Relationship between the function and the location of G1 cyclins in *S. cerevisiae*

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SUMMARY

The *Saccharomyces cerevisiae* cyclin-dependent kinase Cdc28 forms complexes with nine different cyclins to promote cell division. These nine cyclin-Cdc28 complexes have different roles, but share the same catalytic subunit; thus, it is not clear how substrate specificity is achieved. One possible mechanism is specific sub-cellular localization of specific complexes. We investigated the location of two G1 cyclins using fractionation and microscopy. In addition, we developed 'forced localization' cassettes, which direct proteins to particular locations, to test the importance of localization. Cln2 was found in both nucleus and cytoplasm. A substrate of Cln2, Sic1, was also in both compartments. Cytoplasmic Cln2 was concentrated at sites of polarized growth. Forced localization showed that some functions of Cln2 required a cytoplasmic location, while other functions required a nuclear location. In addition, one function apparently required shuttling between the two compartments. The G1 cyclin Cln3 required nuclear localization. An autonomous, nuclear localization sequence was found near the C-terminus of Cln3. Our data supports the hypothesis that Cln2 and Cln3 have distinct functions and locations, and the specificity of cyclin-dependent kinases is mediated in part by subcellular location.

Key words: Cyclin, Localization, Targeting, CDK, Yeast

INTRODUCTION

Yeast relies on a single cyclin-dependent kinase (CDK), Cdc28, as the sole essential cell cycle CDK (Lorincz and Reed, 1984). Many different CDC28-dependent events occur at various times in the cell cycle, so the Cdc28 kinase must have a way of recognizing different substrates at different times. This specificity is mediated, in part, by the different cyclin partners, which bind to and activate Cdc28 (Morgan, 1995). In *S. cerevisiae*, Cdc28 is activated by at least nine different cyclins that can be grouped into three functional classes: G1 cyclins (Cln1, Cln2, Cln3), S-phase cyclins (Clb5, Clb6) and G2 cyclins (Clb1, Clb2, Clb3, Clb4) (Futcher, 1996). These cyclins are expressed at different times by a combination of transcriptional control and protein instability. Thus, the different cyclins are available at different times, and could in principle direct Cdc28 kinase towards different, stage-specific substrates.

The G1 cyclin Cln3, in association with Cdc28, allows cells to commit to a new round of division or 'Start' by activating the SBF and MBF transcription factors (Dirick et al., 1992; Nasmyth and Dirick, 1991). These induce transcription of at least 200 cell-cycle regulated genes in late G1 (Spellman et al., 1998). Among the genes induced by Cln3 are *CLN1*, *CLN2*, *CLB5*, *CLB6*, *PCL1* and *PCL2*. The SBF and MBF transcription factors share the protein Swi6, and a *swi6* mutation is epistatic to a *cln3* mutation (Wijnen and Futcher, personal communication), at least for Start. This suggests that promoting transcription may be the only role of Cln3 at Start.

The closely related cyclins Cln1 and Cln2 act redundantly to promote polarized growth, budding (Lew and Reed, 1993), and spindle pole body duplication (Haase et al., 2001), and to repress the anaphase-promoting complex (APC) and phosphorylate the Cdc28 inhibitors Far1 and Sic1, causing their destruction (Cross, 1995; Henchoz et al., 1997; Peter et al., 1993; Schneider et al., 1996; Schwob et al., 1994; Tyers et al., 1991; Tyers et al., 1993). Ste20, a PAK kinase that affects morphogenesis (Oehlen and Cross, 1998; Wu et al., 1998), is another likely substrate. Unfortunately, the substrates involved in budding, spindle pole duplication and shutting off the APC remain unidentified.

Although the Cln1, Cln2 pair promotes essential events such as budding and the destruction of Sic1, the *cln1 cln2* double mutant is viable. This is because the essential functions of Cln1 and Cln2 can also be undertaken (albeit suboptimally) by other cyclins, such as Clb5 and Clb6 (which activate Cdc28) and Pcl1 and Pcl2 (which activate the Cdc28-related kinase Pho85). For example, the *cln1 cln2 clb5 clb6* and the *cln1 cln2 pcl1 pcl2* quadruple mutants are inviable, and arrest with large unbudded cells and unreplicated DNA (Schwob and Nasmyth, 1993). The *cln1 cln2* mutant is also synthetically lethal with a wide variety of other single mutations, including *bud2* (which affects budding) (Benton et al., 1993; Cvrckova and Nasmyth, 1993), *kar3* (a kinesin-like motor protein involved in spindle function) (Cross, 1995), *msn5* (a nuclear import/export protein) (Alepuz et al., 1999), *swi6*, *pho85*, *alg1*, *cla4*, *gin4* and *rad27* (Benton et al., 1993; Espinoza et al., 1994).

Finally, the *cln1 cln2 cln3* triple mutant is also inviable. This inviability is not because Cln3 has overlapping biochemical functions with Cln1 and Cln2; rather, it is because Cln3

promotes transcription of *CLB5*, *CLB6*, *PCL1*, *PCL2* and perhaps other genes, which do have overlapping functions with *CLN1* and *CLN2*.

Cln3 on the one hand, and Cln1 and Cln2 on the other, clearly have different roles in the cell, despite being classed together as G1 cyclins. The S-phase cyclins Clb5 and Clb6, and the G2 cyclins Clb1, Clb2, Clb3 and Clb4 have even more disparate roles. Although different families of cyclins differ in their temporal expression patterns, this alone is not sufficient to explain their distinct roles (Cross et al., 1999). Therefore, it is believed that the different cyclins somehow impose different substrate specificities on Cdc28, which in turn promotes the completion of specific cell cycle events in the correct order. How do cyclins affect substrate specificity of the kinase complex? This question is particularly acute in yeast, where a single Cdk is used for all phases of the cycle.

A typical CDK of the Cdc2 family phosphorylates a site with the consensus (S/T)-P-X-(K/R), and it appears that this site preference is not significantly affected by the cyclin bound to the CDK. This suggests that there is a higher order of substrate preference imposed by the cyclin (Cross et al., 1999).

One way that cyclins can provide substrate specificity is by binding directly to the substrate (Levine and Cross, 1995). For example, cyclin A binds to the CDK inhibitor p27, thus targeting the cyclin A-Cdk2 complex for inhibition by p27. Cyclin A contains a hydrophobic patch, which is important for targeting cyclin A-Cdk2 complexes to substrates containing RXL motifs, including p27 (Schulman et al., 1998; Takeda et al., 2001).

A second mechanism for substrate specificity is that the cyclin may localize the Cdk to a particular subcellular region and thereby restricts access of the Cdk to potential substrates (Gallant et al., 1995; Takizawa and Morgan, 2000). Cyclin B1 exemplifies this method of regulation (Draviam et al., 2001; Li et al., 1997; Pines and Hunter, 1994). If cyclin B1 translocation from the cytosol to the nucleus is blocked, then its biological activity is blocked even though it can still bind and activate Cdc2. This suggests that the localization of cyclin B1-Cdc2 helps specify interactions with cellular substrates.

Recently, Miller and Cross used subcellular fractionation and fluorescence microscopy to study the localization and function of Cln2 and Cln3 (Miller and Cross, 2000). They found that Cln2 is primarily cytoplasmic, while Cln3 is primarily nuclear, at least in large, budded cells. Miller and Cross also used a single artificial NLS (nuclear localization signal) or NES (nuclear export signal) to evaluate the functional relevance of Cln location. Unfortunately, the single NLS or NES was relatively ineffective in changing the location of full length Cln2 or Cln3, which limited the power of the functional studies.

Here, we describe additional studies of the location of Cln2 and Cln3, and of the functional importance of these locations. Location was determined using both GFP fluorescence and sub-cellular fractionation, while the functional significance of location was determined using two copies of an NLS or two copies of an NES. These double signals efficiently targeted the tagged protein to the desired location. Cln2 is localized in both cytoplasmic and nuclear compartments. Cytoplasmic Cln2 is associated with sites of budding, which is intriguing given that Cln activity is needed for budding. Functional studies confirm that Cln2 has important roles in both the cytoplasm and the nucleus, and there is evidence for a role that requires shuttling between the compartments. Cln3 function requires a nuclear location, and this localization is normally imposed by a bipartite NLS at the C-terminus.

MATERIALS AND METHODS

Media and yeast strain manipulations

Yeast strains were grown in YPD media (2% glucose, 1% yeast extract, 2% peptone) or selective synthetic minimal (SD) media if plasmid maintenance was required. Standard methods were used for sporulation, mating, plasmid transformation, tetrad dissection, and one-step gene replacement (Ausubel, 1987). Strains were made auxotropic by disrupting markers by either the marker swap method (Cross, 1997) or by using the dominant selectable KanMX module (Wach et al., 1994). Yeast strains (Table 1) derived either from the W303a *ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1* [psi+] *ssd1-d* background, or the BF264-15Daub *trp1 leu2 ura3 ade1 his2 SSD1*

Table 1. Yeast strains used in these studies

Name	Relevant genotype*	Derivation/source
BF background		
FC-1254-14D	*MAT **a** cln1 cln2 cln3 leu2:LEU2-GAL1,10::CLN1*	F. Cross
N-80	*MAT α cln1 cln2* p*cl1::HIS3* p*cl2::URA3 leu2::G418^R-GAL1,10::CLN1*	This study
N-104(#31)	*MAT **a** cln1::HIS3 cln2::TRP1 cln3-ura3- GAL::CLN3 cdc34-2*	B. Futcher
N-138	*MAT α cln1 cln2 cln3 leu2::G418^R-GAL1,10::CLN1*	This study
N-139	*MAT **a** cln1 cln2 cln3 leu2::G418^R-GAL1,10::CLN1*	This study
N-140	*MAT **a** cln1 cln2 cln3::CLN3-URA3 clb5::ARG4 clb6::ADE2 leu2::G418^R-GAL1,10::CLN1*	F. Cross
N-141	*MAT α cln1 cln2 cln3::CLN3-URA3 leu2::G418^R-GAL1,10::CLN1*	This study
N-142	*MAT **a** cln1 cln2 cln3::CLN3-URA3 leu2::G418^R-GAL1,10::CLN1*	This study
N-163	*MAT α cln1 cln2 cln3::CLN3-URA3 bud2::LEU2 leu2::G418^R-GAL1,10::CLN1*	N141 transformed with pHP529
W303 background		
ML201:699	*MAT **a** cln3::LEU2*	M. Linksens
N-5	*MAT **a** ADE2*	B. Futcher
N-162	*MAT **a** swi4::HIS3 swi6::HIS3 lys2::HO-lacZ sic1::leu2::G418^{R}YC*p*50-GAL1,10::SWI4-3xHA*	This study
N-182	*MAT α cln1 cln2 kar3::leu2::HIS3 leu2::G418^R-GAL1,10::CLN1 SSD1-v*	This study
N-225	*MAT α swi4::HIS3 swi6::HIS3 YC*p*50-GAL1,10::SWI4-3xHA*	
YHW23	*MAT **a** swi4::HIS3 swi6::HIS3 lys2::HO-lacZ YC*p*50-GAL1,10::SWI4-3xHA*	H. Wijnen

**G418^R* represents the *kan^R* open reading-frame of the *E. coli* transposon *Tn903* fused to transcriptional and translational control sequences of the *TEF* gene of the filamentous fungus *Ashbya gossypii* within the KanMX module of Wach et al.

Table 2. Oligonucleotides used in these studies

Name	Sequence
5-NLS-NCO	5′-ATCGTCGAATCCATGGTCTGCGGCCGCGGTCC-3′
3-NLS-NDE	5′-GAGACTAGTCATATGGCTACTACCTACCTTTCG-3′
5-NES-NCO	5′-GAGGAGTCCATGGGTTTAGCACTTAAATTAGC-3′
3-NESA-NDE	5′-AGTAGTGCGCATATGACTACCGATATCTAAACC-3′
3-NESI-NDE	5′-AGTAGTGCGCATATGACTACCGATATCTGCACC-3′
5′ACTNLS-T7	5′-ACGATCGTCGAATTCGAGCTCTGCGGCCGCGGTC-CAAAGAAAAAGCGCAAGGTGGGTATGGCTTCTAT-GACTGGTGGTCAACAAATG-3′
3′ACTNLS-T7	5′-TTCGACAGACTCGAGACTAGTGCGGCCGCTACTA-CCTACCTTTCGTTTCTTCTTAGGACCCATTTGTT-GACCACCAGTCATAGAAGC-3′
5′INACTNLS-T7	5′-ACGATCGTCGAATTCGAGCTCTGCGGCCGCGGTC-CAAAGACTAAGCGCAAGGTGGGTATGGCTTCTAT-GACTGGTGGTCAACAAATG-3′
3′INACTNLS-T7	5′-TTCGACAGACTCGAGACTAGTGCGGCCGCTACTA-CCTACCTTTCGTTTAGTCTTAGGACCCATTTGTT-GACCACCAGTCATAGAAGC-3′
5PNES-T7-A	5′-GAGCTCTGCGGCCGCGGTTTAGCACTTAAATTAG-CTGGTTTGGACATAGCTAGCATGGCTTCTATGAC-TGGTGGTCAACAAATG-3′
3PNES-T7-A	5′-ACTAGTGCGGCCGCTACTACCGATATCTAAACCT-GCCAATTTCAAAGCAAGTCTAGAACCCATTTGTT-GACCACCAGTCATAGAAGC-3′
5PNES-T7-I	5′-GAGCTCTGCGGCCGCGGTTTAGCACTTAAATTAG-CTGGTGCTGACATAGCTAGCATGGCTTCTATGAC-TGGTGGTCAACAAATG-3′
3PNES-T7-I	5′-ACTAGTGCGGCCGCTACTACCGATATCTGCACCT-GCCAATTTCAAAGCAAGTCTAGAACCCATTTGTT-GACCACCAGTCATAGAAGC-3′
METGFP-DN	5′-AGCTTGTGATCTCTAGCTTGGGTCTCTCTCTGTC-GTAACAGTTGTGATATCGTTCATATGAGTAAAGG-AGAAGAACTTTTCACT-3′
NdeGFP3p	5′-CGGGATCCATGGCCATATGGTGGTGGTGGTGGTG-ACCACCACCTTTGTATAGTTCATCCATGCC-3′
2654C3-*Sph*I	5′-ATGCTCTGCCCGCATGCAACTGAAAAAGAGATCA-ACTTCCTCTGTGGATTGTG-3′
2731C3-*Not*I	5′-CTTGAGGTTGCGGCCGCTTCAATCATTAAAATCA-CAATCCACAGAGGAAGTTGATCTC-3′
2726C3-*Sph*I	5′-CTCAAGAAAACTAGATGAGGC-3′
2740C3-*Not*I	5′-GGCCGCCTCATCTAGTTTTCTTGAGCATG-3′
2761C3-*Not*I	5′-TTTGTCGTTGCGGCCGCCTCAGCGAGTTTTC-3′

Table 3. Plasmids used in these studies

Name	Relevant genotype	Derivation/ source
pUNI-GFP	*GFP*	S. J. Elledge
pHY314-HA	*pCEN-TRP1-GAL::*HA	S. J. Elledge
pGLA	*pCEN-TRP1-GAL::*NLS-A	This study
pGLI	*pCEN-TRP1-GAL::*NLS-I	This study
pGEA	*pCEN-TRP1-GAL::*NES-A	This study
pGEI	*pCEN-TRP1-GAL::*NES-I	This study
pCB1314	*pCEN-LEU2 CLN2*	K. Arndt
pNE104	p*CEN-LEU2 CLN2*-NLS-A	This study
pNE108	p*CEN-LEU2 CLN2*-NLS-I	This study
pNE105	p*CEN-LEU2 CLN2*-NES-A	This study
pNE106	p*CEN-LEU2 CLN2*-NES-I	This study
pM10X series	p*CEN-LEU2-MET3::CLN2*-LOC.CASS.	This study
p314M::C2	p*CEN-TRP1-MET3::CLN2*	This study
pNE12X series	p*CEN-TRP1-MET3::CLN2*-LOC.CASS.	This study
pmgfp6	p*EGFP6* variant	J. Haseloff
pMG31	p*CEN-LEU2-MET3::GF*P *CLN3* (AA 557-572)	This study
pMG40	p*CEN-LEU2-MET3::GF*P *CLN3* (AA 576-580)	This study
pMG61	p*CEN-LEU2-MET3::GF*P *CLN3* (AA 557-580)	This study
pMGH::C2	p*CEN-LEU2-MET3::GF*P-*6HIS::CLN2*	This study
pMGH101	p*CEN-LEU2-MET3::GF*P-*6HIS::CLN2-18xMYC*	This study
pMHG10X series	p*CEN-LEU2-MET3::GF*P-*6HIS::CLN2*-LOC. CASS.	This study
pHW415	*CEN-URA CLN3*	H. Wijnen
pHP529	pint. *bud2::LEU*	H. Oak-Park
pSL46	p*CEN-URA3-GAL1,10::CLN2-3xHA*	C. Wittenberg
pSL122	p*CEN-URA3-GAL1,10::CLN2^{4T3S}-3xHA*	C. Wittenberg
p306-NLS-A	pRS306-PCR NLS-A cassette	This study
p306-NLS-I	pRS306-PCR NLS-I cassette	This study
p306-NES-A	pRS306-PCR NES-A cassette	This study
p306-NES-I	pRS306-PCR NES-I cassette	This study
pBF580	pint.-*URA3-CLN3*	M. Linkens
pBF580HA	pint.-*URA3-CLN3*-3xHA	M. Linkens
pBFΔ528HA	pint.-*URA3-cln3*(Δ528)-3xHA	M. Linkens
pBFΔ528NLS-A	pint.-*URA3 cln3*(Δ528)-NLS-A cassette	This study
pBFΔ528NLS-I	pint.-*URA3- cln3*(Δ528)-NLS-I cassette	This study
pNE110	pint.-*URA3-CLN3*-NES-A	This study
pNE111	pint.-*URA3-CLN3*-NES-I	This study

(Richardson et al., 1989). Strains containing gene fusions to the galactose-inducible promoter *GAL1,10* were first grown in 2% raffinose, and then induced for expression by the addition of 1-2% galactose. Plasmids containing the *MET3* promoter were induced on liquid or solid media lacking methionine, and repressed with 2 mM methionine, unless otherwise noted.

Plasmid construction

Synthetic oligonucleotides used in the construction of plasmids are listed in Table 2. The plasmids used in this study are listed in Table 3.

Forced localization cassettes

The oligonucleotide pairs 5′ACTNLS-T7/3′ACTNLS-T7 and 5′INACTNLS-T7/3′INACTNLS-T7 were annealed, digested with *Eco*RI and *Xho*I, and subcloned into pRS306 (Sikorski and Hieter, 1989) to create the plasmids p306-NLS-A and p306-NLS-I. The plasmids p306-NES-A and p306-NES-I were created similarly using *Not*I digested fragments obtained from the annealing of the oligonucleotide pairs, 5PNES-T7-A/3PNES-T7-A and 5PNES-T7-I/3PNES-T7-I.

GFP-forced localization cassette control fusions

GFP was cloned as a *Not*I fragment into the Univector plasmid pUNI15 (Liu et al., 1998), resulting in the plasmid pUNI-GFP. The forced localization cassettes from the pM10X series of plasmids, using the primer sets 5-NLS-NCO/3-NLS-NDE and 5-NES-NCO/3-NES[(A) or (I)] –NDE, were amplified by PCR, and digested with the restriction enzymes *Nco*I and *Nde*I. The plasmid pHY314-HA (*pGAL*-HA) was digested with *Nde*I and *Nco*I to remove the HA-tag, and received the similarly digested forced localization cassettes via ligation. The resulting plasmids were fused with the plasmid pUNI-GFP as detailed by Liu et al. (Liu et al., 1998), to create the plasmids pGLA (*GAL*-NLS-A-GFP), pGLI (*GAL*-NLS-I-GFP), pGEA (*GAL*-NES-A-GFP), pGEI (*GAL*-NES-I-GFP).

Cln2 plasmids

The forced localization cassettes were introduced to the C-terminus of *CLN2*, under control of the *CLN2* native promoter, as *Not*I fragments into the plasmid pCB1314 (CEN-*LEU-CLN2*) (kindly provided by K. Arndt, Wyeth-Ayerst Research, NJ), to create the pNE10X series, which are listed as follows: pNE104 (NLS-A), pNE108 (NLS-I), pNE105 (NES-A), and pNE106 (NES-I). *CLN2* under control of the *MET3* promoter was provided by the plasmid pNE113. The plasmid pNE113 (CEN-*LEU-MET3::CLN2*) was created by introducing a 2.8 kb *Sal*I-*Not*I cut fragment containing the *MET3* promoter fused to the *CLN2* ORF from pRS313MET::*CLN2* (kindly provided by H. Wijnen, Rockefeller University, NY). The forced localization cassettes were introduced

to the C-terminus of *CLN2* by gap repair of pNE113 to create the following plasmids (pM10X series): pM104 (NLS-A), pM108 (NLS-I), pM105 (NES-A), and pM106 (NES-I). A series of CEN-*TRP-MET3::CLN2* were created by subcloning 2.8 kb *Sal*I-*Cla*I fragments from pM10X series into pRS314 digested with *Xho*I and *Cla*I, to create the following plasmids: pNE124 (NLS-A), pNE128 (NLS-I), pNE125 (NES-A), and pNE126 (NES-I). Green fluorescent protein/six-histidine affinity purification tag was created by PCR using the plasmid pmgfp6 (kindly provided by J. Haseloff, University of Cambridge, UK) as a template, and the primers METGFP-DN and NdeGFP3p. The resulting PCR fragment was introduced into pNE113 by gap repair, resulting in the plasmid pMGH::C2. A *Xho*I fragment from pMGH::C2 containing the green fluorescent protein/six-histidine affinity purification tag was introduced into the pM10X series to create pMGH104(NLS-A), pMGH108(NLS-I), pMGH105(NES-A), and pMGH101(18xMYC). All PCR products were fully sequenced after introduction into the host plasmid to ensure no unwanted mutations were introduced.

Cln3 plasmids

Construction of pBF580, pBF580HA and pBFΔ528HA has been previously described (Yaglom et al., 1995). The plasmids pBFΔ528NLS-A and pBFΔ528NLS-I were created by replacing the HA-tag at the C-terminus with *Not*I fragments containing NLS-A and NLS-I from p306-NLS-A and p306-NLS-I, respectively into pBFΔ528HA. The plasmids pNE110 and pNE111 were created by cloning NES-A and NES-I from *Not*I fragments from p306-NES-A and p306-NES-I into the C-terminus of pBF580HA, thereby removing the HA-tag. These constructs were integrated into yeast strains after digestion with the *Pvu*II restriction enzyme.

GFP-Cln3 putative NLS fusion plasmids

The oligonucleotides 2654C3-*Sph*I and 2731C3-*Not*I were annealed, amplified by PCR, digested with *Sph*I and *Not*I, and subsequently cloned into a similarly digested pMGLA5 (*MET3*-GFP-6HIS) plasmid to create pMG31. The oligonucleotides 2726C3-*Sph*I and 2740C3-*Not*I were annealed and cloned into a *SphI/Not*I-digested pMGLA5 plasmid to create pMG40. The plasmid pMG61 was created by amplifying the C-terminus of *CLN3* using the primers 2654C3-*Sph*I and 2761C3-*Not*I, digesting the product with *Sph*I and *Not*I, and cloning into a similarly digested pMGLA5 plasmid.

Fractionations

Cells containing a galactose-inducible or methionine-repressible *CLN2* fusion plasmid were grown to mid-log phase. *CLN2* message was induced for 30 minutes to one hour, pelleted, and fractionated essentially as previously described (Ausubel, 1987). Western blots were probed with anti-HA monoclonal antibody (12CA5) at 1:10,000-fold dilution, polyclonal anti-ADH antibody (which recognizes the *S. cerevisiae* cytoplasmic alcohol dehydrogenase enzyme EC 1.1.1.1) (1:5000), polyclonal antibody anti-Sic1 (1:100), and/or the monoclonal antibody anti-Nop1 (1:10,000).

Microscopy and GFP imaging

Cln2-GFP was visualized by arresting *cdc34* temperature-sensitive cells (N-104(#31)) at 37°C for 1-3 hours prior to resuspension in mounting media (Pringle et al., 1989). DNA staining was performed by staining with live cell-permeable Hoechst 333642 (Molecular Probes, Eugene, OR). Images were obtained by using a CCD camera (Photometrics Sensys) mounted to a Nikon Microphot FXA with the 100× objective, and captured using Oncor software (Oncor Inc., Gathersburg, MD). Hoechst and DIC images were merged digitally using PhotoShop 4.0 (Adobe Systems Inc.). Standard filter sets were used to visualize fluorescent emissions.

Serial dilutions and plating

Strains containing *CLN2* or *CLN3* fused to the forced localization cassettes were grown to mid-log phase in selective minimal media, then diluted to 1×10^4 cells per μl in sterile distilled water. 5 μl of five ten-fold serial dilutions were plated on solid media under either inducing or noninducing conditions. Negative control platings, to show that the strains used were indeed dependent on Cln expression, were performed, and demonstrated lack of cell proliferation (data not shown). In some of the figures, only three relevant dilutions are shown for simplicity. Plates were incubated in a 30°C incubator unless otherwise noted, and photographed after 2-4 days, as detailed in the figure legends.

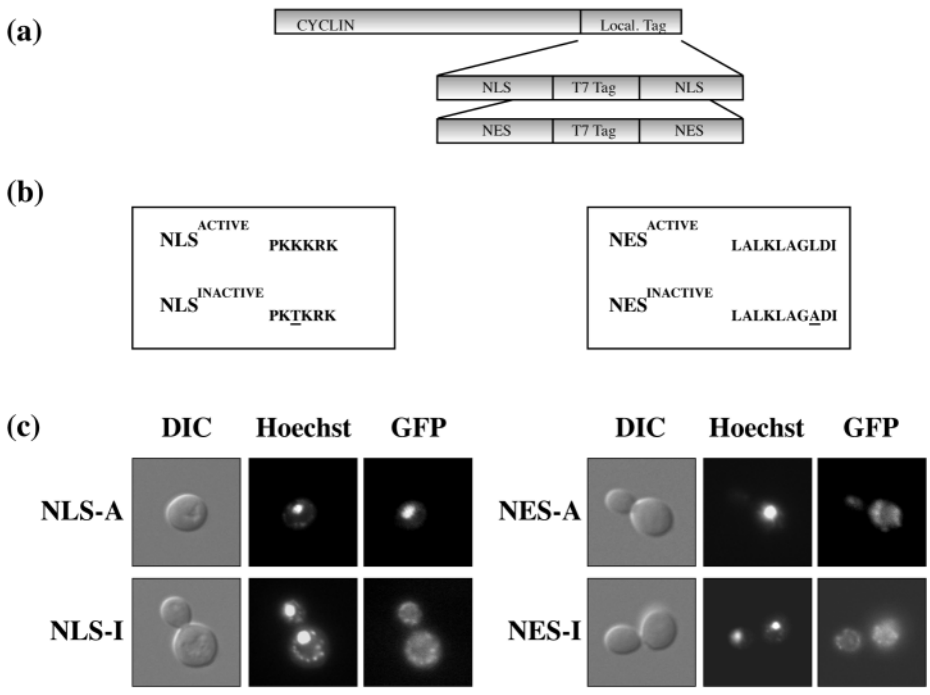

Fig. 1. Construction of the forced localization cassettes. (a) Cassettes containing two NLS or NES localization motifs separated by a T7 epitope tag were added to the C-terminus of Cln2 and Cln3. (b) Active and inactive versions of both a SV40 nuclear localization signal (NLS) and a PKI nuclear export signal (NES) were created. Inactive versions contain point mutations previously shown to prevent transport activity in *S. cerevisiae* (Nelson and Silver, 1989) (Murphy and Wente, 1996). (c) The localization cassettes direct GFP to the expected locations. Forced localization cassettes fused to GFP under control of the *GAL1,10* promoter were grown to mid-log growth phase. GFP expression was induced by the addition of 0.1% galactose for 1-2 hours, and live cells were subsequently stained, observed and digitally captured as described in Materials and Methods. DIC, differential interference contrast; Hoechst represents DNA staining; –A represents the active/functional transport sequences; –I represents the inactive/nonfunctional transport sequences.

RESULTS

Forced localization cassettes to redirect Cln localization

Studies of nuclear transport have led to the discovery of discrete amino acids that can act as targeting motifs that direct protein localization (Gorlich, 1998; Nigg, 1997; Osborne and Silver, 1993). Mutagenesis of these motifs has defined critical residues (Kalderon et al., 1984a; Kalderon et al., 1984b; Lanford and Butel, 1984; Wen et al., 1995). These localization motifs can be fused to other proteins to direct their localization (Stade et al., 1997). We created a forced nuclear localization cassette containing two copies of the SV40 nuclear localization signal separated by a T7 epitope tag. This cassette is referred to as the NLS-A (nuclear localization signal-active) cassette (Fig. 1a). Similarly, we created a forced cytoplasmic localization cassette containing two copies of the nuclear export signal from PKI, again separated by a T7 affinity tag. This cassette is referred to as the NES-A (nuclear export signal-active) (Fig. 1a). Two motifs were included in each cassette in the hope that, at equilibrium, two motifs might maintain the majority of tagged protein in the target location even in the face of a native signal of the opposite type. Finally, we created two control cassettes, each containing a change in a critical single amino acid in each copy of the motif (i.e. two amino acid changes per cassette), thus inactivating the cassette. These are referred to as the NLS-I and NES-I (I for inactive) cassettes (Fig. 1b).

To assay the effectiveness of these cassettes, we fused them to the Green Fluorescent Protein (GFP), and observed the location of the fusion protein by fluorescent microscopy (Fig. 1c). GFP-NLS-A appears to be completely nuclear, while the control, GFP-NLS-I, appears to be both nuclear and cytoplasmic, as expected from the small size of this protein. By contrast, GFP-NES-A is not obviously different from GFP-NES-I, but because some cytoplasm overlays the nucleus in these live, spherical cells, nuclear exclusion of GFP-NES-A could be occurring without being visible. Genetic results suggesting that the NES-A cassette is effective are described below.

Cln2 is located in both the nucleus and cytoplasm

As a first approach to determining the location of Cln2, we used subcellular fractionation of cells overproducing Cln2 tagged with 3×HA (Fig. 2a). Cln2 protein was found at similar concentrations in the cytoplasmic and nuclear fractions, by blotting equivalent amounts of total protein from each fraction (Fig. 2a), and by blotting cellular compartment volume equivalents (data not shown). The fractionation was repeated using a strain that expressed Cln2 from the *CLN3* promoter, which gives essentially wild-type amounts of Cln2. Again, Cln2 protein was found at similar concentrations in both the cytoplasmic and nuclear fractions (data not shown).

Since an essential function of the G1 cyclins in yeast is to phosphorylate Sic1, we also determined the localization of Sic1. We probed subcellular fractions with an anti-Sic1 polyclonal antibody (Fig. 2b), and found that Sic1, like Cln2, is found at similar concentrations in both the cytoplasm and nucleus. Over-expression of Cln2 resulted in the loss of Sic1 from both compartments (Fig. 2b, lanes 2 and 4).

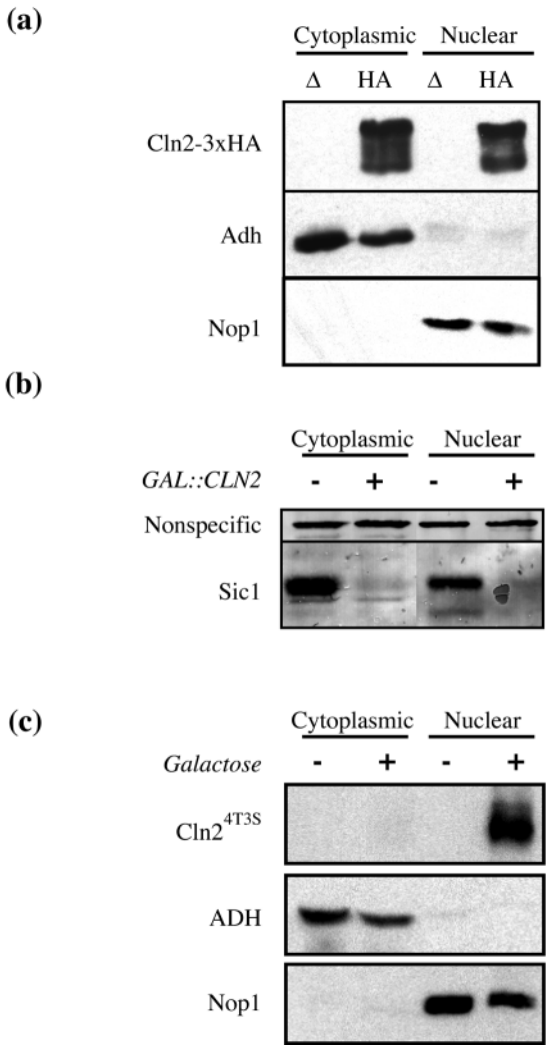

Fig. 2. Cln2 is located in both the nucleus and the cytoplasm. Strain N-5 containing an empty plasmid (Δ) or the plasmid pSL46(*GAL*::*CLN2*-3xHA) (HA) was grown at 30°C in selective media containing 2% raffinose to 5×10^6 cells/ml. The culture was divided in two, and galactose was added to one of them (1% final). All four cultures (+/– *CLN2*-3xHA, and +/– galactose induction) were grown for an additional hour then collected and fractionated. (a) Cytoplasmic and nuclear fractions from induced *cln2*Δ or GAL-*CLN2*-3xHA cells were analyzed using SDS/PAGE and blotted onto a membrane. 20 μg of protein from the cytoplasmic fraction, and 20 μg of protein from the nuclear fraction, were loaded. The western blot was first probed with anti-HA 12CA5 monoclonal antibody to detect Cln2, and subsequently reprobed with an anti-ADH polyclonal antibody as a cytoplasmic control, and a monoclonal antibody that recognizes the nuclear protein Nop1 (a kind gift from J. Aris, University of Florida, FL). (b) Cytoplasmic and nuclear fractions from uninduced (–) or induced (+) cells bearing GAL-*CLN2*-3xHA were analyzed using SDS-PAGE and blotted onto a membrane. 7 μg of protein was loaded. The western blot was probed with an anti-Sic1 polyclonal antibody (kindly provided by J. Donovan, SUNY at Stony Brook, NY). The top panel shows a high molecular weight nonspecific band as an internal loading control. (c) Plasmid pSL122(*GAL*::*CLN2*^{4T3S}-3xHA) was introduced to strain N-5, and treated as in (a). Equal amounts of protein were loaded in each lane. The blot was probed with anti-HA (12CA5) antibody to detect Cln2-3xHA, and subsequently reprobed with an anti-ADH polyclonal antibody, and anti-NOP1 monoclonal antibody.

For some proteins, access to the nucleus is regulated, and is controlled by phosphorylation state. For example, Swi5 (Jans et al., 1995), Swi6 (Sidorova et al., 1995), and Cdc6 (Jong et al., 1996) each have consensus Cdc28 phosphorylation sites close to their NLS. Phosphorylation masks these NLSs, so phosphorylated molecules are preferentially cytoplasmic, and unphosphorylated molecules are nuclear. Since Cln2 is also thought to be phosphorylated by Cdc28, we asked whether this might regulate location. We therefore performed subcellular fractionation of a strain containing *CLN2*^{4T3S}, a mutant lacking Cln2/Cdc28-dependent phosphorylation (Lanker et al., 1996), due to the replacement of serines and threonines with alanine at seven potential CDK sites (Fig. 2c). The nonphosphorylatable Cln2 appeared to be exclusively nuclear, suggesting that phosphorylation either activates nuclear export, or masks an NLS, preventing nuclear import. However, long exposures of the western blot suggest that a very small fraction of *CLN2*^{4T3S} may still reside in the cytoplasm. Fig. 2a shows that wild-type Cln2 is present in the nucleus in a phosphorylated form; perhaps this Cln2 was imported in the unphosphorylated form, and then phosphorylated in the nucleus.

The location of Cln2 was also examined using microscopy. We elected to use GFP-Cln2 fusions, since this allowed examination of live cells. Unfortunately Cln2 is a very unstable protein, and in wild-type cells the GFP moiety of a *MET3*-regulated GFP-*CLN2* fusion does not have time to fold and fluoresce before the fusion protein is degraded. We therefore expressed GFP-*CLN2* from the *MET3* promoter in a *cdc34*^{ts} strain at the nonpermissive temperature, where Cln2 is somewhat stabilized. In agreement with the fractionation experiments, some Cln2 clearly co-localizes with the nucleus, while some Cln2 is cytoplasmic (Fig. 3a).

Interestingly, the cytoplasmic GFP-Cln2 was often punctate (Fig. 3). The number of GFP-Cln2 dots and their location was quantitated (data not shown). In general, these dots tended to be at an apex of the daughter or mother cell (i.e. associated with the cell cortex at a site where budding or apical growth was occurring). Seventy-five percent of the dots were in the mother cell. Seventy-two percent of mothers had one dot, and these were often (58%) located at the apex opposite the daughter. Two dots within a single mother were located at opposite poles 61% of the time, with one dot near the daughter, and one dot opposite the daughter. Dots residing in only the daughter bud were rare (5%). When a dot was present in both the mother and the daughter, the dot within the daughter was almost always (85%) at the apex of the daughter opposite the mother cell. Several typical examples of apical localization of Cln2 can be seen in Fig. 3. Since these studies were performed in a *cdc34*^{ts} strain at nonpermisssive temperature, some cells exhibited elongated, and sometimes multiple, daughter buds.

We then asked whether the forced localization cassettes conferred the expected localization on GFP-Cln2. As shown in Fig. 3b, GFP-Cln2-NLS-A is indeed nuclear, with little or no cytoplasmic staining, while GFP-Cln2-NLS-I is mainly cytoplasmic, with some nuclear staining. GFP-Cln2-NES-A and GFP-NES-I both appear mainly cytoplasmic. It is possible that GFP-Cln2-NES-A causes nuclear exclusion, and that the very weak signal over the nucleus in these cells is due to the over- and underlying cytoplasm. Evidence that Cln2-NES-A, but not NES-I, is largely excluded from the nucleus is presented in genetic studies below (Fig. 4; Fig. 5).

Nuclear and cytoplasmic roles of Cln2

The two forms of Cln2, nuclear and cytoplasmic, were then used in a variety of mutant strains to see which aspects of Cln2

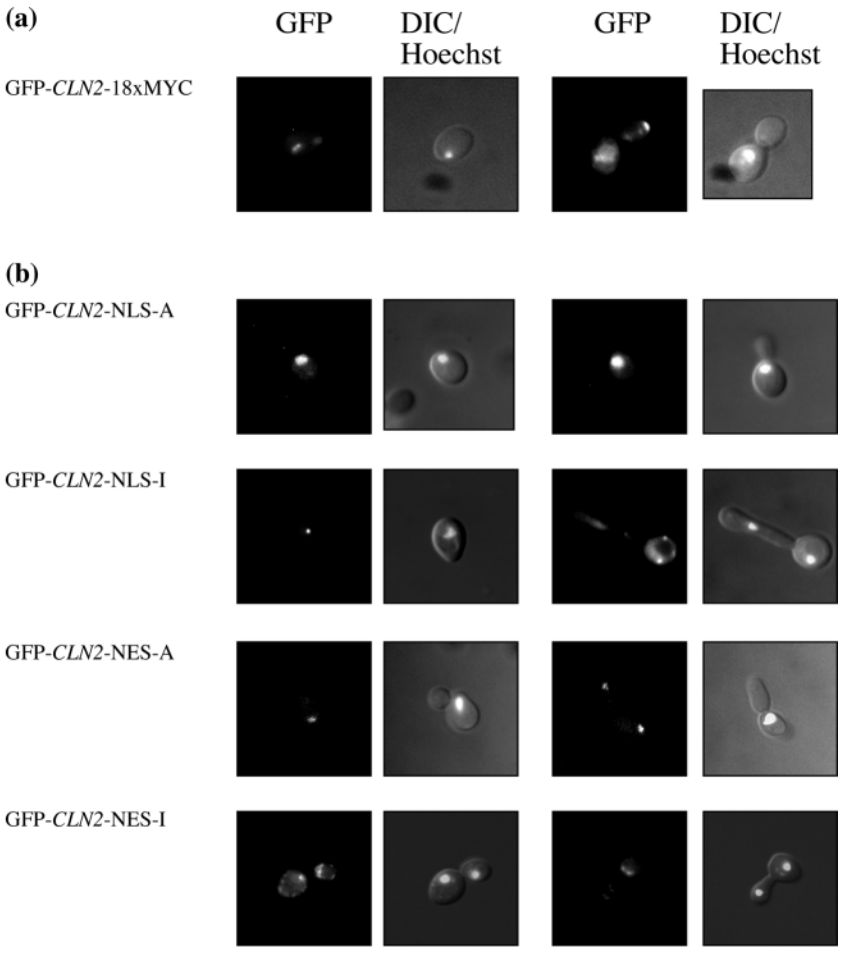

Fig. 3. Cln2 localizes to the cytoplasm and to the nucleus, and the forced localization cassettes can shift Cln2 to specific compartments. **(a)** GFP-*CLN2*-18xMYC (plasmid pMGH101) was introduced to strain N-104(#31) (*cln1 cln2 GAL-CLN3 cdc34-2*), which also contains a centromeric plasmid containing *CLN3* under its own promoter (pHW415). Cells were grown under selective conditions (–met 2% glucose) at 25°C to early log phase, and then shifted to 37°C for 1-3 hours. Live cells were stained with Hoechst 333642, mounted under coverslips in mounting media, and images were captured digitally. The cell on the right rotated slightly between imaging. **(b)** Plasmids (pMGH10X series) containing GFP-*CLN2* fused to the forced localization cassettes at the Cln2 C-terminus were introduced to strain N-104(#31) and analyzed as described in (a).

function depended on which location. In a *cln1 cln2 cln3* strain, *CLN2*-NLS-A was able to complement for viability, whereas the cytoplasmic form, *CLN2*-NES-A, complemented poorly, suggesting that it is the nuclear form that is functionally important (Fig. 4a). However, this experiment has the potential to be misleading: Cln2, like Cln3, can activate SBF and MBF, although this is not the normal role of Cln2 (Dirick et al., 1995; Stuart and Wittenberg, 1995). If nuclear Cln2 could mimic Cln3, and artefactually activate SBF and MBF, this would in turn increase the levels of *CLB5*, *CLB6*, *PCL1*, *PCL2* and many other transcripts, and these might be more directly responsible for the restored viability of the *cln1 cln2 cln3 CLN2*-NLS-A strain.

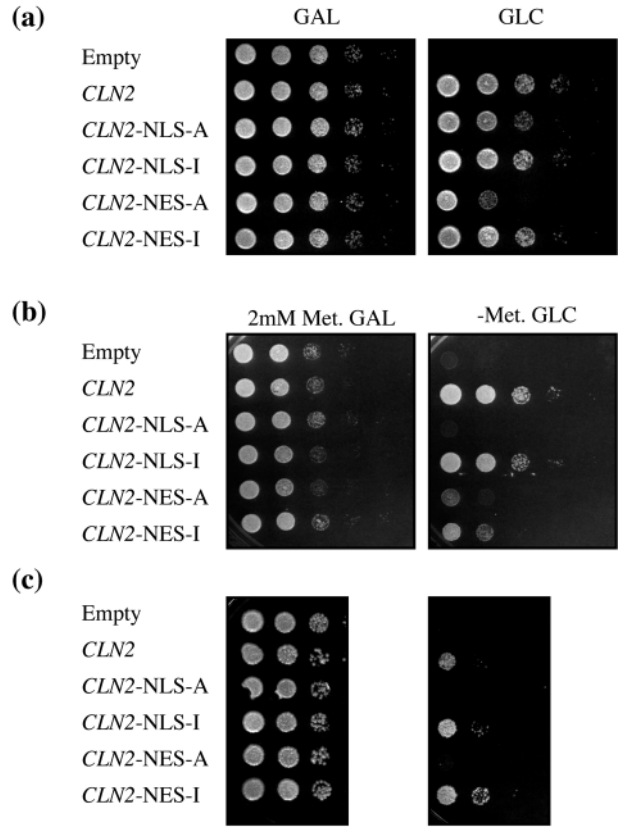

Fig. 4. Cln2 performs important roles in both the nucleus and the cytoplasm. (a) An empty control vector (pRS315), a low-copy plasmid containing *CLN2* with no tag (*CLN2*/pCB1314) and plasmids containing *CLN2* with C-terminal forced localization cassettes under control of the *CLN2* promoter (pNE10X series) were transformed into strain N-138 (*cln1 cln2 cln3 GAL-CLN1*). Cells were serially diluted (see Materials and Methods) onto selective plates containing 2% galactose (GAL) or glucose (GLC), and grown for 2 days at 30°C. Within a single dilution series, the number of cells in each spot differs by tenfold from its neighbor (b) An empty control vector (pRS315), a low copy plasmid containing *CLN2* with no tag (*CLN2*/ pNE113) and plasmids containing *CLN2* with C-terminal forced localization cassettes under control of the methionine-repressible *MET3* promoter (pM10X series) were transformed into strain YHW23 (*swi4 swi6 GAL-SWI4*). Strains were serially diluted as in (a) on plates containing 2 mM methionine or lacking methionine (–MET) and containing 2% galactose or glucose. (c) The same plasmids were used as in (b), but were transformed into strain N-162 (*swi4 swi6 GAL-SWI4 sic1*), and cells were grown for 5 days at 30°C.

To test this possibility, we repeated the experiment in a *swi4 swi6* mutant, where there is no SBF or MBF (Fig. 4b). The viability of this mutant is restored by wild-type *CLN2*, presumably because *CLN2* is the essential transcriptional target of SBF/MBF. In this *swi4 swi6* background, neither *CLN2*-NLS-A nor *CLN2*-NES-A could efficiently restore viability. This result establishes that both the cytoplasmic and nuclear forms of Cln2 have important functions. It strongly suggests that *CLN2*-NLS-A complements a *cln1 cln2 cln3* strain indirectly, by activating transcription of other cyclins (*CLB5*, *CLB6*, *PCL1*, *PCL2*) via SBF and MBF. Finally, the fact that the NLS-A and the NES-A form of Cln2 complement the mutant so poorly (while the *CLN2*, NLS-I and NES-I controls complement) strongly suggests that both localization cassettes, NLS-A and NES-A, are effective at targeting the majority of Cln2 to the desired location.

An essential function of Cln-Cdc28 is to promote phosphorylation of Sic1 (Schneider et al., 1996; Tyers, 1996), thereby targeting it for destruction (Verma et al., 1997). Since Sic1 is present in both the cytoplasm and the nucleus (Fig. 2b), the requirement for both cytoplasmic and nuclear Cln2 could reflect a need to phosphorylate Sic1 in both compartments. However, neither *CLN2*-NLS-A nor *CLN2*-NES-A is able to rescue the viability of a *swi4 swi6 sic1* strain (Fig. 4c). Thus, both forms of Cln2 must have additional important roles beyond the phosphorylation of Sic1.

Potential nuclear roles of Cln2

A *cln1 cln2* double mutant is viable and reasonably healthy, but is synthetically lethal with a large number of other mutations, including *bud2*, *kar3*, *rad27*, *cla4*, *gin4* (Benton et al., 1993; Cross, 1995; Cvrckova and Nasmyth, 1993), *ssd1* (Cvrckova and Nasmyth, 1993), *msn5* (Alepuz et al., 1999), *clb5 clb6* (Schwob and Nasmyth, 1993), and *pcl1 pcl2* (Espinoza et al., 1994). The precise reason for the inviability of these triple or quadruple mutants is not known; however, we presume that Cln2 shares a redundant function with these various proteins. To narrow down the functions of Cln2 that might be involved, we asked whether various synthetic lethal combinations could be complemented by the nuclear form of Cln2, or the cytoplasmic form, via tenfold serial dilutions of the appropriate conditional strains.

Nuclear Cln2 is better than cytoplasmic Cln2 in complementing the *cln1 cln2 clb5 clb6* mutant (Fig. 5a), suggesting it is the nuclear Cln2 that may be important for this overlapping role with the S-phase cyclins. Correspondingly, Clb5 is a nuclear protein by immunofluorescence (Jacobson et al., 2000; Shirayama et al., 1999).

Kar3 is a microtubule-based motor protein important for driving the contraction of the mitotic spindle assembly, promoting nuclear congression during mating, and promoting proper microtubule array structure from Start until anaphase (Hildebrandt and Hoyt, 2000; Rose, 1996). A *cln1 cln2 kar3* mutant strain appears to be inviable due to defects in spindle pole body duplication and/or separation (Cross, 1995). Since Kar3 acts in both the nuclear and cytoplasmic compartments (Cottingham et al., 1999; Page et al., 1994; Zeng et al., 1999), we tested whether the overlapping role of Cln1 and Cln2 with Kar3 was cytoplasmic, nuclear, or both. Fig. 5b shows that a nuclear Cln2 was more efficient than cytoplasmic Cln2 at rescue of the *cln1 cln2 kar3* mutant strain, but nuclear Cln2

was not as efficient as wild-type Cln2, Cln2-NLS-I, or Cln2-NES-I. Thus the nuclear form of Cln2 is probably most important for complementing the Kar3 function, but the cytoplasmic form of Cln2 probably also plays a role.

Potential cytoplasmic roles of Cln2

Pcl1 and Pcl2 are cyclins that associate with the CDK Pho85 (Measday et al., 1997; Moffat et al., 2000). They appear to play a role in cellular morphogenesis, since null mutations in conjunction with *CLN1* and *CLN2* deletions result in cells with a G1 arrest and abnormal morphology (Espinoza et al., 1994). In addition, Pcl1 and Pcl2 physically interact with and phosphorylate the protein Rvs167, a gene involved in endocytosis and organization of the actin cytoskeleton in the cytoplasm (Lee et al., 1998). We found that cytoplasmic Cln2 complements a *cln1 cln2 pcl1 pcl2* mutant as well as wild-type Cln2, but nuclear Cln2 does not (Fig. 5c), suggesting it is the cytoplasmic Cln2 that is important. Accordingly, Pcl1 and Pcl2 appear to be localized in the cytosol (B. Andrews, personal communication).

Bud2 is a GTPase-activating protein that specifies cellular polarity, and is localized at the presumptive bud site during early G1 phase, presumably to direct the GTPase Rsr1 to the incipient bud-site, and to convert Rsr1-GTP to the Rsr1-GDP bound state (Park et al., 1993; Park et al., 1999). It is also required for cellular viability in the absence of *CLN1* and *CLN2* (Benton et al., 1993; Cvrckova and Nasmyth, 1993). Introduction of the Cln2-forced localization cassettes into a conditional strain deleted for *cln1 cln2 bud2* was performed (Fig. 5d). Cytoplasmic Cln2 complements a *bud2* mutation as well as wild-type Cln2, but nuclear Cln2 does not, suggesting it is the cytoplasmic form of Cln2 that is important for budding in the absence of Bud2.

Is shuttling of Cln2 important?

Neither nuclear nor cytoplasmic Cln2 was able to rescue the *swi4 swi6* mutant strain (Fig. 4b), whereas wild-type Cln2 did rescue this strain. These data suggest that Cln2 has substrates in both compartments (Fig. 4c). Thus, simultaneous introduction of both nuclear and cytoplasmic Cln2 may now allow rescue of the *swi4 swi6* strain. We tested this hypothesis by simultaneously introducing *CLN2*-NLS-A and *CLN2*-NES-A into a *swi4 swi6* strain using two plasmids with two different selectable markers (Fig. 6). Surprisingly, *CLN2*-NLS-A plus *CLN2*-NES-A was unable to rescue the *swi4 swi6* mutant efficiently, whereas *CLN2*-NLS-A plus *CLN2*, *CLN2*-NLS-I or *CLN2*-NES-I were all able to rescue. This surprising result may mean that there is some important function of Cln2 for which active shuttling between the nuclear and cytoplasmic compartments is required.

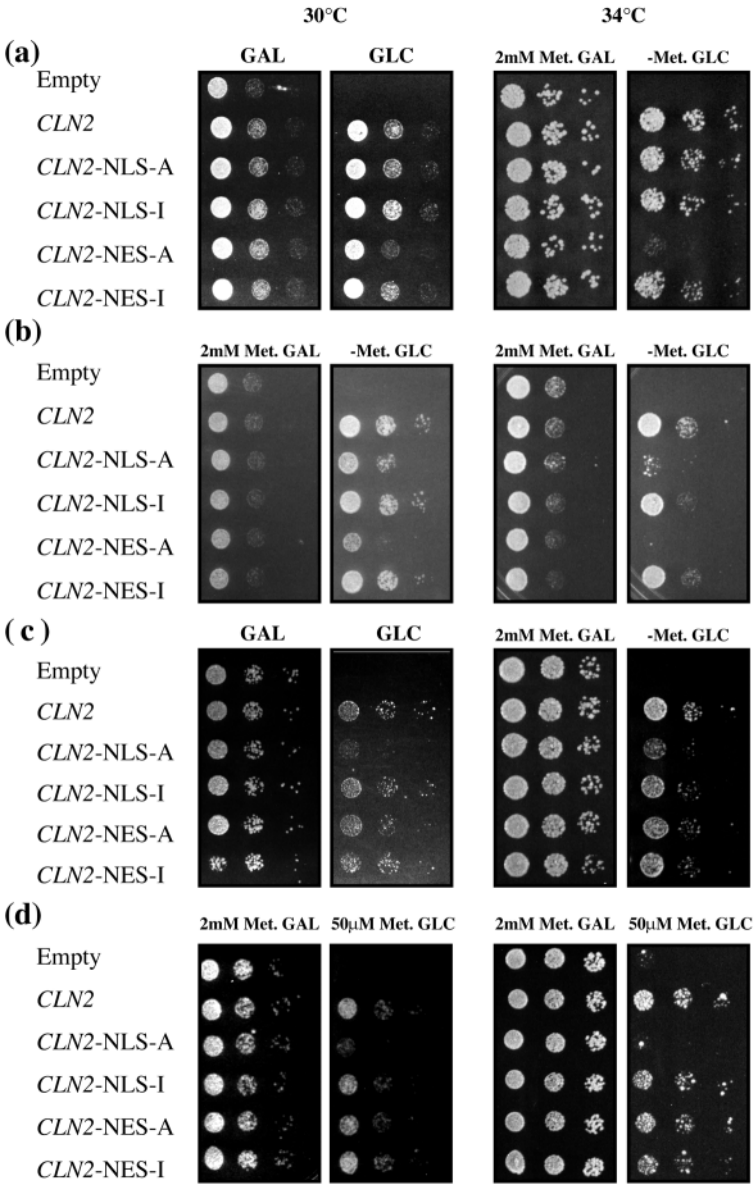

Fig. 5. Cln2 shares nuclear functions with the S-phase cyclins Clb5 and Clb6, and with the kinesin motor protein Kar3, and shares cytoplasmic functions with the cyclin pair Pcl1 and Pcl2, and with GTPase-activating protein Bud2. (a) The same plasmids as in Fig. 4, the pNE10X series and the pM10X series, were introduced into strain N140 (*cln1 cln2 clb5 clb6 GAL-CLN1*), and processed as described in Fig. 4. The left panel are serial dilutions that were incubated at 30°C for 2 days, and the right panel were incubated at 34°C for 3 days. (b) Plasmids containing *CLN2* with C-terminal forced localization cassettes under control of the methionine-repressible *MET3* promoter (pM10X series) were introduced into strain N182 (*cln1 cln2 kar3 GAL-CLN1*). (c) The same plasmids as in (a) were introduced to the strain N80 (*cln1 cln2 pcl1 pcl2 GAL-CLN1*), and processed as described in Fig. 4. (d) An empty control vector (pRS314), a low-copy plasmid containing *CLN2* with no tag (*CLN2*/p314M::C2) and plasmids containing *CLN2* with C-terminal forced localization cassettes under control of the methionine-repressible *MET3* promoter (pNE12X series) were transformed into the strain N163 (*cln1 cln2 bud2 GAL-CLN1*) and processed as previously described.

Cln3 acts in the nucleus

The Cln3-Cdc28 complex activates the transcription factors SBF and MBF in late G1 phase, thus leading to the transcription of about 200 genes in late G1 (Spellman et al., 1998), including *CLN1* and *CLN2*. The fact that Cln3 affects transcription is consistent with the idea that it might be a

nuclear protein, and evidence that Cln3 is nuclear in large, budded cells has been obtained by immunofluorescence (Miller and Cross, 2000).

Deletions of Cln3 have been made from the C-terminus, to define functionally important regions of the protein (Yaglom et al., 1995). A deletion of 54 amino acids from the C-terminus yielded the allele *cln3(528)*, which, in single copy, failed to complement a *cln1 cln2 cln3* triple mutant for viability, and failed to complement a *cln3* null mutant for its large cell phenotype, suggesting that some functional element resides near the C-terminus of Cln3 (Yaglom et al., 1995). This region of the protein does not include any portion of the cyclin homology, but does include the sequences KKR and KKTR separated by 15 amino acids, a sequence resembling a classic Robbins-Dingwall bipartite nuclear localization signal (Robbins et al., 1991). It is possible that this is a relevant NLS, and when this signal is deleted, Cln3 becomes cytoplasmic and non-functional.

To investigate this possibility, we fused an NLS cassette to the C-terminus of *cln3*-528. As shown in Fig. 7a, *cln3*(528)-NLS-A is able to complement the large cell phenotype of a *cln3* null mutant to a degree equivalent to *CLN3*(580)-HA. Importantly, the inactive version of the NLS, *cln3*(528)-NLS-I, has no such effect, even though the cassette differs from NLS-A by only two amino acids. If nuclear localization of Cln3 is essential for function, then forcing full-length Cln3 to the cytoplasm may interfere with its function. This hypothesis was tested by introducing an active NES to an otherwise wild-type Cln3. Fig. 7a shows that *CLN3*(580)-NES-A has a cell size similar to a *cln3* null strain, whereas *CLN3*(580)-NES-I had a wild-type cell size. This result implies not only that Cln3 must be nuclear in order to function, but also that the two NES motifs in the NES-A cassette are dominant over the single Robbins-Dingwall NLS in native Cln3.

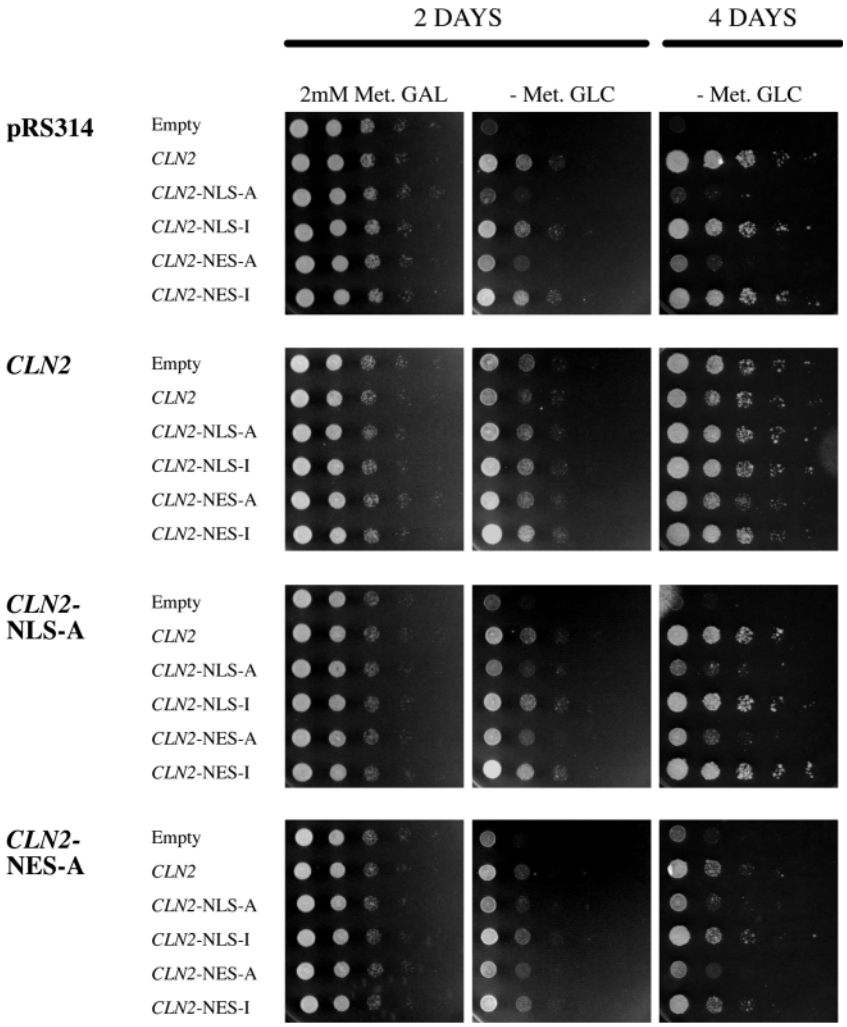

Fig. 6. Cln2 may need to shuttle between nucleus and cytoplasm. Strain N-225 (*swi4 swi6 GAL-SWI4*) was doubly transformed so as to contain two plasmids simultaneously, one of the pNE12X series, and one of the pM10X series. Strains were serially diluted and plated as described in Fig. 4, except that growth was for 2 days or 4 days (panels on right).

We also introduced the same Cln3 fusions constructs into a *cln1 cln2 cln3 GAL*::*CLN1* mutant strain (Fig. 7b). Consistent with the results in Fig. 7a, we find that a cytoplasmic Cln3 (either by deletion of a putative native NLS (*cln3*(528)-NLS-I), or by forced cytoplasmic localization of full length Cln3 (*CLN3*(580)-NES-A)) is unable to complement a triple *cln* null strain. These data again suggest that Cln3 must be nuclear to be functional.

If the Cln3 amino acids from 557 to 580 contain an NLS, then this sequence should be able to act autonomously, and direct a test protein to the nuclear compartment. We made in-frame C-terminal fusions of all or part of this sequence to GFP (Fig. 7c). The predicted full-length NLS sequence resulted in exclusively nuclear fluorescence (pMG61), whereas the pMG40 construct, containing only the last five amino acids, lacked nuclear-restricted fluorescence. Cells containing the pMG31 construct exhibited mainly cytoplasmic fluorescence, although a minority of cells exhibited partial nuclear staining (Fig. 7d). Thus Cln3 contains a functional NLS that directs Cln3 to the nuclear compartment.

DISCUSSION

We have constructed localization cassettes that can force proteins into the nucleus or the cytoplasm. These allow a new approach to studying protein location that complements the previous approaches of subcellular fractionation and fluorescence microscopy. Advantages of the forced localization approach are, first, that it can be carried out even with non-abundant or unstable proteins, and second, that it can be used to assay the functional relevance of location.

Miller and Cross have conducted related studies, but one of the differences between the studies is that Miller and Cross used a single NLS or NES, which unfortunately was only partially effective in redirecting the location of wild-type Cln2

or Cln3 proteins (Miller and Cross, 2000). These single signals were effective when placed on Cln3-1 which, as we show here, lacks the native NLS of Cln3. Thus it appears that a single artificial NLS or NES is effective when it is not competing with any other signal, but may not be effective within a protein that has a native signal of an opposite type. Our studies used localization cassettes containing two NLSs or NESs, and these double signals appeared to overcome the native signals in Cln2 and Cln3. We imagine that a protein bearing, for example, one NLS and two NES signals probably shuttles back and forth between cytoplasm and nucleus, but that the steady-state distribution is biased towards the cytoplasm, since there are twice as many cytoplasmic as nuclear signals. Thus in yeast, protein location may be determined by a democratic method. Possibly a triple NLS or triple NES would be even more generally effective in forcing proteins to a particular location, regardless of what native signals they might bear.

We found that the functionally relevant form of Cln3 is in the nucleus, since Cln3 directed to the nucleus is fully functional, and Cln3 directed to the cytoplasm, or lacking an NLS, is non-functional. This is consistent with the fact that the role of Cln3 is to activate the transcription factors SBF and MBF. We have identified a native NLS of Cln3 located at its extreme C-terminus.

The idea that a C-terminal NLS is important for the function of Cln3 is apparently contradicted by properties of the *CLN3-1* allele. *CLN3-1* encodes a protein lacking the C-terminal third of the wild-type protein, including the putative NLS, yet *CLN3-1* is a hyperactive, dominant allele. However, this last third of the protein includes signals for degradation in addition to the NLS. Thus, although Cln3-1 lacks the NLS, it is also stabilized and therefore relatively abundant. Cln3-1 may have some residual ability to reach the nucleus (Miller and Cross, 2000), and this combined with its increased abundance may be responsible for its Cln-like activity. However, the Cln3-1 protein also mislocalizes to the cytoplasm (data not shown) (Miller and Cross, 2000). Thus the Cln3-1 protein may now perform some of the functions of cytoplasmic Cln2, or even some functions not ordinarily performed by any Cln. This may explain a long-standing puzzle: a classic phenotype of *CLN3-1* is α-factor resistance, yet over-expression of *CLN3* in an otherwise wild-type strain does not yield α-factor resistance (Tyers et al., 1993). By contrast, over-expression of *CLN2* does cause α-factor resistance (Oehlen and Cross, 1994; Tyers et al., 1993). In the light of our present results, we speculate that the α-factor resistance of *CLN3-1* may be a neomorphic, Cln2-like phenotype caused by the cytoplasmic mis-localization of the Cln3-1 protein.

We have shown that Cln2 localizes to both the nucleus and to the cytoplasm by subcellular fractionation and GFP fusion

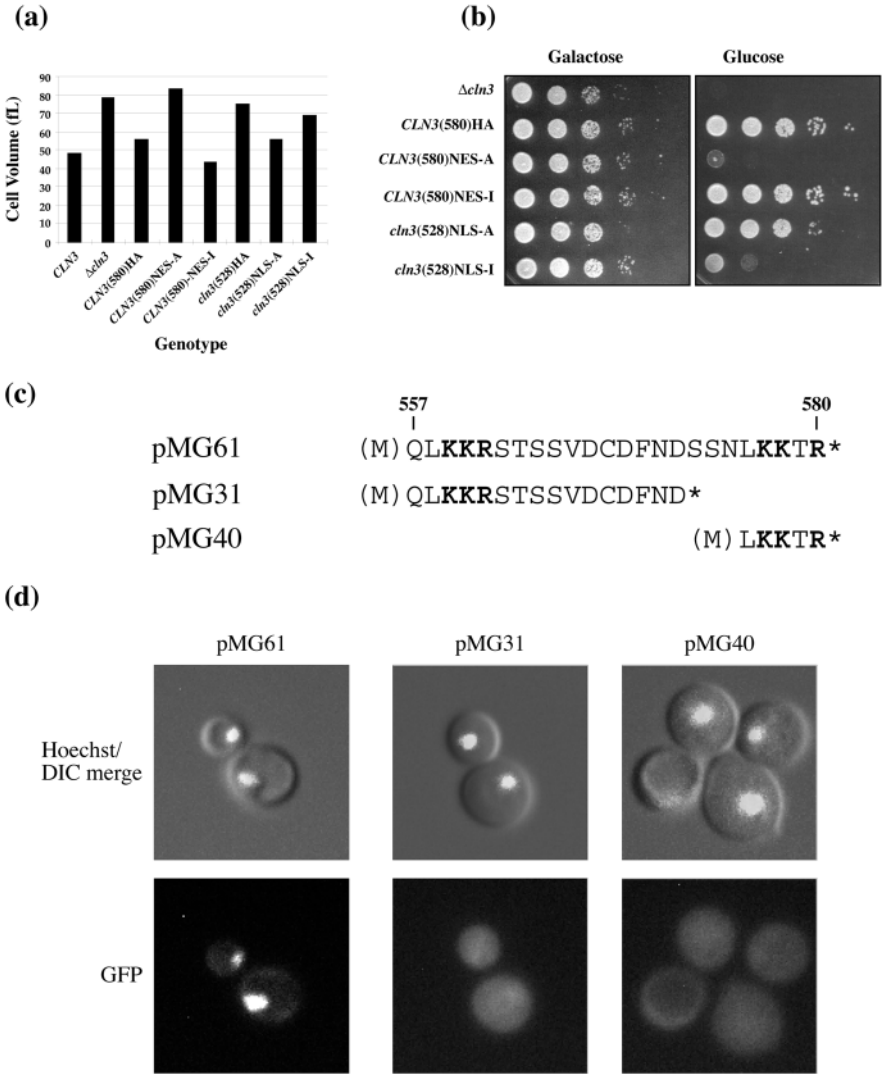

Fig. 7. Cln3 must be in the nucleus to function. (a) Cell volumes are shown in femtoliters. Various versions of *CLN3* (plasmids pBF580, pBF580HA, pNE110, pNE111, pBFΔ528HA, pBFΔ528NLS-A, and pBFΔ528NLS-I) were integrated in single copy at the *CLN3* locus in the parental strain ML201:699(*cln3*::*LEU2*). These strains were grown in YEPD, and cell volumes were measured with a Coulter Channelyzer (Yaglom et al., 1995). The mode volume is reported. (b) The same plasmids containing alleles of *CLN3* used in (a) were integrated in single copy into the strain FC-1254-14D (*cln1 cln2 cln3 GAL-CLN1)*. Cells were grown overnight in YEP 2% galactose, and tenfold serial dilutions were spotted onto YEP plates containing either 2% galactose or 2% glucose. Plates were photographed after 2 days at 30°C. (c) *CLN3* C-terminal amino acids encompassing either the whole (pMG61, 24 aa) or a portion (pMG31, 16 aa; pMG40, 5 aa) of the putative bipartite NLS were fused to GFP under control of the *MET3* promoter. Residues in bold show the NLS. Methionine residues, in parentheses, were introduced to facilitate cloning. (d) Plasmids containing the GFP fusions described in (c) were introduced to strain N-5. Cells were grown to log-phase in medium containing 2 mM methionine and 2% glucose, induced for 3 hours by resuspension in medium lacking methionine, and images were collected.

fluorescence (Fig. 2a; Fig. 3). Two substrates of Cln2, Sic1 (Fig. 2b) and Far1 (Blondel et al., 1999; Nern and Arkowitz, 2000; Shimada et al., 2000), are also in both compartments. Examination of the amino acid sequence of Cln2 did not reveal any homology to previously characterized nuclear export or import motifs. However, the phosphorylation state of Cln2 appears to affect localization, since an unphosphorylatable version of Cln2 was nuclear (Fig. 2c). Strangely, the apparent preference of unphosphorylatable Cln2 for the nucleus is not reflected in the subcellular fractionation data, where all mobility forms of Cln2 were found equally in the nuclear and cytoplasmic fraction. Although many interpretations are possible, this could mean that equilibration between phosphorylated and unphosphorylated forms of Cln2 is relatively rapid compared to nuclear/cytoplasmic transport.

GFP-Cln2 was often seen in one or several cytoplasmic dots in arrested *cdc34* strains. These dots, when located in the daughter cell, were located at the apex most of the time. This is a particularly interesting location, since Cln2 is involved in budding and polarized growth, and many other proteins involved in budding, such as Cdc24 and Cdc42, are also located at the apex. We find that the GFP-Cln2 dots maintain the same localization even after cells have been incubated with latrunculin B to disrupt actin structures (data not shown). Additionally, the GFP-Cln2 dots do not significantly colocalize with actin spots (data not shown). A *cln1 cln2* mutant is synthetically lethal with a *bud2* mutant. Bud2 is also involved in budding, and is located at the incipient bud site. Perhaps the presence of Cln2 at the bud tip is related to the synthetic lethality of *cln1 cln2* with *bud2*, especially since the *cln1 cln2 bud2* mutant is rescued efficiently by the cytoplasmic form of Cln2, but not by the nuclear form. These observations suggest that Cln2 may play a direct role at the bud tip to promote budding.

Since the localization cassettes shifted the location of Cln2 to the expected compartments, we were able to test the consequences of restricted Cln2 localization. Neither nuclear nor cytoplasmic Cln2 was able to rescue a *swi4 swi6* mutant, suggesting that both the nuclear and cytoplasmic pools of Cln2 are functionally important. The nuclear form of Cln2 was best at complementing the *kar3* defect and the *clb5 clb6* defect. Since Kar3 is a spindle motor protein, and since *cln1 cln2 kar3* mutant strains have defects in SPB duplication/separation (Cross, 1995), nuclear Cln2 may also have a role in spindle pole body or spindle function. There are many possibilities for collaboration between nuclear Cln2 and Clb5/Clb6. For instance, Clb5 has also been implicated in spindle function and SPB duplication (Haase et al., 2001; Segal et al., 2000a; Segal et al., 2000b; Segal et al., 1998); alternatively, nuclear Cln2 and Clb5/Clb6 could have overlapping roles in the phosphorylation and destruction of Sic1 or Cdc6.

The cytoplasmic form of Cln2 was best at complementing the *pcl1 pcl2* defect and the *bud2* defect. There are several ways in which cytoplasmic Cln2 could collaborate with these proteins (Cvrckova and Nasmyth, 1993), especially since we now show that Cln2 has the ability to localize to the bud tip.

In the complementation studies using *kar3*, *bud2*, *pcl1 pcl2* and *clb5 clb6*, one form of Cln2 complemented better than the other, but both complemented to some extent. This may mean that the NLS/NES cassettes are not completely effective at forcing localization, and that only a trace of nuclear or cytoplasmic Cln2 is needed for complementing these defects. Alternatively, it could be that some truncated protein molecules are produced, perhaps because of proteolysis, or errors in transcription or translation, and these truncated molecules, which would lack the localization cassettes, allow weak complementation.

Wild-type Cln2 rescues the *swi4 swi6* double mutant. Surprisingly, the simultaneous presence of both nuclear and cytoplasmic Cln2 does not rescue this mutant. This suggests that Cln2 must shuttle back and forth between nucleus and cytoplasm to fulfill at least one of its functions. Recently it has been shown that Ste5, Far1, and Cdc24 shuttle between nucleus and cytoplasm, and each of these proteins has a function related to Cln2. In the case of Ste5, shuttling is essential for function; the delivery of Ste5 to the appropriate site on the plasma membrane depends on the prior residence of Ste5 in the nucleus (Mahanty et al., 1999; Pryciak and Huntress, 1998). It is therefore possible that the association of Cln2 with regions of the cortex (see above) could likewise be dependent on shuttling.

Far1, which has a region of similarity to Ste5, also shuttles between the nucleus (where it sequesters Cdc24) and the cytoplasm (from where, like Ste5, it can interact with Ste4 at the plasma membrane) (Blondel et al., 1999; Butty et al., 1998; Nern and Arkowitz, 1999; Nern and Arkowitz, 2000; Shimada et al., 2000; Toenjes et al., 1999). Cdc24, the GTP-GDP exchange factor for the small G-protein Cdc42, shuttles because of an association with Far1 (O'Shea and Herskowitz, 2000). Cdc24 is essential for budding, and is found at the bud site. In small G1 phase haploid cells, Cdc24 is sequestered in the nucleus by Far1. As cells grow, they express Cln2, and this leads to the phosphorylation of nuclear and cytoplasmic Far1, and the nuclear destruction of Far1, which releases Cdc24 into the cytoplasm, and thence to the bud site (Blondel et al., 2000). Interestingly, Cln2, like Cdc24, is known to associate with Far1 (Peter et al., 1993), and Cln2 is also known to be associated with an unidentified protein of 94 kDa (Tyers and Futcher, 1993), which is about the size of Cdc24. The membrane recruitment of Cdc24 requires Cln2-Cdc28 activity, although this effect is apparently not mediated via a direct phosphorylation of Cdc24 by Cln2-Cdc28 (Gulli et al., 2000). Finally, Ste5 and Far1 are transported via the exporter Msn5, and *msn5* is synthetically lethal with *cln1 cln2* (Alepuz et al., 1999). On the basis of these connections between Cln2, Far1 and Cdc24, several models for the mechanism and role of shuttling of Cln2 could be imagined.

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