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The Ccr4-Pop2-NOT mRNA Deadenylase Contributes to Septin Organization in *Saccharomyces cerevisiae*

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ABSTRACT

In yeast, assembly of the septins at the cell cortex is required for a series of key cell cycle events: bud-site selection, the morphogenesis and mitotic exit checkpoints, and cytokinesis. Here we establish that the Ccr4-Pop2-NOT mRNA deadenylase contributes to septin organization. mRNAs encoding regulators of septin assembly (Ccd42, Cdc24, Rga1, Rga2, Bem3, Gin4, Cla4, and Elm1) presented with short poly(A) tails at steady state in wild-type (wt) cells, suggesting their translation could be restricted by deadenylation. Deadenylation of septin regulators was dependent on the major cellular mRNA deadenylase Ccr4-Pop2-NOT, whereas the alternative deadenylase Pan2 played a minor role. Consistent with deadenylation of septin regulators being important for function, deletion of deadenylase subunits *CCR4* or *POP2*, but not *PAN2*, resulted in septin morphology defects (*e.g.*, ectopic bud-localized septin rings), particularly upon activation of the Cdc28-inhibitory kinase Swe1. Aberrant septin staining was also observed in the deadenylase-dead *ccr4-1* mutant, demonstrating the deadenylase activity of Ccr4-Pop2 is required. Moreover, *ccr4*Δ, *pop2*Δ, and *ccr4-1* mutants showed aberrant cell morphology previously observed in septin assembly mutants and exhibited genetic interactions with mutations that compromise septin assembly (*shs1*Δ, *cla4*Δ, *elm1*Δ, and *gin4*Δ). Mutations in the Not subunits of Ccr4-Pop2-NOT, which are thought to predominantly function in transcriptional control, also resulted in septin organization defects. Therefore, both mRNA deadenylase and transcriptional functions of Ccr4-Pop2-NOT contribute to septin organization in yeast.

THE septins are evolutionarily conserved filament-forming proteins, whose functions and assembly are best understood in the model yeast *Saccharomyces cerevisiae*. The mitotic septins Cdc3, Cdc10, Cdc11, Cdc12, and Shs1 assemble a ring at the mother-bud neck just before bud emergence, which expands into a collar as the cell cycle progresses and is split between the mother and daughter cells at cytokinesis (Ford and Pringle 1991; Kim *et al.* 1991; reviewed in Douglas *et al.* 2005). The septins are thought to function as scaffolds to recruit proteins to specific parts of the cell cortex and as membrane diffusion barriers that enable asymmetric distribution of proteins between the mother and daughter cells (Douglas *et al.* 2005). The cellular functions of the septins include cytokinesis, bud-site selection, cell wall deposition, and mitotic checkpoints related to bud emergence and exit from mitosis (reviewed in Douglas *et al.* 2005; Park and Bi 2007).

Septin assembly is controlled at the post-translational level in a pathway coordinated by the small GTPase Cdc42 (reviewed in Douglas *et al.* 2005; Park and Bi 2007). Proposed roles for Cdc42 include a direct role in septin recruitment to the cortex for assembly into the ring (Gladfelter *et al.* 2002; Caviston *et al.* 2003), as well as regulation of effector proteins (Douglas *et al.* 2005; Park and Bi 2007). Cdc42 regulators and/or effectors are also required for proper septin assembly. Mutations in the Cdc42 GTP exchange factor (GEF) Cdc24 and the GTPase activating proteins (GAPs) Rga1, Rga2, and Bem3 cause defects in septin assembly, as do mutations in Cdc42 effectors Gic1 and Gic2 and the kinases Cla4, Gin4, and Elm1 (Cvrckova *et al.* 1995; Longtine *et al.* 1998; Caviston *et al.* 2003; Versele and Thorner 2004; Iwase *et al.* 2006; reviewed in Douglas *et al.* 2005; Park and Bi 2007). Cla4 and Gin4

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phosphorylate the septins to stabilize their association (Dobbelaere *et al.* 2003; Versele and Thorner 2004), whereas Elm1 phosphorylates Gin4 to activate it and cause Gin4-dependent phosphorylation of the septin Shs1 (Asano *et al.* 2006).

Post-transcriptional shortening of mRNA poly(A) tails (deadenylation) is an important mechanism in regulation of gene expression (Parker and Song 2004; Goldstrohm and Wickens 2008). mRNA poly(A) tail length can affect both transcript stability and translation; long tails positively correlate with translation efficiency (Preiss *et al.* 1998), while tail shortening by mRNA deadenylation serves as an entry point into mRNA decay (Parker and Song 2004; Goldstrohm and Wickens 2008). During mRNA maturation in yeast, poly(A) length is set by the Pan2 mRNA deadenylase, which trims the tails following nuclear polyadenylation. The cytoplasmic mRNA deadenylase Ccr4-Pop2-NOT shortens the tail further to a length of $\sim(A)_{10}$, and the mRNA then becomes the substrate for decapping and $5'\rightarrow3'$ exonucleolytic decay. These generic processes of mRNA ageing and decay are thought to operate on most cellular mRNAs (Parker and Song 2004; Goldstrohm and Wickens 2008). Nevertheless, different mRNA species can display distinct spectra of poly(A) tail lengths at steady state that reflect mRNA-specific control of deadenylation and decay, and such post-transcriptional control is typically driven by elements in the mRNA 3′-untranslated region (3′-UTR).

Microarray-based analyses of mRNA polyadenylation state uncovered widespread use of mRNA-specific poly(A) tail length control in the *S. cerevisiae* and *Schizosaccharomyces pombe* transcriptomes (Beilharz and Preiss 2007; Lackner *et al.* 2007). Of note here, many mRNAs encoding cell cycle, cell polarity, and morphogenesis-related functions including septin assembly, were found to be present with mostly short oligo(A) tails in nonsynchronized, exponentially growing wild-type (wt) cell cultures. These data suggested that rapid deadenylation by Ccr4-Pop2-NOT of such "short-tailed" mRNAs serves as a mechanism to restrict their translation to a tight time window during the cell cycle (Beilharz and Preiss 2007). Thus, even though the Ccr4-Pop2-NOT deadenylase shortens the poly(A) tails of virtually all mRNAs (Parker and Song 2004; Woolstencroft *et al.* 2006; Beilharz and Preiss 2007; Goldstrohm and Wickens 2008), its effects on the group of "short-tailed" mRNAs are particularly critical for cell function, and their deregulation in deadenylase mutant cells is therefore likely to contribute to observable phenotypes.

Our transcriptome-wide analysis had indicated that mRNAs that preferentially possess short poly(A) tails are enriched in gene ontology (GO) terms such as bud neck, cell cortex, establishment and maintenance of cell polarity, and cellular morphogenesis, and deadenylase mutant strains showed morphology defects (*e.g.*, wide necks and elongated cells) that could result from problems in septin organization (Beilharz and Preiss 2007). We therefore decided to examine the role of the Ccr4-Pop2-NOT mRNA deadenylase in septin organization. We show that a short poly(A) tail is a property of all tested mRNAs coding for major septin assembly factors and that Ccr4-Pop2-NOT contributes to septin organization in yeast.

MATERIALS AND METHODS

Yeast strains and growth conditions: The strains used in this study are listed in Table 1. All strains are isogenic to KY803 (*MAT***a** *trp1*Δ*1 ura3-52 gcn4 leu2::PET56*). Deletion mutants were created by standard gene replacement methods. GFP was fused to the C terminus of Cdc3 at the endogenous locus using the GFP-KANMX6 cassette. Standard growth conditions were YPD (2% glucose, 2% peptone, 1% yeast extract) media at 30°. For assaying growth at 37°, cells were pregrown in YPD at 30° and then 2.5 μl of 10-fold serial dilution starting from $OD_{600} = 0.5$ were plated on YPD plates and incubated at 37° for 2–3 days. For assaying suppression by sorbitol, sorbitol was added to the YPD plates at 1 M concentration.

Double *ccr4*Δ*/pop2*Δ and *cla4*Δ or *elm1*Δ mutants were obtained from dissection of tetrads after sporulation of the respective heterozygote diploid strains. Diploid strains were sporulated using standard methods and spores were dissected onto YPD plates using a Zeiss dissecting microscope. The spores were genotyped by plating on selective plates for the markers used to delete the relevant genes (see Table 1).

Cell morphology assays and microscopy: To assay cell morphologies in the presence or absence of hydroxyurea (HU), cells were grown to early-to-mid logarithmic phase in YPD at 30° and then treated with or without 0.2 M HU for 16 hr. We observed that the morphology defects of the mutants (particularly *ccr4*Δ) decreased with chronological age (when cultures were inoculated from plates kept at 4° for >2 weeks), and therefore plates from strains freshly streaked from stocks were used in all experiments. For microscopy, cells were fixed with 70% ethanol for 30 min–1 hr. After fixation, cells were rehydrated in 1× PBS (phosphate-buffered saline) and samples were kept at 4° until viewing. Cells were viewed with a Zeiss Axiovert 25 microscope using a 100× magnification objective. Photographs were taken on Kodak film at an original magnification of 250× or with an AxioCam MRc camera using the Axiovision Rel. 4.6 software. Typically between 100 and 300 cells were scored for each strain, for each independent culture. Averages are from at least three independent cultures and the experimental error represents the standard deviation.

For immunofluorescence microscopy, cells were fixed for 1 hr in 3.7% formaldehyde at 30°. Cell walls were digested by treatment with zymolyase 20T (250 μg/ml) for 10–20 min and cells were applied to polylysine-coated slides. All antibody incubations and blocking were done in 3% BSA (bovine serum albumin) in PBS. The anti-Cdc11 antibody (Santa Cruz Biotechnologies) was used at 1:100 dilution, followed by 1:1000 dilution of secondary anti-rabbit antibody conjugated to Alexa488. Cover slips were sealed with clear nail polish and slides were stored at −20°. The slides were viewed and photographed with the Zeiss Axiovert 25 microscope using the Axiocam MRc camera, and the Axiovision Rel. 4.6 software. For Figures 3B and 4B, cells were viewed with an Olympus BX51 microscope and photographs were taken with the DP Controller software.

TABLE 1

Yeast strains

Strain	Genotype	Reference
Y136 (KY803)	*MAT***a** *trp1Δ1 ura3-52 gcn4 leu2::PET56*	Denis *et al.* (2001)
Y181 (KY804)	AS Y136 but *MAT*α	Denis *et al.* (2001)
Y215	AS Y136 but *not1-1*	Denis *et al.* (2001)
Y214	AS Y136 but *not4Δ::URA3*	Denis *et al.* (2001)
Y294	AS Y136 but *ccr4Δ::klURA*	Traven *et al.* (2005)
Y297	AS Y136 but *pop2Δ::klURA*	Traven *et al.* (2005)
Y298	AS Y136 but *not2Δ::klURA*	Traven *et al.* (2005)
Y299	AS Y136 but *not5Δ::klURA*	Traven *et al.* (2005)
Y310	AS Y136 but *not3Δ::klURA*	Traven *et al.* (2005)
Y369	(ccr4-1) AS Y136 but *ccr4-E556A*	Traven *et al.* (2005)
Y763	AS Y136 but *swe1Δ::KAN*	This study
Y816	AS Y136 but *pop2Δ::klURA swe1Δ::KAN*	This study
Y956	AS Y136 but *cla4Δ::KAN*	This study
Y960	AS Y181 but *cla4Δ::KAN*	This study
Y961	AS Y136 *pop2Δ::klURA cla4Δ::KAN*	This study
Y968	AS Y181 but *elm1Δ::KAN*	This study
Y972	AS Y136 but *shs1Δ::KAN*	This study
Y976	AS Y136 but *pop2Δ::klURA shs1Δ::KAN*	This study
Y980	AS Y136 but *ccr4-E556A shs1Δ::KAN*	This study
Y982	AS Y136 but *ccr4Δ::klURA shs1Δ::KAN*	This study
Y989	AS Y136 but *CDC3-GFP-KAN*	This study
Y992	AS Y136 but *ccr4Δ::klURA* CDC-GFP-KAN	This study
Y993	AS Y136 but *pop2Δ::klURA* CDC3-GFP-KAN	This study
Y1013	AS Y136 *ccr4Δ::klURA cla4Δ::KAN*	This study
Y1126	AS Y136 but *ccr4Δ::klURA swe1Δ::KAN*	This study
Y1128	AS Y136 but *ccr4-E556A swe1Δ::KAN*	This study
Y1172	AS Y136 but *pan2Δ*	Beilharz *et al.* (2007)
Y1173	AS Y136 but *ccr4-E556A pan2Δ*	Beilharz *et al.* (2007)
YAT164	AS Y136 but *gin4Δ::KAN*	This study
YAT165	AS Y136 but *ccr4Δ::klURA gin4Δ::KAN*	This study
YAT170	AS Y136 but *pop2Δ::klURA gin4Δ::KAN*	This study

klURA-Kluyveromyces lactis URA

Western and Northern blotting: For Western blots, cells were grown overnight, diluted to $OD_{600} = 0.2–0.3$, grown for 3 hr at 30°, and then treated with 0.2 M HU for the times indicated in the figures. Protein extracts were prepared by precipitation of proteins with trichloro acetic acid (TCA), followed by resolubilization in Laemli buffer. The antibodies were used at the following dilutions: anti-actin 1:4000 (Chemicon) and anti-Swe1 1:200 (Santa Cruz Biotechnologies). The secondary antibodies were conjugated to horseradish peroxidase and detection was performed with the ECL reagent (Amersham).

Northern analysis of gene expression (supporting information, Figure S1) was performed from wild type, *ccr4*Δ, *ccr4-1*, and *pop2*Δ cultures not treated by HU. RNA was isolated by the hot phenol method and processed for Northern blot analysis as described (Hammet *et al.* 2002). Probes were obtained by PCR with ORF-specific primers from genomic DNA of wild-type KY803 yeast and were labeled with ^{32}P. The signals were quantified with the ImageQuant software after exposure on Phosphorimager screens. The expression levels of the relevant genes were normalized to *ACT1* levels and are expressed as fold change compared to expression in the wild type (with wild-type levels set to 1).

Ligation-mediated polyadenytation test: Ligation-mediated polyadenytation test (LM-PAT) was performed as described previously (Beilharz and Preiss 2007). The TVN-PAT samples (see Figure 1) were obtained from wt cDNA using a PAT-T12-VN reverse transcription primer (5′ GCGAGCTCCGC GGCCGCGTTTTTTTTTTTVN; where V is any nucleotide except T and N is any nucleotide). The PAT-T12-VN binds at the 3′-UTR and poly(A) junction, and therefore the TVN-PAT samples show the shortest PCR product detected by the assay. The shared 5′ sequence between the regular LM-PAT reverse primer and the TVN-PAT primer allows parallel PCR amplification from these control samples and the PAT cDNAs that report on the poly(A) length.

RESULTS

mRNAs encoding septin assembly factors are short-tailed and their deadenylation is controlled by Ccr4-Pop2: Gene ontology analysis of microarray data (Beilharz and Preiss 2007) suggested that terms such as "establishment and maintenance of cell polarity," "cellular morphogenesis," "bud neck," and "cell cortex" are prevalent in the group of short-tailed mRNAs. Among the identified short-tailed genes were several with known roles in septin assembly, such as the septin *SHS1*, the kinase *GIN4*, the Cdc42 GAPs *RGA1* and

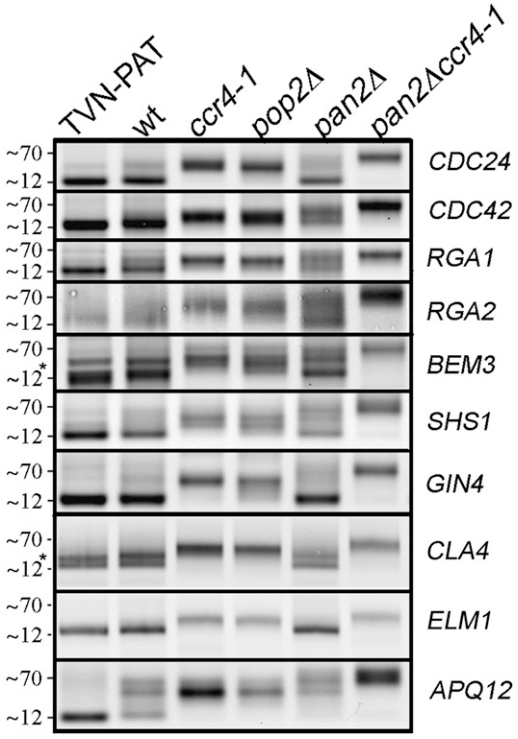

Figure 1.—Transcripts encoding septin regulators have short mRNA poly(A) tails and their deadenylation is controlled by Ccr4-Pop2. LM-PAT was performed on cDNAs from the indicated strains using gene-specific primers. The TVN-PAT samples represent the shortest products detected by the assay. The lengths, ~12 and ~70, of the poly(A) tail are approximate lengths, and the * indicates alternate 3′-UTR usage in the *BEM3* and *CLA4* transcripts.

RGA2, as well as other genes implicated in septin organization (*eg.*, *BNI1*, *BNI4*, and *BUD3*; Gladfelter *et al.* 2005).

We wanted to address whether a short poly(A) tail was a general property of mRNAs encoding proteins required for septin assembly. To that end we analyzed poly(A) tail length distribution on transcripts encoding the central regulator of septin assembly, the small GTPase Cdc42, as well as its regulators and effectors: the Cdc42 GEF Cdc24, the Cdc42 GAPs Rga1, Rga2, and Bem3, and the kinases Gin4, Cla4, and Elm1. *GIN4*, *RGA1*, and *RGA2* were assigned to the short-tailed group in our whole transcriptome analysis and here we verified their poly(A) tail length distribution by direct assessment on the individual mRNAs. *CDC42*, *CDC24*, *BEM3*, *CLA4*, and *ELM1* had not been assigned to a tail length category by microarray analysis (Beilharz and Preiss 2007). We also looked at the mRNA encoding the septin Shs1, as it was found to be short-tailed by microarray analysis (Beilharz and Preiss 2007).

The lengths of the mRNA poly(A) tails were assessed by ligation-mediated poly(A) test (LM-PAT). LM-PAT is a reverse-transcription/PCR assay where product sizes reflect the distribution of poly(A) tail lengths on the tested mRNAs (Sallés and Strickland 1995; Beilharz and Preiss 2007). As a control for the shortest possible tail lengths in each case, we performed PCRs from cDNAs generated with a primer that anneals to 3′-UTR and poly(A) junction (TVN-PAT lanes in Figure 1, see materials and methods). Whether the mRNA poly(A) tail on the tested mRNAs was short or long in wild-type cells was determined by comparison to the TVN-PAT samples. The mRNA poly(A) tail lengths were analyzed in the wild type and in mutants in the subunits of Ccr4-Pop2-NOT that are essential for mRNA deadenylase activity: the exonuclease-inactive *ccr4-1* and *pop2*Δ mutants (Tucker *et al.* 2001, 2002; Chen *et al.* 2002). We also analyzed mutants deleted for the alternative mRNA deadenylase *PAN2*, and the *pan2*Δ *ccr4-1* double mutant was included to determine the maximal poly(A) tail length (Figure 1).

All septin assembly factors, as well as the septin *SHS1*, were predominantly short-tailed mRNAs in the wild-type cells at steady state, barely differing from the minimally short TVN-PAT samples that reflect the shortest detectable product in these assays (Figure 1). In the mRNA deadenylase mutants *ccr4-1* and *pop2*Δ the tails on the tested mRNAs increased dramatically in size, confirming that their deadenylation is dependent on Ccr4-Pop2 (Figure 1). As a "long-tailed" control we used the *APQ12* mRNA because, in addition to some short-tailed species, this transcript presents with long poly(A) tails at steady state. Although there was less *APQ12* length heterogeneity in the *ccr4-pop2* mutants with an apparent loss of the short-tailed fraction observed in the wild type, the majority of the transcripts were not altered in length by loss of Ccr4-Pop2 activity.

Longer poly(A) tails on mRNAs for septins and septin assembly factors could lead to stabilization and higher mRNA levels and/or increased translation of the transcripts. mRNA levels for the septins and septin assembly factors that we tested were not elevated in *ccr4* and *pop2* mutants (Figure S1), suggesting that, as it has been previously suggested for the Ccr4 target gene *CRT1* (Woolstencroft *et al.* 2006), translational rates, rather than mRNA stability is affected by longer poly(A) tails. We tested protein levels of some proteins for which antibodies were commercially available (Cdc42 and the septin Cdc11); however we did not observe higher levels in *ccr4* and *pop2* mutants (data not shown), indicating that perhaps some of the other mRNAs are the relevant targets.

In contrast to the tightly stabilized longer mRNA poly(A) tails (which were devoid of short-tailed molecules and were of a uniform longer size) observed in the *ccr4-1* and *pop2*Δ mutants, the alternative mRNA deadenylase Pan2 had a less dramatic effect on poly(A) tail lengths for transcripts encoding septin assembly factors (see *pan2*Δ mutants in Figure 1). This result is consistent with previous transcriptome data that showed the short-tailed mRNAs strongly depended on Ccr4 to establish appropriate tail length control (Beilharz and Preiss

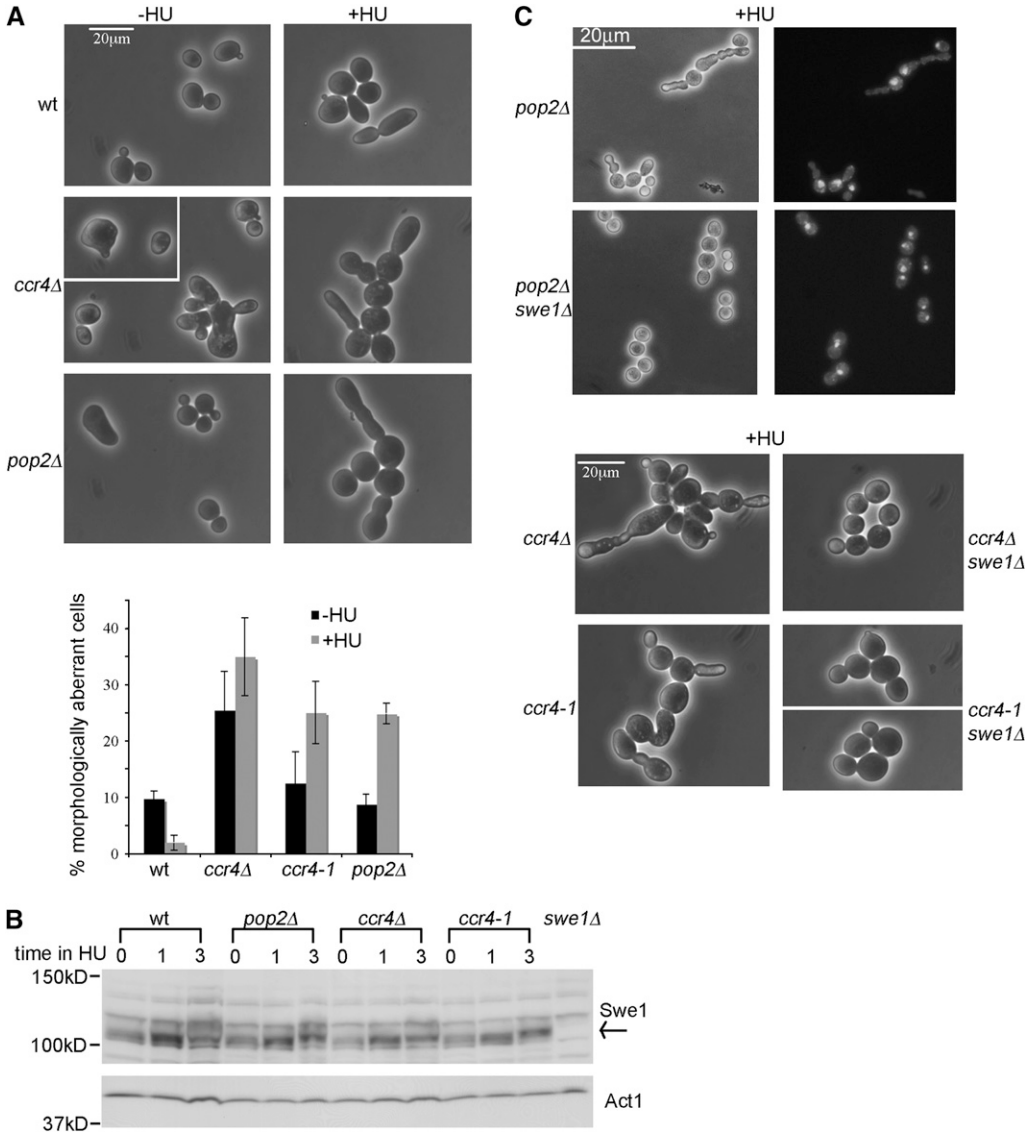

FIGURE 2.—*ccr4* and *pop2* mutants show Swe1-dependent aberrant cell morphology. (A) Cells were treated with or without 0.2 M HU for 16 hr. The graph represents averages from at least three independent cultures for each strain. Error bars depict the standard deviation. (B) Cell extracts were from cells treated with 0.2 M HU for the indicated times (hours). Membranes were probed with the antibody against Swe1, followed by the antibody against actin (loading control). (C) Cells from the indicated mutants were treated with 0.2 M HU for 16 hr. Nuclear DNA was stained with DAPI.

2007). Some transcripts (*eg.*, *CDC24*, *GIN4*, *CLA4*, and *ELM1*) were not affected by loss of Pan2. In other cases (for example *RGA1*, *BEM3*, and *SHS1*), the mRNAs did have longer tails in *pan2*Δ mutants. However, only a subset of the molecules for each affected mRNA had a longer tail in the *pan2*Δ cells, and shorter tails could also be observed, including tails as short as in the wild type (Figure 1).

Ccr4 and Pop2 are required for normal morphogenesis and septin ring organization: If Ccr4-Pop2-dependent deadenylation of septin assembly factors (*ie.*, their physiologically short mRNA poly(A) tails) is functionally relevant, *ccr4* and *pop2* mutants should display aberrant septin morphology and/or localization and, consequently, also cell morphology defects (for examples see BARRAL *et al.* 1999; LONGTINE *et al.* 2000; GLADFELTER *et al.* 2004, 2005).

The *ccr4*Δ mutant indeed displayed various morphology defects, such as bigger and misshapen cells and elongated and/or misshapen buds (the −HU panel in Figure 2A and data not shown), whereas the *pop2*Δ strain and the deadenylase-dead *ccr4-1* mutant had a milder phenotype with occasionally bigger, multi-budded cells or cells with wide necks and misshapen and/or elongated buds (see the −HU left panel in Figure 2A and data not shown).

It has been previously shown that activation of the Cdc28-inhibitory kinase Swe1 increases the morphology defects of septin assembly mutants (GLADFELTER *et al.* 2005; ENSERINK *et al.* 2006; KEATON and LEW 2006; LIU and WANG 2006; SMOLKA *et al.* 2006). Therefore, we activated Swe1 by treatment with the DNA replication inhibitor HU (LIU and WANG 2006), and then observed the morphology of the deadenylase mutant strains. Figure 2B shows that Swe1 was activated by HU in the wild type and *ccr4* and *pop2* mutants, as evidenced by HU-induced Swe1 stabilization and change in mobility toward slower migrating forms by immunoblot analyses.

Consistent with the hypothesis that Ccr4-Pop2 contributes to septin organization, the morphology defects

of the *ccr4*Δ, *pop2*Δ, and *ccr4-1* mutants became more pronounced after HU treatment (Figure 2A, +HU panel on the right; the *ccr4-1* mutant is shown in Figure 2C). Similar to previous observations for septin assembly mutants (Gladfelter *et al.* 2005), HU treatment caused a bud-chain phenotype in the *ccr4*Δ, *pop2*Δ, and *ccr4-1* strains: the cells displayed elongated and misshapen buds, which often had constrictions along the length of the bud (see the +HU samples in Figure 2, A and C). DAPI staining of HU-treated *pop2*Δ mutants showed one nucleus in the cells with bud chains (Figure 2C), suggesting uncoupling of budding from nuclear division. In addition to bud chains, HU-treated *ccr4*Δ mutants (and to a lesser extent the *pop2*Δ and *ccr4-1* mutants) also displayed formation of cell aggregates (clumps), which could be dispersed by brief sonication, as well as some cell chains.

To confirm that the effects of HU on the morphology of the *ccr4* and *pop2* mutants were due to Swe1 activation, we deleted *SWE1* in the *ccr4*Δ, *pop2*Δ, and *ccr4-1* mutants and asked whether that could suppress the morphology defects (Figure 2C). Deletion of *SWE1* suppressed the elongated bud-chain morphology defect of HU-treated *pop2*Δ cells by approximately threefold (38.1 ± 6.5% reduced to 11.8 ± 4.5%, averages ± standard deviation from three independent cultures), resulting in predominantly large budded cells (Figure 2C). *swe1*Δ also suppressed the elongation and bud morphology defects of the *ccr4*Δ and *ccr4-1* mutants (Figure 2C, note that the double mutants still formed clumps and chains). Therefore, Swe1-dependent mechanisms are at least in part responsible for the morphology defects of Ccr4-Pop2 deadenylase mutants, particularly for formation of elongated buds and bud chains.

The Swe1-dependent morphology defect of the *ccr4* and *pop2* mutants (in particular formation of bud chains) was a strong indication that the mutants have problems with septin organization (Gladfelter *et al.* 2005). To test this directly, we stained the septins in the wild type and *ccr4*Δ, *pop2*Δ, and *ccr4-1* mutants, using an antibody against the Cdc11 septin. As shown in Figure 3A, wild-type cells mostly displayed normal septin staining at the neck after HU treatment, whereas *ccr4*Δ, *pop2*Δ, and *ccr4-1* showed aberrant septin staining. The mutants formed additional septin rings within the elongated buds, and absent or diffuse septin staining could also be observed, particularly in bigger and misshapen cells and cells with wide necks (4.8% of HU-treated wild-type cells showed aberrant septin staining and this was increased to 23, 30.3, and 18.8% in the *ccr4*Δ, *pop2*Δ, and *ccr4-1* mutants, respectively; quantification is from single cultures, $n \geq 200$). Consistent with what we observed in the morphology assays, Swe1 was required for formation of ectopic bud-localized rings in *ccr4*Δ and *pop2*Δ mutants in HU-treated cells (Figure 3B). In those experiments, 21 and 17.6% of cells from *ccr4*Δ and *pop2*Δ cultures, respectively, showed aberrant

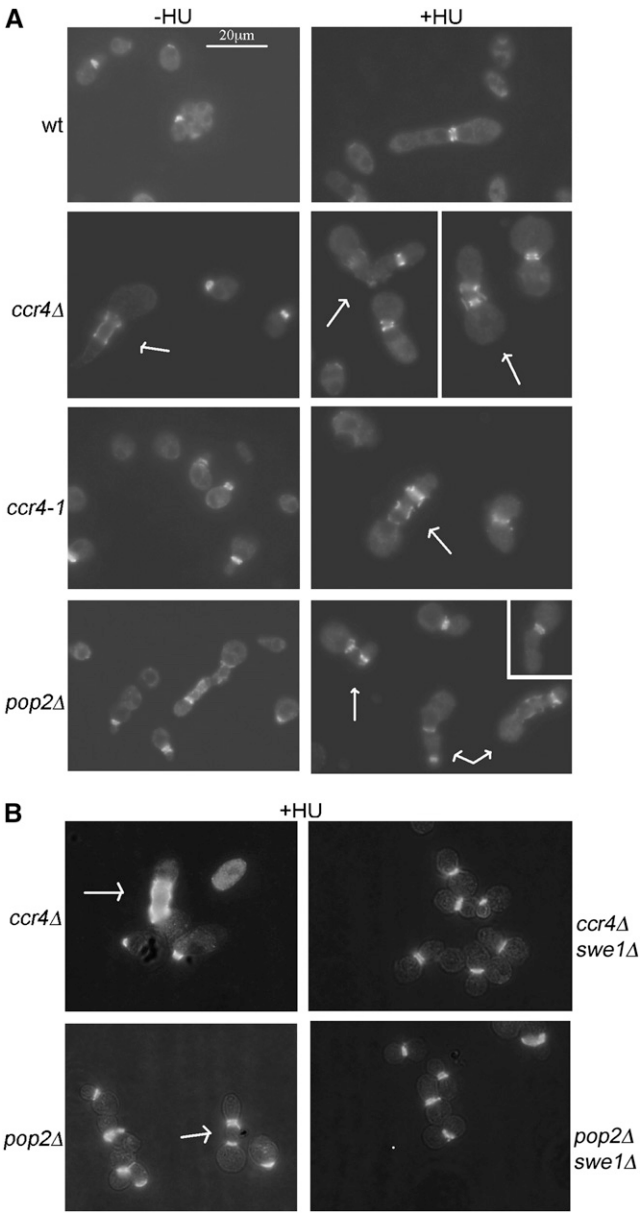

Figure 3.—Ccr4 and Pop2 are required for proper septin organization. Septins were stained in HU-treated and untreated cultures by immunofluorescence with the anti-Cdc11 antibody. The arrows represent cells with ectopic septin rings in the buds.

septin staining (predominantly ectopic septin rings in the bud), whereas in the double *ccr4*Δ *swe1*Δ and *pop2*Δ *swe1*Δ mutants ≤1.5% cells showed ectopic septin rings and most cells were large budded with wild-type septin staining at the mother-bud neck ($n \geq 200$ for all strains, the wild-type and swe1Δ mutants displayed ≤2% aberrant septin staining).

Roles of the alternative mRNA deadenylase Pan2 in septin assembly: Pan2 had a less pronounced role in deadenylation of septin assembly factors than Ccr4-Pop2 (Figure 1), and we next tested whether Pan2 was required for septin assembly. We found that *pan2*Δ mutants did not show bud chains in response to Swe1

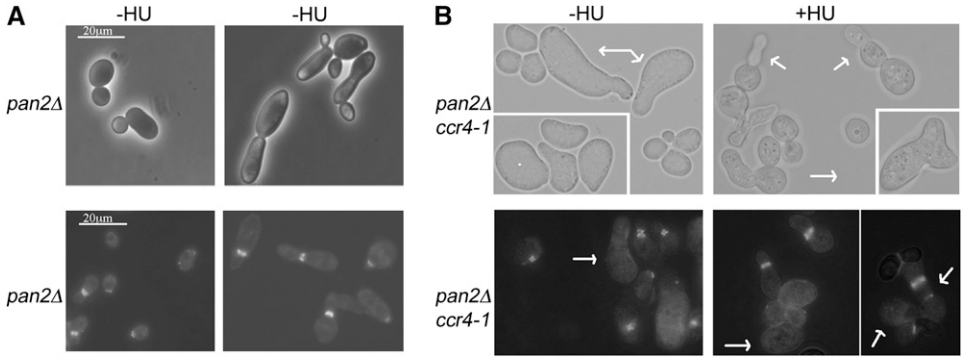

Figure 4.—The Pan2 mRNA deadenylase is dispensable for septin organization. (A) Upper panel: Cells were treated with or without 0.2 M HU for 16 hr and processed for microscopy as in Figure 2A. Lower panel: Immunofluorescence with the anti-Cdc11 antibody in *pan2*Δ cultures, with or without treatment with 0.2 M HU for 16 hr. (B) Double *pan2*Δ *ccr4-1* mutants were either from log phase cultures (−HU) or after treatment with 0.2 M HU for 16 hr. Cells were processed for microscopy and immunofluorescence as in Figures 2 and 3. The arrows indicate the big elongated cells that did not stain for septins, as well as cells displaying bud chains and ectopic septin rings.

activation by HU (even though the cells did display an elongated cell morphology/pseudohyphal phenotype, see upper panel in Figure 4A; compare with *ccr4* and *pop2* mutants in Figure 1). Furthermore, the mutants displayed normal septin staining at the neck, in the presence or absence of HU treatment (Figure 4A), indicating that, unlike Ccr4-Pop2, Pan2 is not required for septin organization.

The role of Pan2 in deadenylation of transcripts encoding septin assembly regulators becomes more pronounced in the absence of Ccr4 activity (double *pan2*Δ *ccr4-1* mutants displayed longer poly(A) tails on mRNAs for septin assembly factors than *ccr4-1* single mutants, Figure 1). Therefore, we also tested the morphology and septin organization in *pan2*Δ *ccr4-1 vs. ccr4-1* mutant strains. As shown in Figure 4B, cultures from strains in which both mRNA deadenylases are inactivated displayed a proportion of very large, sometimes elongated cells, which were not observed in cultures from single *ccr4-1* or *pan2*Δ mutants. These cells did not show any detectable septin staining with the anti-Cdc11 antibody (Figure 4B): 23.63 ± 8.8% of cells in *pan2*Δ *ccr4-1* did not stain with the anti-Cdc11 antibody, compared to 7.4 ± 3.3% in *ccr4-1* single mutants (the cells that showed absence of septin staining were bigger and/or elongated or had wide mother-bud necks), showing the number of cells with no septin staining increased in the absence of both Ccr4 and Pan2 activity compared to cells in which only Ccr4 is inactivated. Upon treatment with 0.2 M HU, a proportion of cells from *pan2*Δ *ccr4-1* mutant cultures displayed bud chains, and these cells showed formation of ectopic septin rings in immunofluorescence experiments with the anti-Cdc11 antibody (Figure 4B). Counting of cells with septin staining defects (cells forming ectopic bud-localized septin rings, as well as big cells and cells with wide necks displaying no septin staining) showed that HU-treated *pan2*Δ *ccr4-1* mutants have a comparable number of cells with aberrant septin staining to *ccr4-1* single mutants (17.45 ± 2.5% in double mutants, 14.8% in single *ccr4-1* mutants, $n \geq 200$ per individual culture).

Because the morphology defects in the *pan2*Δ *ccr4-1* mutants are somewhat different than *ccr4-1* cells, it is hard to directly compare the defects in septin assembly. For example, it is not clear what the etiology of the big cells in the double *pan2*Δ *ccr4-1* mutants is and whether the absence of normal septin organization is a cause or consequence of the aberrant morphology in those cells. However, overall the data suggest that, even though *per se* Pan2 does not play a detectable role in septin organization, in the absence of Ccr4 activity, the function of Pan2 in determination of morphology/cell size and possibly also septin organization becomes more important.

***CCR4 and POP2* genetically interact with genes encoding the septins and septin assembly kinases Cla4, Elm1, and Gin4:** To further evaluate the role for the Ccr4-Pop2 deadenylase in septin organization, we tested if *ccr4*Δ, *ccr4-1*, and *pop2*Δ mutations genetically interacted with mutations that compromised septin ring structure. All mitotic septins except Shs1 are essential in *S. cerevisiae*, but we took advantage of the fact that fusion of GFP to the C terminus of septins can affect their function (for an example see Enserink *et al.* 2006) to test the genetic interaction with an essential septin gene. We used a *CDC3-GFP* allele to test the effects of lower Cdc3 function in the deadenylase mutant strains. In agreement with diminished Cdc3 function, we observed an increase in aberrant morphology of wild-type cells upon fusion of GFP to Cdc3 (~20% of cells were aberrant, compared to 5% in the absence of GFP and the cells were also more prone to lysis during ethanol fixation, Figure 5A and data not shown). We found that fusion of GFP to the C terminus of Cdc3 resulted in slower growth of *ccr4*Δ and *pop2*Δ cells (*ccr4*Δ mutants were considerably more affected) and the synthetic growth defect was stronger at 37° (Figure 5A). *ccr4*Δ, *pop2*Δ, and the catalytically inactive *ccr4-1* allele also showed a synthetic slow growth phenotype with deletion of the only nonessential mitotic septin *SHS1*. Again, *ccr4*Δ showed significantly stronger slow growth phenotype with *shs1*Δ than *pop2*Δ or the catalyt-

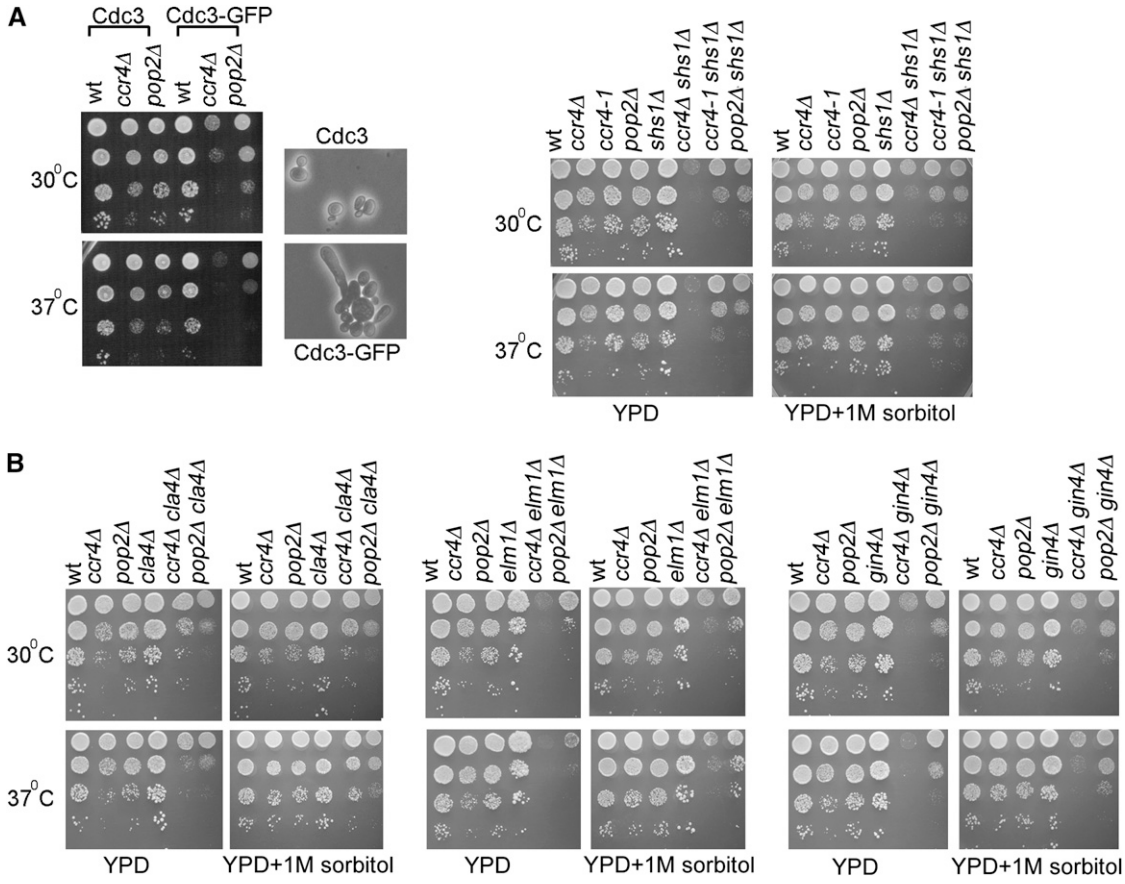

FIGURE 5.—*CCR4* and *POP2* show genetic interactions with mutations affecting septin assembly. (A) Tenfold serial dilutions of wild-type or mutant cultures were spotted on YPD plates with or without 1 M sorbitol and incubated for 2–3 days at 30° or 37°. The micrographs are from wild-type cultures at 30°, with or without GFP fused C terminally to Cdc3. (B) Tenfold serial dilutions of cells from the indicated strains were plated on YPD plates with or without 1 M sorbitol and incubated for 2 days at 30° or 37°.

ically inactive *ccr4-1* mutant and the synthetic growth defects of double *ccr4*Δ *shs1*Δ mutants were slightly more pronounced at 37° (Figure 5A).

Next we tested the genetic interactions of *ccr4*Δ and *pop2*Δ with deletions of the genes encoding the septin assembly kinases *CLA4*, *ELM1*, and *GIN4* (DOUGLAS *et al.* 2005). With *cla4*Δ, *pop2*Δ, but not *ccr4*Δ, showed a slight synthetic growth defect already at 30°, whereas at 37° both *ccr4*Δ *cla4*Δ and *pop2*Δ *cla4*Δ mutants showed a synthetic growth defect (Figure 5B). The synthetic growth defects of *ccr4*Δ *cla4*Δ and *pop2*Δ *cla4*Δ were the weakest of all the double mutants analyzed. *CLA4* is a short-tailed transcript and its deadenylation depends on Ccr4-Pop2 (Figure 1). The weaker synthetic interactions between *CCR4/POP2* and *CLA4* compared to the other tested genes, indicates that *CLA4* could be a relevant target of the deadenylase for septin organization.

The *ccr4*Δ *elm1*Δ and the *pop2*Δ *elm1*Δ double mutants also displayed a synthetic growth defect (the strongest growth defect of all double mutants tested) and the defects were again stronger at 37° (Figure 5B). *ccr4*Δ and *pop2*Δ were also synthetic sick with *gin4*Δ and again the slow growth phenotype of the double mutants was more pronounced at 37°.

Mutants in the Ccr4-Pop2-NOT complex have phenotypes consistent with cell wall defects (see CHEN *et al.* 2002) and the septins are also required for wall biogenesis: septins are needed for deposition of chitin in the cell wall (DEMARINI *et al.* 1997) and mutants in the septins and septin assembly regulators have cell wall defects in *S. cerevisiae* and *Candida albicans* (GONZALES-NOVO *et al.* 2006; SCHMIDT *et al.* 2008). Therefore, we wondered if the synthetic slow growth phenotype of cells in which both the Ccr4-Pop2 deadenylase and septin assembly are compromised is due to increased cell wall defects. To address this, we tested whether the synthetic growth defects of the double mutants could be suppressed by addition of the osmostabilizer sorbitol to the media (Figure 5B).

The synthetic growth defects of double *ccr4*Δ *elm1*Δ and *pop2*Δ *elm1*Δ, as well as *ccr4*Δ *gin4*Δ and *pop2*Δ *gin4*Δ could be partially suppressed by sorbitol (Figure 5B). However, the double mutants still grew slower than the single mutants, suggesting the synthetic growth defects are due to cell wall-dependent and -independent functions. This was also true in the case of double *ccr4/pop2* mutants with deletion of the *SHS1* septin: the growth of *ccr4*Δ *shs1*Δ mutants was moderately improved by

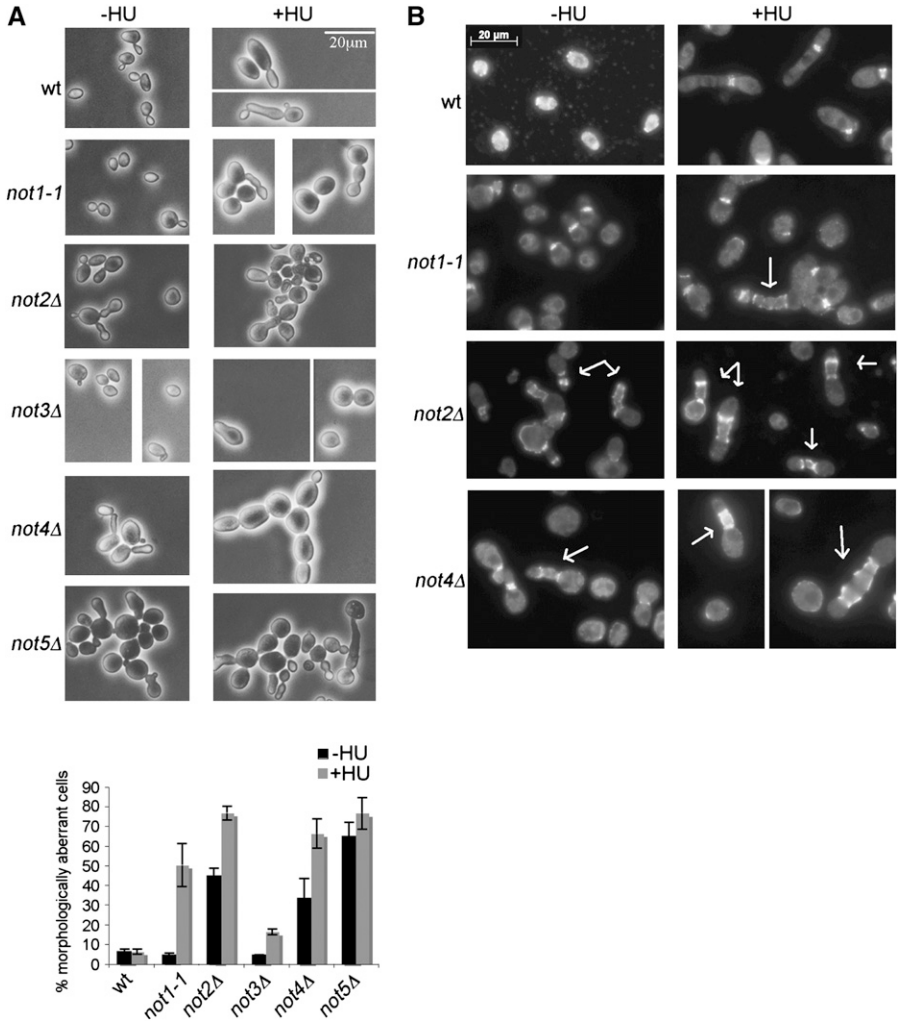

FIGURE 6.—The Not subunits of Ccr4-NOT perform roles in septin assembly and cell morphology. (A) Cells were treated with or without 0.2 M HU for 16 hr and processed for microscopy after fixation in 70% ethanol. The graph shows averages from three independent cultures for each of the strains. The error bar is the standard deviation. (B) Immunofluorescence with the anti-Cdc11 antibody in HU-treated and -untreated strains. Arrows indicate cells that formed ectopic septin rings in the elongated buds.

addition of sorbitol (but not completely rescued), whereas the modest growth defect of double *ccr4-1 shs1*Δ and *pop2*Δ *shs1*Δ was not affected by sorbitol (Figure 5A).

Supression of growth defects by sorbitol was most pronounced for double *ccr4*Δ *cla4*Δ and *pop2*Δ *cla4*Δ mutants, for which sorbitol could restore growth almost completely to levels of single *ccr4*Δ and *pop2*Δ mutants at 37° (Figure 5B). This indicates that the slow growth of the *ccr4*Δ *cla4*Δ and *pop2*Δ *cla4*Δ mutants at 37° is largely due to increased cell wall defects. Of note, the slower growth of *pop2*Δ *cla4*Δ at 30° could not be suppressed by sorbitol, suggesting it is not a consequence of augmented cell wall biogenesis defects.

In conclusion, the synthetic sick genetic interactions between *CCR4/POP2* and *SHS1*, *CDC3*, *CLA4*, *ELM1*, and *GIN4* support a role for Ccr4-Pop2 in septin assembly. Addition of sorbitol can partially suppress the synthetic growth defect of the double mutants, indicating that in part the synthetic genetic interactions are due to the roles of septins and Ccr4-Pop2 in cell wall biogenesis. Other cell wall-unrelated functions of Ccr4-Pop2 in septin organization also contribute to the observed genetic interactions.

The Not subunits of Ccr4-Pop2-NOT also contribute to morphogenesis and septin organization: In addition to the mRNA deadenylase catalytic subunits Ccr4 and Pop2, the Ccr4-Pop2-NOT complex also contains five NOT proteins (Not1–Not5). The Not proteins have overlapping, but also separate functional roles from Ccr4-Pop2 and they are thought to predominantly function in transcriptional control (TUCKER *et al.* 2002; COLLART 2003; TRAVEN *et al.* 2005; LARIBEE *et al.* 2007; MULDER *et al.* 2007). We wanted to address if the NOT subunits of Ccr4-Pop2-NOT were also required for septin organization and cellular morphogenesis.

As shown in Figure 6A, mutations in the *NOT* genes resulted in aberrant cell morphology and the defects were again exacerbated by Swe1 activation in response to HU treatment. The morphological defects in the *not* mutants were similar to the defects of *ccr4* and *pop2* strains and were characterized by the presence of long, often irregularly shaped buds and formation of bud chains. *not2*Δ and particularly *not5*Δ mutants had the strongest morphological defect, consistent with the very slow growth of cells deleted for *NOT2* or *NOT5*.

Immunofluorescence with anti-Cdc11 demonstrated that the *not1-1*, *not2*Δ, and *not4*Δ mutants also mislocalized the septins (Figure 6B; *not5*Δ were very sensitive to zymolyase treatment and were therefore excluded from immunofluorescence analysis of septin organization). Similarly to what we observed in *ccr4* and *pop2* strains, the *not* mutants also displayed formation of additional, bud-localized septin rings in the elongated buds (Figure 6B). Other septin morphology defects in the mutants included diffused or absent staining particularly on cells with wide necks. In conclusion, our data shows that the NOT subunits of Ccr4-Pop2-NOT are also required for septin assembly and establishment of wild-type cell morphology.

DISCUSSION

mRNA deadenylation affects both transcript stability and translation and is therefore a crucial mechanism in the control of gene expression. Because each mRNA deadenylase can in principle regulate a large number of genes, it remains poorly understood which cellular processes are particularly dependent on proper deadenylase activity.

In the present report, we documented short poly(A) tails on all tested mRNAs encoding septins (Shs1) and septin assembly factors (Cdc42, Cdc24, Rga1, Rga2, Bem3, Gin4, Cla4, and Elm1) and we therefore conclude that a short poly(A) tail is very likely a general property of mRNA encoding proteins required for septin assembly. The tails were subject to Ccr4-Pop2-dependent deadenylation, as the tested mRNAs displayed tightly stabilized longer tails in deadenylase mutant strains *ccr4-1* and *pop2*Δ.

In agreement with mRNA deadenylation being important for function of the septin regulators, the Ccr4-Pop2 mRNA deadenylase contributes to septin organization as follows: (i) *ccr4*Δ and *pop2*Δ mutants display morphogenesis defects (*eg.*, bud chains) particularly in response to activation of the kinase Swe1, a phenotype previously reported for mutants with compromised septin structure (Gladfelter *et al.* 2005); (ii) *ccr4*Δ and *pop2*Δ mutants showed synthetic sick genetic interaction with mutations that compromise septin assembly (*shs1*Δ, *cla4*Δ, *elm1*Δ, *gin4*Δ, and a fusion of GFP to Cdc3); and (iii) septin staining demonstrated septin organization defects in the *ccr4*Δ and *pop2*Δ mutant strains, such as formation of ectopic bud-localized septin rings. The ectopic septin rings are thought to form in the presence of an unstable initial ring at the mother-bud neck and therefore their formation is indicative of defects in primary septin ring organization (Gladfelter *et al.* 2005). The exonuclease inactive *ccr4-1* mutant also showed Swe1-dependent morphogenesis defects and aberrant septin organization, demonstrating that the exonuclease activity of Ccr4-Pop2-NOT contributes to proper septin assembly.

Mutants in the alternative yeast mRNA deadenylase Pan2 displayed wild-type septin staining at the mother-bud neck, even after activation of Swe1 by HU. This is in contrast to similar phenotypes for mutants in the two deadenylases for other cellular functions such as the DNA damage response (Hammet *et al.* 2002; Traven *et al.* 2005). A possible explanation for the phenotypic difference in septin organization could lie in the differential effects on mRNA poly(A) tail lengths seen for the two deadenylase mutants. The length of the poly(A) tail on mRNAs for some of the tested transcripts (*eg.*, *GIN4*, *CLA4*, and *ELM1*) was not affected at all by loss of Pan2. Even for the mRNAs that did accumulate some "longer-tailed" forms in *pan2*Δ cells (for example *RGA1*, *RGA2*, *BEM3*, and *SHS1*), the length of the tail was less tightly stabilized than in *ccr4-1* and *pop2*Δ cells, and tails as short as in the wild type still predominated. However, Pan2 could compensate for the absence of Ccr4: the poly(A) tails on the short-tailed septin assembly regulators were longer in *pan2*Δ *ccr4-1* than in *ccr4-1* mutants, and *pan2*Δ *ccr4-1* double mutants displayed bigger and elongated cells (not seen in *pan2*Δ or *ccr4-1* single mutants), which showed no septin staining, but it remains to be determined if this is a cause or a consequence of the cell size/morphology defect.

How does Ccr4-Pop2 contribute to septin assembly? We suggest that post-transcriptional control of the expression of septins and septin assembly factors by Ccr4-Pop2-NOT-dependent deadenylation contributes to proper septin organization. Longer mRNA poly(A) tails on transcripts encoding septin assembly factors likely lead to higher mRNA levels and/or more efficient translation. Alternatively timely expression during the cell cycle might be compromised (Beilharz and Preiss 2007). The process of septin assembly is sensitive to protein levels, for example overexpression of the septins compromises cell morphology and septin organization (Sopko *et al.* 2006; Iwase *et al.* 2007) and therefore higher levels of septins and/or septin assembly factors could lead to septin organization defects.

It remains to be determined how longer mRNA poly(A) tails on septins and septin assembly factors control their expression. Our data suggest that translation rather than mRNA stability is affected, a result consistent with a previous report for the Ccr4 target gene *CRT1* (Woolstencroft *et al.* 2006). We tested protein levels for a few of the septins and septin assembly regulators for which antibodies were available to us (Cdc42 and Cdc11), but did not observe higher levels in *ccr4* and *pop2* mutants, indicating that perhaps these are not the relevant targets. However, the experiments addressing mRNA and protein levels of the septins and septin assembly factors in cultures of *ccr4* and *pop2* mutant are complicated by the fact that only a proportion of the cells in the deadenylase mutant cultures display septin defects (generally around 15–30% in HU-treated samples, see Figure 3). Therefore the change in expression

levels in the affected cells could be buffered by the remaining cells that display normal septin morphology, making it difficult to address the effects of Ccr4-Pop2-NOT. The heterogeneity of deadenylase mutant cultures suggests the presence of alternative or redundant pathways that compensate for absence of Ccr4-Pop2 in regulating the expression of genes that contribute to septin assembly.

Because Ccr4-Pop2-NOT affects a large number of genes, it is likely its effects on septin assembly are complex and, in addition to regulation of expression of septins and septin assembly factors, they include effects on other cellular processes, such as the cell cycle (Westmoreland *et al.* 2004; Traven *et al.* 2005; Woolstencroft *et al.* 2006; Manukyan *et al.* 2008). An obvious connection to test was between Ccr4 and G1 cyclin expression: G1 cyclin activity is required for proper septin ring assembly (Moffat and Andrews 2004; Gladfelter *et al.* 2005) and a recent report demonstrated that timely expression of *CLN1* and *CLN2* in the cell cycle is compromised in *ccr4*Δ mutants, leading to increased cell size (Manukyan *et al.* 2008). However, even though overexpression of Cln2 was enough to suppress the cell size defects of *ccr4*Δ (Manukyan *et al.* 2008), we found that it did not suppress the morphology defects (data not shown), suggesting perturbations in G1 cyclins activity are unlikely to be the reason for the observed septin assembly defects in *ccr4*Δ mutants.

Our data also indicate that, in addition to the mRNA deadenylase catalytic activity, other functions of Ccr4-Pop2-NOT are required for regulation of septin organization and morphogenesis: (i) the *ccr4*Δ mutant showed a more pronounced morphology defect and stronger synthetic interactions with mutations in the septins than the exonuclease-deficient *ccr4-1* strain, even though *ccr4*Δ and *ccr4-1* mutants show no difference in their deadenylation defects for all of the transcripts tested so far (T. H. Beilharz and T. Preiss, unpublished data) and (ii) mutants in the *NOT* genes, which have minor deadenylation defects (Tucker *et al.* 2002), also displayed morphogenesis and septin defects. In particular, the morphology defects seen in *not2*Δ and *not5*Δ mutants were more pronounced than the defects seen in the absence of Ccr4-Pop2 deadenylase activity.

What is the deadenylase-independent function of Ccr4-Pop2-NOT? Ccr4 might have roles in post-transcriptional control that are additional to its catalytic role as an exonuclease; for example, it might facilitate recruitment of factors to target mRNAs by protein–protein interactions. A prime candidate is the translational repressor and decapping activator Dhh1, which makes direct physical contact with Ccr4-Pop2-NOT complex via Not1, Not4, and Caf40 (Tarassov *et al.* 2008). Interestingly, it has been reported that deletion of the *RRP6* and *RRP44* exonuclease subunits of the exosome (a nuclear and cytoplasmic RNA degradation complex) also leads to a stronger phenotype than inactivation of their nuclease activity, possibly because of requirements for complex stability and/or scaffold functions for interactions with cofactors (Schmid and Jensen 2008).

The NOT subunits of Ccr4-Pop2-NOT are thought to predominantly function in transcriptional control, affecting transcription initiation and chromatin modification (Collart 2003; Laribee *et al.* 2007; Mulder *et al.* 2007). Therefore, the morphogenesis and septin defects in the *not* mutants suggest that transcriptional functions of Ccr4-Pop2-NOT also contribute to septin assembly.

The septins perform diverse and essential cellular roles in lower and higher eukaryotes including humans, and thus how their organization and function is controlled is an important issue. We have identified that the Ccr4-Pop2-NOT complex contributes to septin organization in yeast. The mRNA deadenylase subunits Ccr4 and Pop2 contribute to septin organization, as do the NOT subunits, which have additional functions in transcriptional regulation. Future studies will be aimed at identifying the Ccr4-Pop2-NOT-targeted mRNAs relevant for septin organization.

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The Ccr4-Pop2-NOT mRNA Deadenylase Contributes to Septin Organization in *Saccharomyces cerevisiae*

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Expression levels mutant/wt

	CDC42	*CDC24*	*RGA1*	*RGA2*	*BEM3*	*BEM2*	*BNI4*	*CDC3*	*CDC12*	*SHS1*
ccr4Δ	1.1	1.0	0.6	0.9	0.6	0.7	1.7	ND	ND	ND
ccr4–1	1.1	1.1	0.8	1.0	0.9	0.5	2.0	0.7	1.0	1.3
pop2Δ	1.0	1.3	0.7	1.4	1.0	0.8	1.6	1.2	1.0	1.1

FIGURE S1.—The mRNA levels for septin and septin assembly factors are not increased in cells lacking Ccr4-Pop2 activity. Northern blots were performed with probes specific for the indicated genes. Signals were quantified using ImageQuant software and normalized to levels of *ACT1*. ND-not determined