Brief Report

Nucleocytoplasmic Shuttling of the Cdc42p Exchange Factor Cdc24p

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Abstract. Cdc24p, the GDP/GTP exchange factor for the regulator of actin cytoskeleton Cdc42p, localizes to sites of polarized growth. Here we show that Cdc24p shuttles in and out of the yeast nucleus during vegetative growth. Far1p is necessary and sufficient for nuclear accumulation of Cdc24p, suggesting that its nuclear import occurs via an association with Far1p. Nuclear export is triggered either by entry into the cell cycle or by mating pheromone. As Far1p is degraded upon entry into the cell cycle, cell cycle–dependent export of Cdc24p occurs in the absence of Far1p, whereas during mating similar export kinetics indicate that a Cdc24p–Far1p complex is exported. Our results suggest that the nucleus serves as a store of preformed Cdc24p–Far1p complex which is required for chemotropism.

Key words: nucleocytoplasmic shuttling • GDP/GTP exchange factor • Far1p • Cdc24p • polarized growth

Introduction

Subcellular relocalization of signaling proteins in response to external signals is critical for recruitment of signaling cascades and efficient signaling. In yeast, mating pheromones result in the redistribution of several proteins required for responses to this signal including the MAP kinase scaffold protein Ste5 and the cyclin dependent kinase inhibitor Far1 (Butty et al., 1998; Blondel et al., 1999; Mahanty et al., 1999). Both of these proteins appear to shuttle in and out of the nucleus during vegetative growth. Pheromone triggers nuclear export of these proteins (Butty et al., 1998; Blondel et al., 1999; Mahanty et al., 1999). In response to pheromone, the GDP-GTP exchange factor Cdc24p, which is necessary for oriented growth, forms a complex with Far1p and Gβγ and is localized to the shmoo tip (Nern and Arkowitz, 1998, 1999). Cdc24p is a member of a ubiquitous class of small G protein regulators that are critical for cell growth and polarity in virtually all eukaryotes. Recently, Cdc24p has been shown to localize to the nucleus of budding cells (Toenjes et al., 1999). Here we show that Far1p is necessary and sufficient for Cdc24p nuclear localization and that Cdc24p is exported from the nucleus upon either entry into the cell cycle or in response to pheromone. Our results suggest that Cdc24p nuclear export in budding cells is triggered by degradation of Far1p, whereas in response to pheromone a complex of Cdc24p–Far1p is exported.

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Materials and Methods

Standard techniques were used for yeast manipulation (Rose et al., 1991). Unless otherwise indicated, yeast cells were grown at 30°C and temperature-sensitive strains at 25°C. The strains used are listed in Table I. Deletion mutants were constructed by PCR-mediated gene replacement as described (Nern and Arkowitz, 1999) and verified by PCR and mating defects. p416Cdc24HAGFP and p416Cdc24-m1HAGFP are the pRS416 versions of p414Cdc24HAGFP and p414Cdc24-m1HAGFP (Nern and Arkowitz, 1999). p2μCDC24GFP and p2μcdc24-m1GFP are 2μ plasmids in which Cdc24GFP expression is driven by the triose phosphate isomerase (TPI) promoter. For regulated expression of Cdc24GFP, p416Gal Cdc24HAGFP, which was constructed by cloning a Cdc24HAGFP fragment into a pRS416 *GAL1/10* plasmid, was used. Cdc24p was targeted to the nucleus using a *GAL4* nuclear localization sequence NLS (MDKAELIPEPPKKKRKVEL) followed by an HA epitope using p2μATNLSHA or p413TNLSHA vectors in which expression is from the TPI promoter (Nern and Arkowitz, 1999). Plasmids pCMP63 and pCMP68 were used for expression of Far1-22p and Far1GFP (Henchoz et al., 1997), respectively. *Far1-D1* (Gly 646 to Asp and Pro 671 to Leu), D1a (Gly 646 to Asp), and D1b (Pro 671 to Leu) were constructed by DpnI mutagenesis of pCMP63. The coding sequences of *FAR1* and ΔN FAR1 (lacking codons 1–393 which encode the first 131 amino acids) were amplified by PCR and cloned into pRSR424 with the *GAL1/10* promoter and a modified multiple cloning site.

Cells for microscopy were grown in appropriate selective media supplemented with 55 μg/ml adenine sulphate. For galactose induction, cells

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
381CG28a6	*Matα, ade6, his4, lys2, trp1, cdc28-13*	CSH Yeast Genetics Course
JY429	*Matα, trp1-Δ1, ura3-52, cyh2, fus1-Δ1, fus2-Δ1*	CSH Yeast Genetics Course
K699	*Mat**a**, ura3, leu2-3,-112, trp1-1, ade2-1, can1-100, his3-11,-15, ssd1-Δ2*	K. Nasmyth (IMP, Vienna)
K842	*Mat**a**/α, ura3/ura3, leu2-3,-112/leu2-3,-112, trp1-1/trp1-1, ade2-1/ade2-1, can1-100/can1-100, his3-11,-15/his3-11,-15, ssd1-Δ2/ssd1-Δ2*	K. Nasmyth (IMP, Vienna)
MS10	*Mat**a**, ura3-52, ade2-101, leu2-3,-112*	CSH Yeast Genetics Course
MS10*cdc28*	*Mat**a**, ura3-52, ade2-101, leu2-3,-112, cdc28-13*	CSH Yeast Genetics Course
SEY6211	*Mat**a**, leu2-3,-112, ura3-52, his3-Δ200, trp1-Δ901, ade2, suc2-Δ9*	S. Emr (University of California San Diego)
RAY1034	Same as SEY6211 with *cdc24::TRP1 CDC24*	Nern and Arkowitz, 1998
RAY1135	*Mat**a**, ura3-52, his3-Δ200, trp1-Δ901, ade2, suc2-Δ*	Nern and Arkowitz, 1999
RAY1254	Same as K699 with *cdc24::TRP1 3xmyc CDC24*	Nern and Arkowitz, 1999
RAY1256	Same as K699 with *cdc24::TRP1 3xmyc cdc24-m1*	This study
RAY1361	Same as SEY6211 with *cdc24Δ-1::HIS5* with p414Cdc24HAGFP	Nern and Arkowitz, 1999
RAY1373	Same as SEY6211 with *cdc24Δ-1::HIS5* with p414Cdc24-m1HAGFP	This study
RAY1378	Same as RAY1254 with *far1-Δ1::LoxPHis5LoxP*	This study
RAY1380	Same as RAY1256 with *far1-Δ1::LoxPHis5LoxP*	This study
RAY1423	Same as RAY1034 with *far1::far1-H7 LoxPHis5LoxP*	Nern and Arkowitz, submitted for publication
RAY1475	Same as K699 with *far1-Δ1::LoxPHis5LoxP*	This study
RAY1519	Same as SEY6211 with *msn5Δ-1::LoxPHis5LoxP*	This study
RAY1562*	*MAT**a**, ura3-52, trp1-Δ901, cdc28-13, msn5Δ-1::LoxPHis5LoxP*	This study

HIS5p refers to *HIS5* from *S. pombe*.
*Made by crossing RAY1519 and 381CG28a6 followed by sporulation.

were grown overnight in media with either 4% raffinose or 2% fructose, collected by centrifugation, and resuspended in medium with 2% galactose and 2% raffinose for further incubation. Cdc24HAGFP was immunoprecipitated with anti-HA 12CA5 from pulse-labeled cells to confirm glucose repression. For quantitation of Cdc24GFP localization from cells at different cell cycle stages and different strains (Fig. 1) all cells from confocal images were counted. For quantitation of the localization of Cdc24GFP and Far1GFP in nuclear export experiments, cells with GFP fluorescence above background were classified as either nuclear, growth site, or cytoplasmically localized. Cells with GFP fluorescence localized to both the growth site and nucleus were counted in both nuclear and growth site categories. For determination of the amount of total Cdc24GFP in the nucleus the intensity of a fixed area from the nucleus, cytoplasm, intercellular region of cells ($n = 100$) with a Cdc24GFP, or a control plasmid was determined from the central confocal optical section using NIH Image. Background values and the volume difference between the cytoplasm and nucleus were corrected for. The ratio of nuclear/cytoplasmic fluorescence shown in Fig. 3 D was not corrected for background cellular fluorescence. BioRad MRC-600 and Radiance Plus confocal microscopes were used for imaging of GFP fusions in live cells.

Mating assays were carried out as described (Nern and Arkowitz, 1998, 1999). DAPI was added to the growth medium at 2.5 μg/ml ~3 h before microscopy for nuclear staining of live cells. α-Factor was added to 24 μM in growth media. Expression levels were compared by SDS-PAGE of glass bead lysed cell extracts (Nern and Arkowitz, 1999), immunoblotting, followed by ECL visualization. Anti-GFP rabbit polyclonal serum (used at 1:5,000), anti-Far1p rabbit polyclonal serum (used at 1:1,000), and anti-HA mAb (12CA5) tissue culture supernatant (used at 1:40) were used for immunodetection.

Results and Discussion

Previously we observed Cdc24GFP localized to sites of polarized growth, including the bud tip, shmoo tip, and the mother-daughter neck (Nern and Arkowitz, 1999). Further examination revealed fluorescence in what appeared to be the nucleus in some cells. This was confirmed by colocalization with 4′,6-diamidino-2-phenylindole (DAPI) stained nuclear DNA (99% colocalization, $n = 110$ M/G1 cells). Nuclear Cdc24GFP was observed in early G1 and late M phase cells yet was rarely visible in budded cells (Fig. 1 A). In contrast, throughout the cell cycle 60–80% of the cells had Cdc24GFP localized to sites of polarized growth. Consistent with cell cycle–dependent nuclear accumulation, an increase in the number of cells with nuclear localized Cdc24p was seen in *cdc28-13* cells, which arrest at G1 at the nonpermissive temperature (90% at 37°C for 1 h 40 min; compared with 40% at 25°C; $n = 135$). Quantitation of the intensity of GFP fluorescence revealed that under such conditions ≥2/3 of the Cdc24GFP was in the nucleus. Nuclear accumulation of Cdc24GFP was observed in haploid Δcdc24 (Fig. 1 A) and *CDC24* but not diploid cells despite the similar localization of Cdc24p to growth sites in both cell types (Fig. 1 B). These results suggest that Cdc24p nuclear localization is dependent on a haploid specific protein whose level varies during the cell cycle.

Far1p accumulates in the nucleus of haploid, but not diploid, cells in the G1 cell cycle phase (Henchoz et al., 1997). Because Far1p interacts both in vivo and in vitro with Cdc24p (Butty et al., 1998; Nern and Arkowitz, 1999) we examined whether Far1p was necessary for Cdc24p nuclear localization. Cdc24p was not observed in the nuclei of *Δfar1* cells and furthermore the Cdc24p mutant Cdc24-m1p, which is defective in Far1p binding, was not localized to the nucleus in either *Δcdc24* or *CDC24* cells (Fig. 1 B and data not shown). In both cases Cdc24p growth site localization and expression levels were similar to wild-type strains (Fig. 1 B and data not shown). In addition, cells with the *far1* mutation, *far1-H7*, which is defective in Cdc24p binding, had a reduction in the percentage of cells with nuclear Cdc24p. Together, these results demonstrate that Far1p and its ability to bind Cdc24p are necessary for Cdc24p nuclear localization, either by promoting nuclear retention or by cytoplasmic association and subsequent nuclear import.

As Far1p is not expressed in diploids, we expressed a stabilized mutant of Far1p, Far1-22p (Henchoz et al., 1997), to determine if it was sufficient to direct Cdc24p to

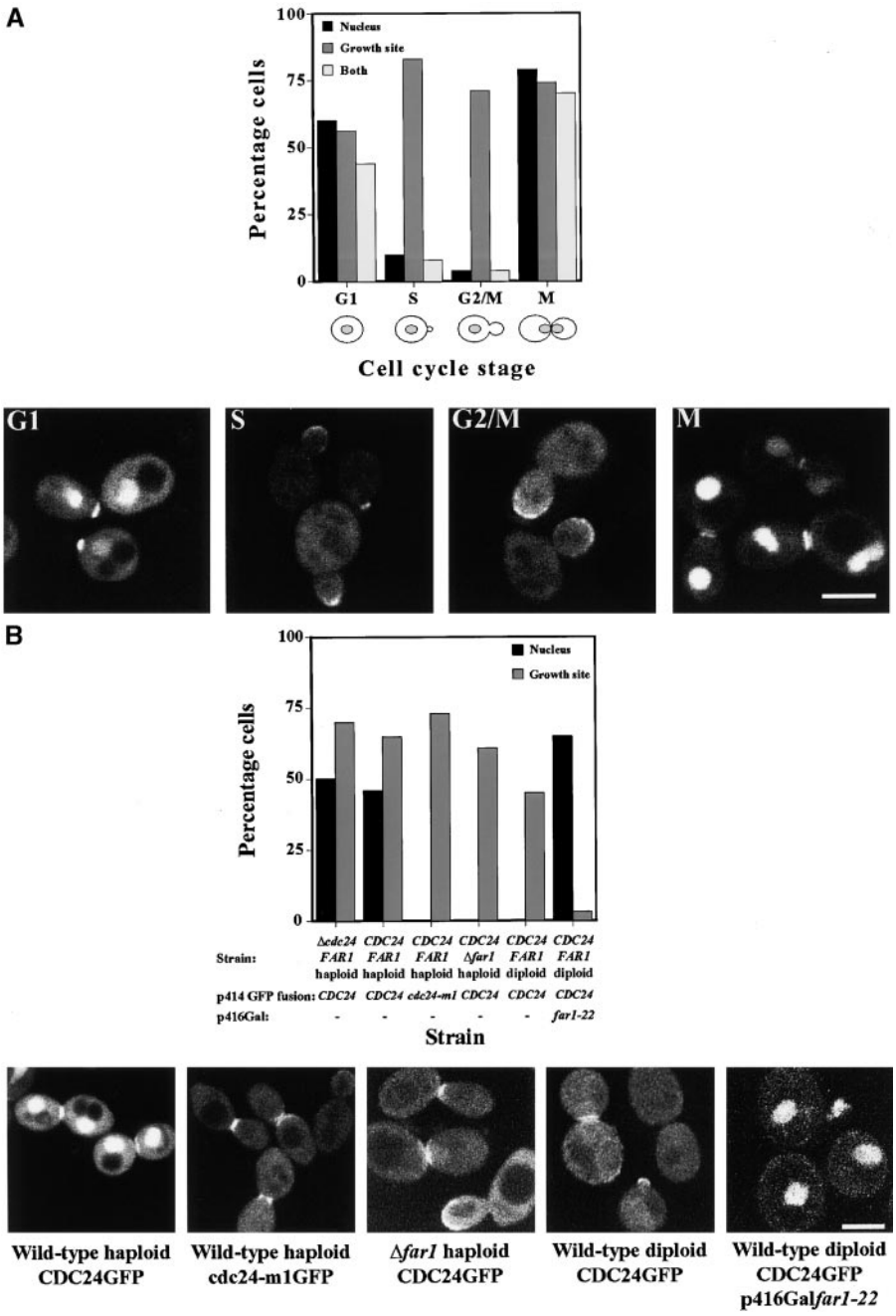

Figure 1. Far1p is necessary and sufficient for Cdc24p nuclear localization. (A) Cdc24GFP localizes to the nucleus of G1 phase cells. Nuclear and growth site localized Cdc24GFP were counted from images of Δcdc24 cells carrying p414Cdc24HAGFP (RAY1361) (n = 450). (B) Cdc24p Far1p interaction is necessary and sufficient for Cdc24p nuclear localization. The percentage of cells with nuclear and growth site localized Cdc24p was quantitated (n = 150–175 for each strain). Diploid cells expressing p416Gal *far1-22* were grown in galactose for 3 h and Cdc24GFP was observed in the nucleus at all cell cycle stages although under these conditions, many cells were in the G1 phase. Bars, 5 μm.

diploid cell nuclei. Expression of Far1-22p from a *GAL* promoter for 3 h resulted in Cdc24GFP nuclear localization in 65% of diploid cells (Fig. 1 B). There was a significant decrease in cells with growth site localized Cdc24GFP, suggesting that nuclear accumulation of Far1p depletes cytoplasmic Cdc24GFP. In contrast, expression of Far1-22p did not shift Cdc24-m1GFP to the nucleus (data not shown). These results demonstrate that Far1p is necessary in haploids and sufficient in diploids for Cdc24p nuclear localization.

Overexpression of Far1-22p is toxic, resulting in G1 cell cycle arrest, which has been suggested to be due to Cln/Cdc28p inhibition by Far1p (Henchoz et al., 1997). However, the localization of Cdc24GFP in cells expressing Far1-22p raised the possibility that Far1-22p toxicity might instead be the result of nuclear sequestration of this exchange factor that would remove it from sites of polarized growth thereby physically separating it from the Rho G protein Cdc42p which it normally activates. To test this hypothesis we first determined if mutations in Far1p which block Cdc24p binding (Butty et al., 1998; Nern and Arkowitz, 1999) alleviated the toxicity of Far1-22p overexpression. Strikingly the combination of two different Far1p mutations, either Far1-H7p or Far1-D1p, with Far1-22p completely abolished its toxicity (Fig. 2 A). Far1-D1p is a mutant with two amino acid changes, Gly 646 to Asp (referred to as D1A) and Pro 671 to Leu (referred to as D1B; Valtz et al., 1995). The combination of Far1-22p with each of these single amino acid changes revealed that only the D1A mutation blocked the Far1-22p toxicity (Fig. 2 A). Similarly, Far1-22p overexpression in *cdc24-m1* cells

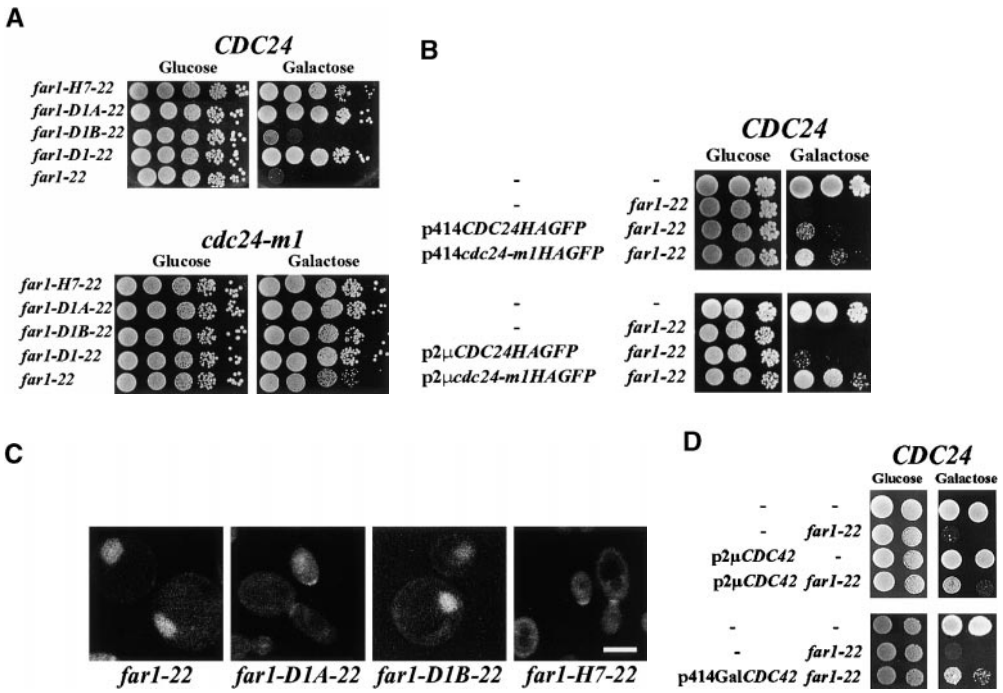

Figure 2. Far1-22 toxicity *via* nuclear sequestration of Cdc24p. (A) *Far1-22* toxicity is blocked by mutations in *far1-22* or *CDC24* which prevent Cdc24p Far1p binding. Serial dilutions of *CDC24* (RAY1254) or *cdc24-m1* (RAY1256) cells with p416-Gal *far1-22* mutants were spotted on glucose and galactose containing plates and grown for 2 days at 30°C. (B) Overexpression of *cdc24-m1* suppresses *far1-22* toxicity. K699 cells carrying the indicated plasmids (− indicates empty plasmid) were spotted on plates as described above. (C) Cdc24GFP localizes to the nucleus in cells expressing Far1-22p which can bind Cdc24p. Diploid cells (K842) expressing *far1-22* mutants and Cdc24GFP were grown in galactose for 4 h. (D) Overexpression of *CDC42* suppresses *far1-22* toxicity. K699 cells carrying the indicated plasmids were spotted on plates as described above. p2μ*CDC42* is a plasmid from a multicopy genomic DNA library which suppress a *cdc24* temperature-sensitive mutant and p414Gal*CDC42* will be described elsewhere. Bar, 5 μm.

resulted in little to no toxicity. The effects of Far1p mutations and *cdc24-m1* on Far1-22p toxicity were also observed in *Δfar1* strains (data not shown). In diploid cells expressing of Far1-D1A-22p or Far1-H7-22p, Cdc24GFP was still observed at growth sites in contrast to Far1-22p and Far1-D1B-22p which resulted in predominantly nuclear localization (Fig. 2 C). Immunoblot analyses indicated that the different Far1-22p mutants were all expressed and that their levels were similar in wild-type and *cdc24-m1* cells (data not shown). Far1p localized to the nucleus in *cdc24-m1* cells (see below) arguing against an indirect effect of *cdc24-m1* on Far1p localization.

The mating defect of *cdc24-m1* is recessive to *CDC24* (Nern and Arkowitz, 1998), but because Cdc24-m1p is unable to bind Far1p it should rescue the Far1-22p toxicity in spite of the presence of a wild-type copy of *CDC24*. Cdc24-m1GFP expression from its own promoter on a CEN plasmid partially restored growth of Far1-22p expressing cells, whereas overexpression of this fusion resulted in a substantial recovery of growth (Fig. 2 B). In contrast Cdc24GFP expression was much less effective in alleviating Far1-22p toxicity. As Cdc42p overexpression suppresses a *cdc24* temperature-sensitive mutant (Bender and Pringle, 1989) we reasoned that increased levels of Cdc42p might also reduce Far1-22p toxicity. *CDC42* on a multicopy plasmid or driven by a *GAL* promoter partially recovered Far1-22p toxicity (Fig. 2 D). Together, these results indicate that the toxicity of Far1-22p overexpression is predominantly the result of nuclear sequestration Cdc24p and not due to inhibition of Cln/Cdc28p. Such sequestration suggests that export of Cdc24p in the nucleus is crucial for cell viability and initiation of the cell cycle.

To examine whether nuclear Cdc24p could be exported and subsequently targeted to sites of growth we used a *cdc28* strain to accumulate Cdc24GFP in G1 cell nuclei at 37°C and followed its localization upon a shift to 25°C (Fig. 3 A). Within 15 min when Cdc24p nuclear fluorescence was still observable, Cdc24GFP was seen at the plasma membrane as a crescent before bud emergence. By 30 min, small buds with Cdc24p localized as a tight patch were observable in some cells and an increase in cytoplasmic fluorescence was apparent. After another 30 min, the majority of the cells were budded with Cdc24p localized to the tips and nuclear Cdc24p fluorescence was undetectable in most cells. These results suggest that nuclear export and localization of Cdc24p to the bud tip occur in similar time frames. Far1p degradation is likely to occur in the nucleus (Henchoz et al., 1997) providing a possible trigger for Cdc24p export upon G1 exit in the absence of pheromone.

In contrast, pheromone treatment results in Far1p export from the nucleus (Butty et al., 1998), suggesting that in these conditions a Cdc24p–Far1p complex might be exported from the nucleus. To examine pheromone-dependent nuclear exit, G1-arrested *cdc28* cells were α-factor treated (Fig. 3 B). Within 5 min, the level of Cdc24p in the nucleus decreased and Cdc24p appeared on the plasma membrane. As cells became pear-shaped a tight patch of shmoo tip Cdc24p was seen. In contrast, cells not treated with pheromone had little Cdc24p associated with the plasma membrane. To address the possibility that pheromone treatment results in the degradation of nuclear Cdc24p and instead newly synthesized Cdc24p localizes to the site of growth, similar experiments were carried out using the protein synthesis inhibitor cycloheximide (Fig. 3

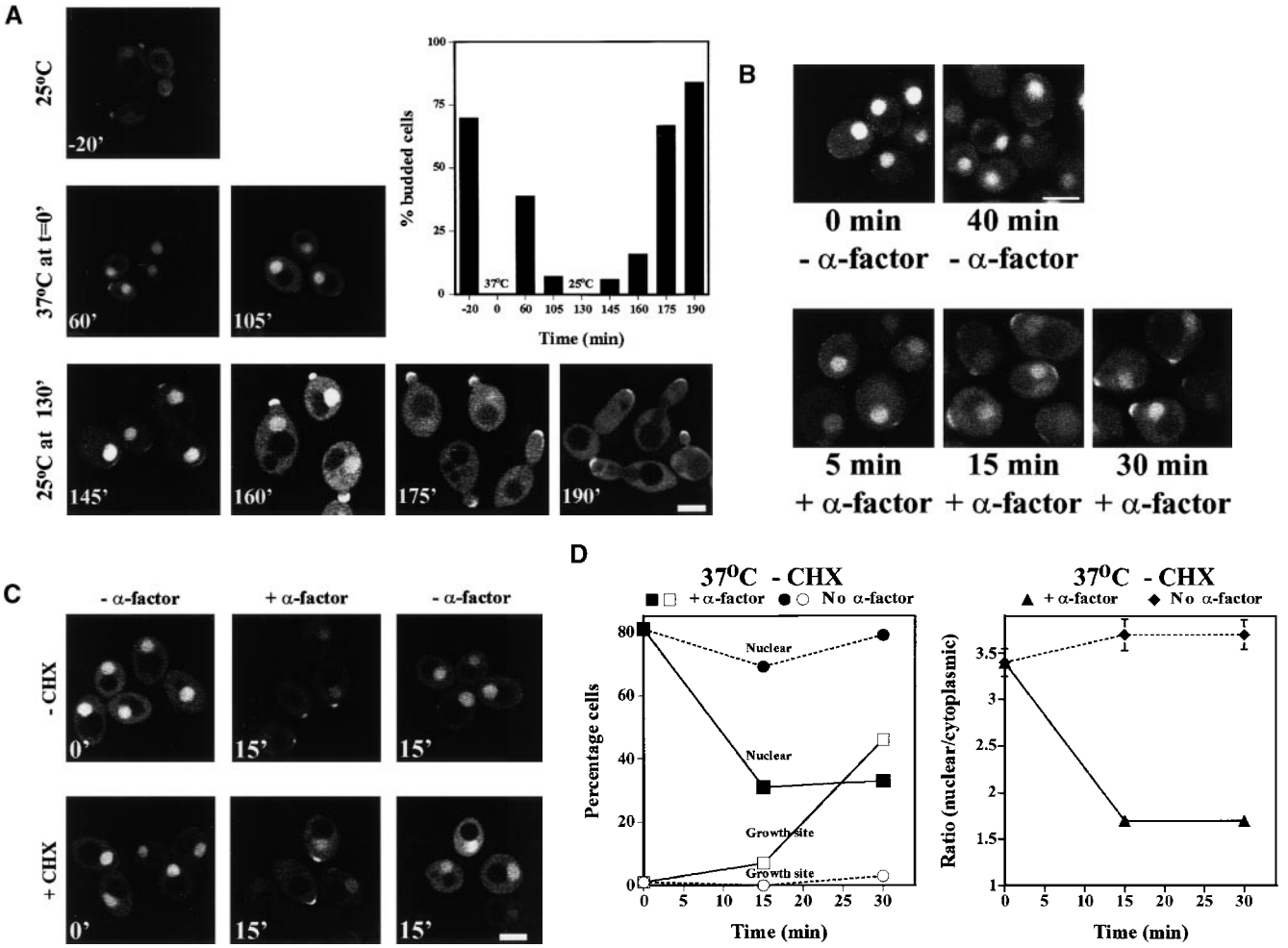

Figure 3. Nuclear Cdc24p is exported upon bud and shmoo formation. (A) Nuclear Cdc24GFP is exported and targeted to the bud. *Cdc28* cells (MS10*cdc28*) carrying p416Cdc24GFP were grown at 37°C for 130 min, then shifted to 25°C for 60 min, and at indicated times imaged. Percentage budded cells were counted from DIC images (n = 100 at each time). (B) Nuclear Cdc24GFP is exported and targeted to the shmoo. *Cdc28* cells (MS10*cdc28*) carrying p416Cdc24GFP were grown at 37°C for 2 h, pheromone was added, and at the indicated times cells were removed for microscopy. (C) Nuclear Cdc24GFP is exported and targeted to the shmoo in the presence of cycloheximide. Cycloheximide (20 μg/ml) was added to *cdc28* cells (MS10*cdc28*) carrying p416Cdc24GFP after 110 min at 37°C and after a further 10 min pheromone was added where indicated. (D) Quantitation of Cdc24p redistribution in response to pheromone. Cdc24p localization in the absence of cycloheximide (Fig. 3 C), filled (■,●) and open (□,○) symbols indicate percentage cells (n = 200) with nuclear and growth site localized Cdc24p, respectively. Ratio of nuclear to cytoplasmic fluorescence intensity (n = 100) was determined for each time (values were not corrected for background cellular fluorescence) and errors bars signify SEM. Bars, 5 μm.

C). In the presence of cycloheximide, pheromone triggered the export of Cdc24p from the nucleus and localization to growth sites on the plasma membrane. However, at longer incubation times, Cdc24p was no longer visible at the plasma membrane, perhaps due to the general toxicity of cycloheximide (data not shown). In the absence of cycloheximide, the number of cells with nuclear Cdc24GFP decreased approximately threefold after 15 min with pheromone, whereas a substantial increase in shmoo site localized Cdc24GFP was not observed until 30 min (Fig. 3, C and D). Quantification of the levels of nuclear and cytoplasmic fluorescence also showed a decrease in the ratio of nuclear to cytoplasmic fluorescence with pheromone (Fig. 3 D). These results indicate that preexisting Cdc24GFP is exported from the nucleus upon pheromone addition and subsequently localizes to sites of growth.

To directly confirm that the nuclear Cdc24p relocalized to sites of growth we used a strain in which Cdc24GFP expression was regulated. Gal Cdc24GFP *cdc28* cells were grown in galactose, shifted to 37°C, and after 1 h resuspended in glucose containing media to repress Cdc24p expression. Pulse labeling demonstrated that Cdc24GFP was no longer synthesized after 1 h in glucose (data not shown). Either the addition of pheromone or a temperature shift to 25°C resulted in the export of Cdc24GFP and localization to growth sites (Fig. 4). After 30 min in pheromone ~50% of cells showed shmoo tip localization, whereas only 10% of the cells had nuclear fluorescence. Similarly after a 60-min shift to 25°C, >70% of cells showed a bud tip localization and only 10% showed nuclear fluorescence. In a second temperature shift experiment we observed Cdc24GFP, which was synthesized be-

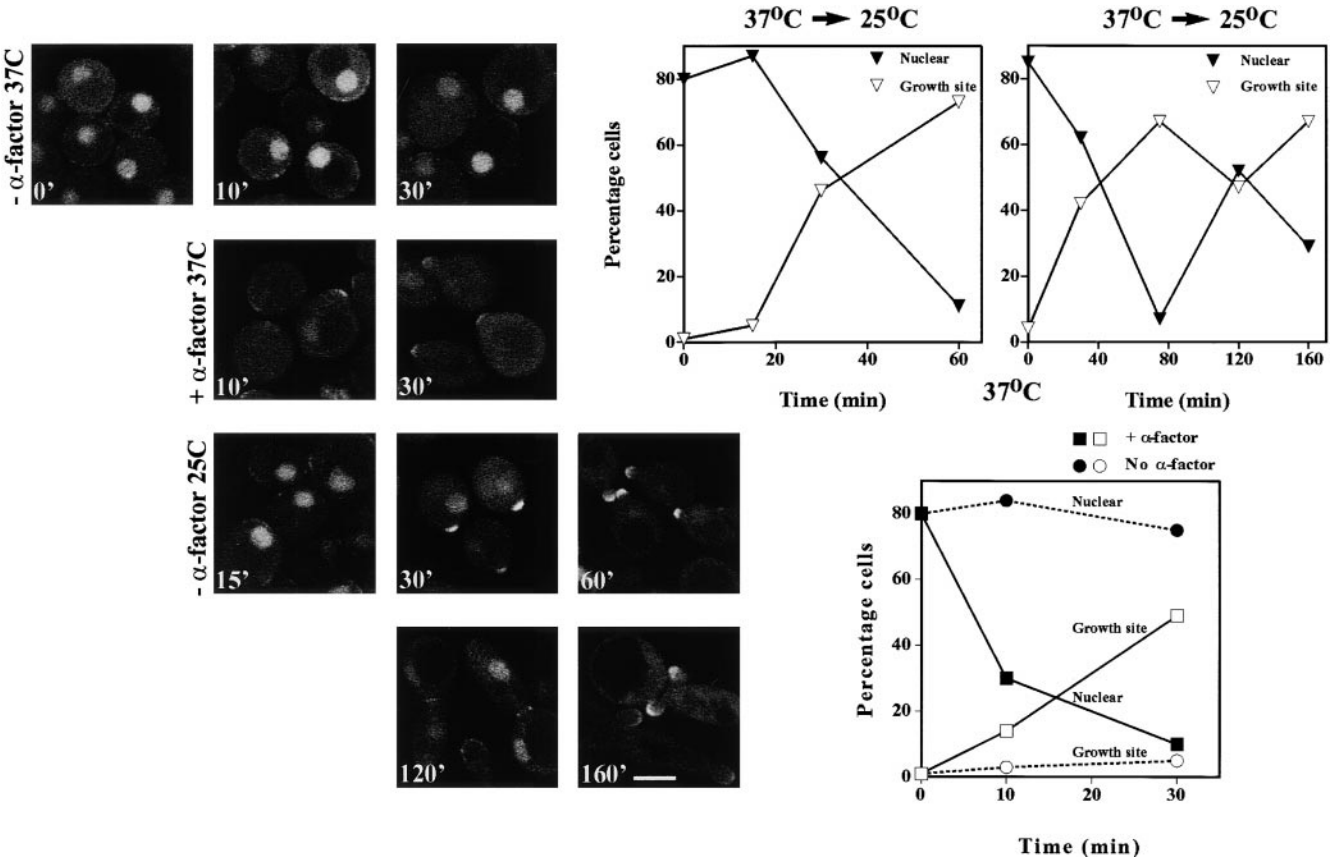

Figure 4. Cdc24GFP shuttles repeatedly between the nucleus and cytoplasm. *Cdc28* cells (MS10*cdc28*) carrying p416GalCdc24GFP were grown in galactose for 3 h, shifted to 37°C for an additional hour, collected by centrifugation, resuspended in medium with 2% glucose, and grown for another hour at 37°C. Where indicated, α-factor was added and cells were grown at 37°C or shifted to 25°C. Images from the first three times of the 25°C time course are from one experiment (quantitation upper left graph) and the last two images are from a second experiment (quantitation upper right graph). Cells (n = 100–120) were quantitated for growth site or nuclear fluorescence at each time point. Bar, 5 μm.

fore the temperature shift, shuttled out of the nucleus after approximately 1 h, back into the nucleus after 2 h, and subsequently at 2 h 40 min out of the nucleus again. Each nuclear export was accompanied by a corresponding increase in growth site localization. These experiments demonstrate that the nuclear localized Cdc24p is recruited to the site of growth on the plasma membrane upon export and that Cdc24p can cycle in and out of the nucleus several times.

Msn5p/Ste21p, a nuclear export factor, is required for efficient cell mating (Blondel et al., 1999; Mahanty et al., 1999). Removal of *MSN5/STE21* results in an increased nuclear and reduced cytoplasmic levels of Ste5p and Far1p. Hence we examined the effect of *Δmsn5/ste21* on Cdc24p localization. Surprisingly, *Δmsn5/ste21* resulted in a threefold reduction in the number of cells with nuclear Cdc24p yet did not affect the localization of Cdc24p to the mother-daughter bud neck (Fig. 5 A and data not shown). Immunoblot analyses indicated that although cellular levels of Cdc24p were unchanged, Far1p levels in *Δmsn5/ste21* cells were lower (Fig. 5 B), providing an explanation for the reduction in nuclear Cdc24p. Consistent with this explanation, an increase in Far1p levels upon pheromone treatment (Fig. 5 B) led to an increase in nuclear Cdc24GFP (Fig. 5 A). The localization of Cdc24GFP to the shmoo tip in *Δmsn5/ste21* (Fig. 5 A) and *cdc28 Δmsn5/ste21* (data not shown) strains appeared reduced, suggesting that similar to Ste5p and Far1p, Cdc24p nuclear export is defective in *Δmsn5/ste21*.

In the absence of Cdc24p Far1p binding, Cdc24p was not observed in the nucleus. Therefore, we examined whether the mating defect of *cdc24-m1*, which was previously attributed to inability to form the Cdc24p-Gβγ-Far1p complex (Butty et al., 1998; Nern and Arkowitz, 1999), might be due to the absence of nuclear Cdc24p. A nuclear localization signal was fused to Cdc24-m1GFP which resulted in an increase in nuclear localization, however, growth site localized Cdc24p was still observable (80% of cells showed nuclear and 30% growth site localization; n = 200 in wild-type and *cdc24-m1* cells). NLS-Cdc24p, but not NLS-Cdc24-m1p complemented the *cdc24-m1* mating defect (data not shown) demonstrating that the lack of nuclear localization is not responsible for this mating defect. Both of these NLS fusions complemented the growth defect of a *cdc24* temperature-sensitive mutant (data not shown) indicating they were functional. Overexpression of a truncated version of Far1p lacking its amino-terminal 131 amino acids including two nuclear localization signals

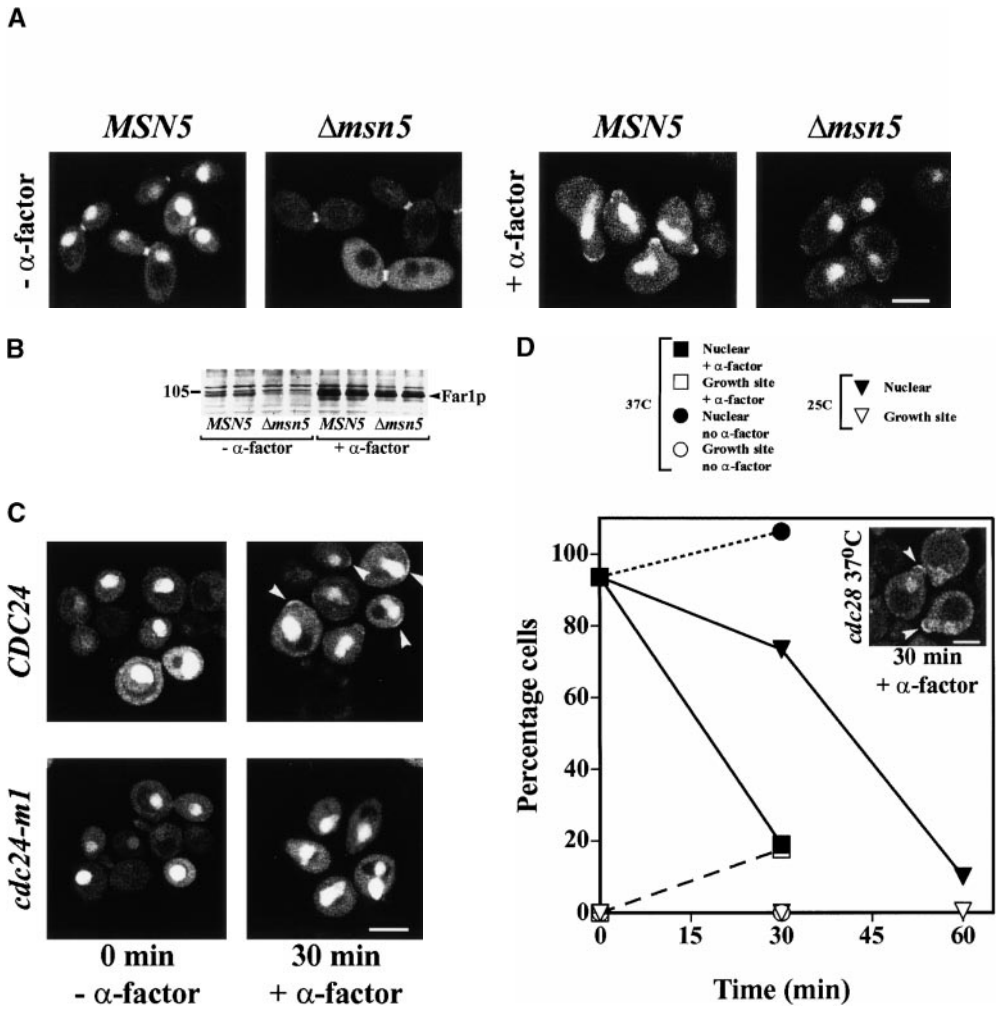

Figure 5. Requirements for Cdc24p and Far1p localization. (A) Cdc24GFP localization in *Δmsn5* cells. *MSN5* (SEY6211) and *Δmsn5* (RAY1519) cells expressing p416Cdc24GFP either grown vegetatively or treated with α-factor for 1.5 h were imaged by confocal microscopy. (B) *Δmsn5* results in a decreased level of Far1p. Immunoblot analysis of cellular Far1p levels in the absence and presence of α-factor (2 h). Cdc24GFP levels were similar in both strains with and without α-factor. (C) Cdc24p–Far1p interaction is necessary for targeting of Far1p to the shmoo tip. *CDC24* (RAY1254) or *cdc24-m1* (RAY1256) cells with pCMP68 (GalFar1GFP) grown in galactose containing media for 4 h were treated with α-factor as indicated. (D) Nuclear Far1p relocalizes to the shmoo site. Experiments identical to those described in Figure 4 except cells are carrying pCMP68. Localization of Far1GFP from 80–100 cells with discernible GFP fluorescence was scored. Bars, 5 μm.

(Blondel et al., 1999) yet still functional for Cdc24p and Gβγ binding (Butty et al., 1998; Nern and Arkowitz, 1999), rescued the mating defect of *Δfar1* cells (data not shown) yet did not accumulate Cdc24GFP in the nucleus (0% nuclear and 33% at growth site compared with 24% nuclear and 39% growth site with full-length Far1p, n = 300). These results suggest that it is the cytoplasmic Cdc24p–Far1p complex that is necessary for mating.

As Far1p was required for Cdc24p nuclear accumulation, we determined conversely, whether Cdc24p played a role in Far1p localization. In response to pheromone, Far1p relocates from the nucleus to the cytoplasm, however, its localization to the shmoo tip has not been reported (Butty et al., 1998). Far1GFP cell cycle–dependent nuclear localization and expression levels were similar in *CDC24* and *cdc24-m1* cells (Fig. 5 C and data not shown). Pheromone addition resulted in the redistribution of Far1GFP to the cytoplasm in both strains, however, *CDC24* cells had an enrichment of Far1p at the plasma membrane in both round cells and shmoo tips. Far1p at the cell periphery was difficult to observe due to high cytoplasmic fluorescence. Experiments with Far1GFP identical to Gal Cdc24GFP experiments (Fig. 4) showed that Far1p was exported and appeared at the shmoo tip with similar kinetics as Cdc24p (Fig. 5 D). Strikingly, Far1GFP localization to the plasma membrane was greatly reduced in pheromone-treated *cdc24-m1* cells (52% of *CDC24* cells had Far1GFP at the membrane compared with 8% of *cdc24-m1* cells; n = 80) indicating that Far1p requires *CDC24* for efficient delivery to or stabilization at the shmoo growth site.

Taken together, our data are consistent with the nuclear import of a preformed Cdc24p–Far1p complex and the degradation of Far1p during vegetative growth triggering the export of Cdc24p. In contrast, upon exposure to mating pheromone and completion of the cell cycle, this Cdc24p–Far1p complex is exported providing a source of this complex for targeting to Gβγ. Both Ste5p and Far1p shuttle in and out of the nucleus during vegetative growth and are exported from the nucleus upon pheromone treatment (Blondel et al., 1999; Mahanty et al., 1999). Our results demonstrate that similar nucleocytoplasmic shuttling occurs with the GDP/GTP exchange factor Cdc24p. Recently, overexpressed Cdc24p was shown to localize to the nucleus in a cell cycle–dependent fashion (Toenjes et al., 1999) in agreement with our results. Cells in which Cdc24p does not accumulate in the nucleus appear normal for vegetative growth, suggesting that this cycling per se does not have a critical function during budding. Cytoplasmic Far1p is functional for cell mating indicating that nuclear import and export of Cdc24p–Far1p is not required for mating. However, as the normal pheromone-dependent cell cycle

arrest function of Far1p is in the nucleus (Blondel et al., 1999) and the Cdc24p–Far1p growth orientation function is in the cytoplasm, nuclear export of this complex is required for efficient mating. The nuclear localization of Cdc24p upon mating pheromone treatment, due to increased levels of Far1p, may prevent inappropriate localization and activation of Cdc24p during completion of the cell cycle before shmoo formation and at the same time allow a fast response to pheromone. It is likely that nucleocytoplasmic shuttling of G protein exchange factors is a conserved feature of this class of G protein regulators as *S. pombe* Cdc24 (scd1; Chen et al., 1999) and very recently the human Rho G protein exchange factor Ect2 also localize to the nucleus in a cell cycle–dependent fashion (Tatsumoto et al., 1999). This suggests that the intracellular distribution of exchange factors might also be regulated by interacting proteins analogous to Far1p.

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References

Bender, A., and J.R. Pringle. 1989. Multicopy suppression of the *cdc24* budding defect in yeast by *CDC42* and three newly identified genes including the ras-related gene *RSR1*. *Proc. Natl. Acad. Sci. USA*. 86:9976–9980.

Blondel, M., P.M. Alepuz, L.S. Huang, S. Shaham, G. Ammerer, and M. Peter. 1999. Nuclear export of Far1p in response to pheromones requires the export receptor Msn5p/Ste21p. *Genes Dev*. 13:2284–2300.

Butty, A.C., P.M. Pryciak, L.S. Huang, I. Herskowitz, and M. Peter. 1998. The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. *Science*. 282:1511–1516.

Chen, C., Y. Li Chen, J.M. Hou, P. Papadaki, and E. Chang. 1999. Moe1, a conserved protein in *Schizosaccharomyces pombe*, interacts with a Ras effector, Scd1, to affect proper spindle formation. *Proc. Natl. Acad. Sci. USA*. 96:517–522.

Henchoz, S., Y. Chi, B. Catarin, I. Herskowitz, R.J. Deshaies, and M. Peter. 1997. Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast. *Genes Dev*. 11:3046–3060.

Mahanty, S.K., Y. Wang, F.W. Farley, and E.A. Elion. 1999. Nuclear shuttling of yeast scaffold Ste5 is required for its recruitment to the plasma membrane and activation of the mating MAPK cascade. *Cell*. 98:501–512.

Nern, A., and R.A. Arkowitz. 1998. A GTP-exchange factor required for cell orientation. *Nature*. 391:195–198.

Nern, A., and R.A. Arkowitz. 1999. A Cdc24p, Far1p, Gβγ protein complex required for yeast orientation during mating. *J. Cell Biol*. 144:1187–1202.

Rose, M.D., F. Winston, and P. Hieter. 1991. Methods in Yeast Genetics: A Laboratory Course Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Tatsumoto, T., X. Xie, R. Blumenthal, I. Okamoto, and T. Miki. 1999. Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. *J. Cell Biol*. 147:921–927.

Toenjes, K., M. Sawyer, and D. Johnson. 1999. The guanine nucleotide exchange factor Cdc24p is targeted to the nucleus and polarized growth sites. *Curr. Biol*. 9:1183–1186.

Valtz, N., M. Peter, and I. Herskowitz. 1995. *FAR1* is required for oriented polarization of yeast cells in response to mating pheromones. *J. Cell Biol*. 131: 863–873.