCL5aa1-180-GFP (CEN)
100 23 11 4
100 75 24 32
+PHO801-73-PCL579-178-PHO80170-294-GFP (2µm)
100 100 1 0
100 100 58 18
+PHO801-73-PCL579-178-GFP (2µm)
100 8 0 0
100 59 38 5
+PCL5prom-PHO801-73-PCL579-178-GFP (CEN)
100 32 4 2
100 100 20 2
% Gcn4 remaining

loss of function. The rationale for these experiments was the question whether nuclear localization represents a distinct domain or whether it overlaps with other functions of the cyclin. Recently, it was shown that a Pho80-Pcl5-Pho80 hybrid is able to mediate Gcn4 degradation (2). The cyclin Pho80 normally acts as the specificity factor of Pho85 for the transcription factor Pho4 and is unable to promote Gcn4 degradation (52). Since the 49 carboxy-terminal amino acids of Pcl5 are required for nuclear localization, we first analyzed whether a Pho80-Pcl5 hybrid carrying the N and C termini of Pho80 is able to mediate nuclear localization of the central Pcl5 domain. The functional Pho80-Pcl5-Pho80-GFP fusion protein (Fig. 5) is localized in the yeast nucleus in contrast to the $Pcl5_{aa61-180}$-GFP and $Pcl5_{aa79-178}$-GFP hybrid (Fig. 3A). Furthermore, we analyzed which part of this Pho80-Pcl5-Pho80 hybrid, which carries aa 79 to 178 of Pcl5 to provide Gcn4 specificity to the CDK, is responsible for nuclear localization. Fusions of either the Pho80 N or C terminus to this Pcl5 fragment were analyzed for their subcellular localization (N-Pho80-Pcl5-GFP and Pcl5-Pho80-C-GFP). Strikingly, only the Pho80-Pcl5-GFP-fusion carrying the Pho80 N terminus is localized in the nucleus (Fig. 3A) and is able to suppress the overexpression toxicity of Gcn4 (Fig. 5A). Accordingly, Gcn4 is degraded in the presence of *PHO80-PCL5* encoding $Pho80_{aa1-73}$-$Pcl5_{aa79-178}$-GFP, even when expressed from a low-copy (*CEN*) plasmid under the control of the *PCL5* promoter (Fig. 5B). In contrast, a Pcl5-Pho80-GFP hybrid containing only the Pho80 C terminus is found as aggregates over the whole cell, similar to the localization of the Pcl5 central part alone (data not shown and Fig. 3A). Thus, in contrast to Pcl5, which primarily depends on a C-terminal NLS, Pho80 is transported into the nucleus via an N-terminal NLS. Our data demonstrate that the C-terminal Pcl5 NLS can be replaced by the N-terminal Pho80 NLS without loss of substrate specificity, and therefore the nuclear localization regions of cyclins Pcl5 or Pho80 represent distinct domains, which can be combined in a modular fashion and exchanged.

DISCUSSION

We show here that the highly unstable Pho85-dependent cyclin Pcl5 of the yeast *S. cerevisiae* is a constitutively nuclear protein due to a C-terminal NLS. Transport is mediated by the interaction of the cyclin with the β-importin Kap95. The C-terminal part of Pcl5 contains the motif PVKRPRESD that is reminiscent of the c-myc NLS (31). Amino acid substitutions of this sequence resulting in changes of the basic cluster into alanine residues lead to increased cytoplasmic localization of Pcl5-GFP. The motif therefore seems to be important for nuclear localization of Pcl5 but requires additional sequences of the C terminus for nuclear import, because the motif alone is not sufficient for directing another cytoplasmic protein in a fusion construct into the nucleus. A construct where the Pcl5 cyclin box domain is fused to a classical NLS motif of the SV40 large tumor antigen is not localized to the nucleus and does not promote Gcn4 degradation, suggesting that adjacent sequences are required for nuclear localization and proper function. This is supported by the finding that mutation of the NLS does not prevent the interaction of Pcl5 and Kap95 or that the C-terminal domain alone (aa 181 to 229) does not interact to Kap95 under the same conditions (Fig. 4C). A similar situation has been described for the motif PAKKSFLRRLFD of the lymphoid specific factor RAG2 essential for the diversification of antigen receptors on B and T lymphocytes. A 37-aa minimal region containing this motif is necessary for nuclear targeting of RAG2 (6). In the case of Pcl5 the 49 C-terminal aa 181 to 229, which are sufficient for directing GFP into the nucleus, represent the nonclassical Pcl5 NLS.

The N- or C-terminal domains of Pcl5, although not essential for Pcl5 function, might contribute to the function of the protein, either at the level of substrate recognition, by maintenance of the structural integrity of the cyclin box domain, or by a residual nuclear import capacity. This is supported by the $Pcl5_{aa1-180}$-GFP, $Pcl5_{aa1-229***}$-GFP, and $Pcl5_{aa61-229***}$-GFP hybrids, which all lack any strong NLS but show some Gcn4 degradation when overexpressed (Fig. 5 and data not shown). The N terminus may contribute to nuclear localization by way of an ancillary NLS. $Pcl5_{aa1-180}$ lacking the C terminus and its NLS shows some nuclear enrichment (Fig. 3B). The N-terminal domain contains eight R/K residues (including one RKK stretch) that may represent in combination with the cyclin box an ancillary nuclear localization motif. This would be similar to the substrate Gcn4 that contains two NLS motifs, of which NLS2 is the essential and specific transport signal, NLS1 playing only an auxiliary role (38).

The amino-terminal portion of Pcl5 without the cyclin box domain targets GFP to a distinct plasma membrane localization. This unexpected localization might represent an artifact of hybrid protein construction, or it might reflect a second yet-unexplored function of Pcl5 at the membrane. It is conceivable that full-length Pcl5 contains a membrane-targeted subpopulation, which is normally masked by the prominent nuclear localization of this protein, and only observed when

FIG. 5. Functional analysis of Pcl5-Pho80 hybrids fused to GFP. (A) The Pho80-$Pcl5_{aa79-178}$-Pho80 hybrid is able to suppress the overexpression toxicity of Gcn4 like full-length-Pcl5 in *pcl5* yeast cells. Wild-type cells (RH3237) and *pcl5* mutant cells (RH3238) expressing *myc*^{3}*-GCN4* from the *GAL1* promoter (pME2848), together with *MET25-PCL5*$_{aa61-229}$*-GFP* (pME2854, 2μm), *MET25-PCL5*$_{aa61-180}$*-GFP* (pME2858, 2μm), *MET25-PCL5*$_{aa1-180}$*-GFP* (pME2853, 2μm), *MET25-PCL5*$_{aa1-180}$*-GFP* (pME3573, *CEN*), *MET25-PHO80*$_{aa1-73}$*-PCL5*$_{aa79-178}$*-PHO80*$_{aa170-294}$*-GFP* (pME2860, 2μm), or *MET25-PHO80*$_{aa1-73}$*-PCL5*$_{aa79-178}$*-GFP* (pME2948, 2μm), and *PCL5prom-PHO80*$_{aa1-73}$*-PCL5*$_{aa79-178}$*-GFP* (pME3576, *CEN*) were spotted in 10-fold dilutions on glucose and galactose to induce expression of *GCN4* driven by the *GAL1* promoter. The plates were incubated for 3 days at 30°C. Furthermore, these cells were used for growth tests in liquid culture with glucose or galactose as a carbon source, and the doubling times of three or more independent cultures were determined. (B) The Pho80-$Pcl5_{aa79-178}$-Pho80 hybrid is able to promote Gcn4 degradation like full-length Pcl5 in *pcl5* yeast cells. Protein levels of myc^3-Gcn4, Cdc28, or eIF-2 were determined by Western blotting at the indicated time points after the *GAL1* promoter shutoff in the same transformed yeast cells as described in panel A. The numbers given below each lane indicate the remaining Gcn4 percentage compared to Cdc28 or eIF-2 as internal standard quantified with a Kodak imaging station of the gel shown.

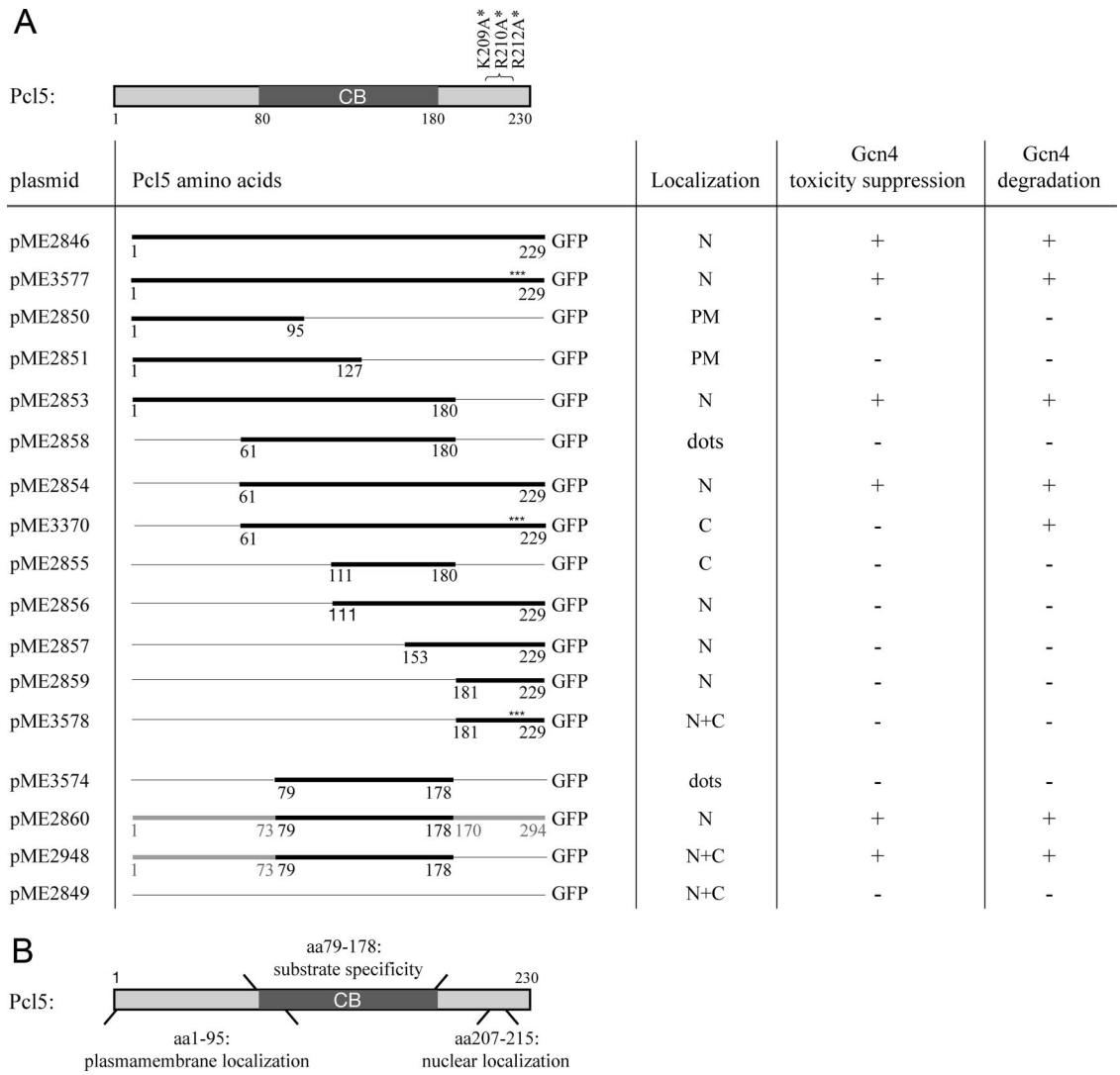


FIG. 6. Domain analysis of yeast cyclin Pcl5. (A) On the left side of the panel are listed Pcl5 fragments fused to the N terminus of GFP. pME2846 ($Pcl5_{aa1-229}$), pME3577 ($Pcl5_{aa1-229***}$ containing the three amino acid substitutions K209A, R210A, and R212A marked by asterisks), pME2850 ($Pcl5_{aa1-95}$), pME2851 ($Pcl5_{aa1-127}$), pME2853 ($Pcl5_{aa1-180}$), pME2858 ($Pcl5_{aa61-180}$), pME2854 ($Pcl5_{aa61-229}$), pME3370 ($Pcl5_{aa61-229***}$ containing the three amino acid substitutions K209A, R210A, and R212A), pME2855 ($Pcl5_{aa111-180}$), pME2856 ($Pcl5_{aa111-229}$), pME2857 ($Pcl5_{aa153-229}$), pME2859 ($Pcl5_{aa181-229}$), pME3578 ($Pcl5_{aa181-229***}$ containing the three amino acid substitutions K209A, R210A, and R212A), pME3574 ($Pcl5_{aa79-178}$), pME2860 ($Pho80_{aa1-73}$-$Pcl5_{aa79-178}$-$Pho80_{aa170-294}$), pME2948 ($Pho80_{aa1-73}$-$Pcl5_{aa79-178}$), and pME2849 (GFP alone). A summary of the results is shown on the right. The columns indicate the subcellular localization of the different *PCL5* or *PCL5-PHO80* deletions (Fig. 3) and their ability to complement the *pcl5* phenotype of Gcn4 toxicity or to promote Gcn4 degradation (Fig. 5). CB, cyclin box; N, nuclear; PM, plasma membrane; C, cytoplasm; +, yeast cells are able to complement the *pcl5* phenotype or to degrade Gcn4, respectively; −, yeast cells are not able to complement the *pcl5* phenotype or rather to degrade Gcn4. (B) Scheme of identified Pcl5 domains. The relative positions of the different domains within the full-length Pcl5 protein are shown. The region from aa 1 to 95 represents a putative plasma membrane binding site motif, and the middle part consisting of aa 79 to 178 is required for the right substrate specificity. The Pcl5 carboxyl terminus of aa 207 to 215 is required for nuclear localization. CB, cyclin box.

the C-terminal NLS is removed. An interesting possibility is that a small membrane-associated Pcl5 population, shielded from autophosphorylation and degradation, is poised for activity in the case of increased amino acid influx.

The cyclin box domain of Pcl5 alone shows a aggregation phenotype with dot-like structures covering the whole cell. The fact that the N- and C-terminal domains which contain the degradation signals for Pcl5 turnover (2) are missing and the fact that the localization was analyzed under overexpression conditions might be the reason for the aggregation. Interestingly, there are similarities to the localization pattern of α-synuclein expressed on different levels in yeast. Expression of one copy shows plasma membrane localization of α-synuclein, and expression of two copies leads to large cytoplasmic inclusions (aggregation). When α-synuclein was expressed from a 2μm plasmid it was first accumulated at the plasma membrane and was later recruited away into cytoplasmic inclusions (42).

For some cyclins it has been shown that mislocalization to another cellular compartment is sufficient to switch cyclin func-

tion. If the normally nuclear G1 cyclin Cln3 is mislocalized to the cytoplasm, where Cln2 normally resides, this Cln3 mutant is able to assume Cln2-like functions (34). These observations suggest that G1 cyclins are mainly required to activate Cdc28, rather than to target the CDK to specific substrates. This hypothesis is further supported by the isolation of a Cdc28 mutant that is independent of G1 cyclin activity (30). Thus, in some instances, the key role of the cyclin may be to activate the CDK in a proper temporal and spatial setting rather than to confer substrate selectivity.

This is clearly not the case here, where Pcl5 and Pho80 are both nuclear cyclins. Exclusion of Pcl5 from the nucleus inhibits Pho85 activity to initiate Gcn4 degradation. Mislocalization of Pcl5 to the cytoplasm by the addition of an NES leads to a strong stabilization of Gcn4. Stabilization of Gcn4 in a *pcl5* strain expressing *GFP* or *PCL5-GFP-NES* has no impact on either the basal transcription of the Gcn4 target genes *HIS4* and *ARO4* or the level of induction after 3-AT (3-amino-1,2,4-triazole) treatment compared to a *PCL5* wild-type strain or a *pcl5* mutant strain expressing *PCL5-GFP* (data not shown). We cannot exclude that this way of mislocalization also affects Pcl5 function but assume that mislocalized cytoplasmic Pcl5 would still be able to mediate Gcn4 phosphorylation, when Pho85 and Gcn4 are also localized in the cytoplasm. A Pcl5 construct that still contains the entire cyclin box domain and that therefore would be expected to recognize and activate Pho85 and to recognize its substrate is also unable to mediate Gcn4 degradation due to the lack of NLSs. Consistently, a fusion of the cyclin box domain to either the native C-terminal NLS of Pcl5 or the N-terminal NLS of Pho80 rescues Pcl5 function in Gcn4 degradation. Therefore, it is possible to transform the substrate specificity of Pho80, which normally does not affect Gcn4 stability by substituting its cyclin box for the corresponding Pcl5 cyclin box domain. This strongly supports the importance of these Pho85 cyclin subunits in substrate selectivity within the corresponding cyclin-dependent kinase complexes. Correct subcellular localization represents an additional, independent requirement for the function of the cyclin.

In summary, we were able to identify the subcellular localization domains of the Pho85 cyclins Pcl5 and Pho80 as distinct domains, which can be separated from the substrate selectivity domain. This module-like manner defines a major difference in comparison to the cyclins Cln2 or Cln3 of the kinase Cdc28, where localization is a predominant determinant of substrate specificity.

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