The *Saccharomyces* Homolog of Mammalian RACK1, Cpc2/Asc1p, Is Required for *FLO11*-dependent Adhesive Growth and Dimorphism*[S]

Oliver Valerius‡, Malte Kleinschmidt‡§, Nicole Rachfall, Florian Schulze, Sarai López Marín, Michael Hoppert, Katrin Streckfuss-Bömeke¶, Claudia Fischer‖, and Gerhard H. Braus**

Nutrient starvation results in the interaction of *Saccharomyces cerevisiae* cells with each other and with surfaces. Adhesive growth requires the expression of the *FLO11* gene regulated by the Ras/cAMP/cAMP-dependent protein kinase, the Kss1p/MAPK, and the Gcn4p/general amino acid control pathway, respectively. Proteomics two-dimensional DIGE experiments revealed post-transcriptionally regulated proteins in response to amino acid starvation including the ribosomal protein Cpc2p/Asc1p. This putative translational regulator is highly conserved throughout the eukaryotic kingdom and orthologous to mammalian RACK1. Deletion of *CPC2/ASC1* abolished amino acid starvation-induced adhesive growth and impaired basal expression of *FLO11* and its activation upon starvation in haploid cells. In addition, the diploid Flo11p-dependent pseudohyphal growth during nitrogen limitation was *CPC2/ASC1*-dependent. A more detailed analysis revealed that a *CPC2/ASC1* deletion caused increased sensitivity to cell wall drugs suggesting that the gene is required for general cell wall integrity. Phosphoproteome and Western hybridization data indicate that Cpc2p/Asc1p affected the phosphorylation of the translational initiation factors eIF2α and eIF4A and the ribosome-associated complex RAC. A crucial role of Cpc2p/Asc1p at the ribosomal interface coordinating signal transduction, translation initiation, and transcription factor formation was corroborated. *Molecular & Cellular Proteomics 6: 1968–1979, 2007.*

Cell-cell and cell-surface adherence are required for developmental processes such as haploid invasive growth (1, 2) or diploid pseudohyphae formation of *Saccharomyces cerevisiae* (3, 4). These growth forms are induced by distinct environmental signals. The lack of glucose triggers invasive growth of haploid yeast cells (5). Limitation of utilizable nitrogen compounds in diploid cells induces a switch from the unicellular yeast growth form to polarized filament-like growth (5–8). Both developmental processes, adhesion and dimorphism, depend on the expression of the cell surface adhesin Flo11p (8), which is controlled by various transcription factors as MAP[1] kinase (Kss1p)-regulated Flo8p, Ras/cAMP/PKA-controlled Ste12p/Tec1p (9–12), and Mss1p (13), respectively.

Amino acid starvation is a further signal for adhesive growth of *S. cerevisiae*. In amino acid-starved haploid and diploid cells, adhesion and *FLO11* expression are induced even in the presence of glucose or ammonium, which normally act as inhibitors of adhesive growth (14). The cAMP-dependent PKA pathway, but not the Kss1p-MAP kinase pathway, is required for amino acid starvation-induced adherence. In addition, the transcriptional activator Gcn4p (15) and the amino acid sensor kinase Gcn2p (16) of the general amino acid control pathway are required for this *FLO11* regulation (14). Transcriptional profiling experiments of the adhesion-deficient *S. cerevisiae* S288c (17) in comparison with the adherent Σ1278b background (18) revealed significant differences in the hundreds of Gcn4p-controlled genes. During adhesion, Gcn4p induces additional genes that might be required for haploid invasive growth and pseudohyphal formation upon amino acid limitation. A corresponding comparison of additional adherence-specific post-transcriptional changes in the yeast proteome has not yet been performed because only the proteome of the non-adherent S288c strain has been analyzed in response to amino acid starvation conditions (19).

Uncharged tRNA molecules accumulate within the cell subsequent to amino acid starvation. The sensor kinase Gcn2p includes an N-terminal protein kinase domain and a C-terminal histidyl-tRNA synthetase-related domain, which recognizes these uncharged tRNAs. The activated kinase phosphorylates the α-subunit of the eukaryotic translation factor eIF2

From the Institute of Microbiology and Genetics, Georg August University, D-37077 Göttingen, Germany

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[1] The abbreviations used are: MAP, mitogen-activated protein; PKA, cAMP-dependent protein kinase; RAC, ribosome-associated complex; 2D, two-dimensional; YNB, yeast nitrogen base; 3AT, 3-aminotriazole; YPD, yeast extract-peptone-dextrose; SLAD, synthetic low ammonia dextrose.

TABLE I
S. cerevisiae strains used in this work

Strain	Genotype	Ref.
RH2817	*MATα ura3-52 trp1::hisG*	This work
RH2819	*MATα ura3-52 leu2::hisG*	82
RH2656	*MATa/α ura3-52/ura3-5 trp1::hisG/TRP1*	14
RH2661	*MATa/α ura3-52/ura3-52 flo11Δ::kanR/flo11Δ::kanR trp1::hisG/TRP1*	14
RH2662	*MATa ura3-52 flo11Δ::kanR*	14
RH3220	*MATα ura3-52 sfl1Δ::kanR trp1::hisG*	This work
RH3222	*MATa/α ura3-52/ura3-52 sfl1Δ::kanR/sfl1Δ::kanR trp1::hisG/TRP1*	This work
RH3263	*MATα ura3-52 trp1::hisG leu2::hisG cpc2Δ::LEU2*	This work
RH3264	*MATa/α GCRE6-lacZ::URA3/ura3-52 trp1::hisG/ trp1::hisG leu2::hisG/leu2::hisG cpc2Δ::LEU2/cpc2Δ::LEU*	This work
RH3328	*MATα ura3-52 leu2::hisG cpc2Δ::kanR*	This work
RH2649	*MATa ura3-52 leu2::hisG gcn2Δ::LEU2*	14
RH3329	*MATα ura3-52 trp1::hisG leu2::hisG cpc2Δ::LEU2 gcn2Δ::kanR*	This work

on serine 51 (20–22). The mechanism of amino acid limitation sensing by Gcn2p is highly conserved from yeast to the cortex of the human brain (23). Phosphorylation of eIF2 results in a reduced level of ternary complex, eIF2-GTP-Met-tRNA$_i^{Met}$, within the cell and ultimately in an overall reduced translation efficiency. Only a limited number of mRNAs carrying specific regulatory elements continue to be translated. One of these mRNAs is the *GCN4* mRNA, which carries four short upstream ORFs in its 5′ leader sequence, thus inhibiting translation under non-starvation conditions (24). At a reduced level of ternary complex ribosomes scan past the upstream ORFs and start translating the mRNA from the genuine *GCN4* ORF. In addition to this translational control of *GCN4* expression, the Gcn4p stability in the nucleus at amino acid starvation increases from a half-life of about 5 min to up to 20 min (25–27).

In this study, post-transcriptionally regulated proteins in amino acid-starved adhesive *S. cerevisiae* cells are presented that were found by the comparison of 2D DIGE data with previous transcriptome data. The mRNA of the Cpc2 protein, one representative of this group of proteins, was not more abundant under amino acid starvation suggesting that Cpc2p is a post-transcriptionally regulated protein. *CPC2* encodes a Gβ-like ribosome-associated trypotophan-aspartate repeats-containing (WD) protein (28–30), which is highly conserved and includes as counterparts *cpc-2* of *Neurospora crassa* (31, 32) or human *RACK1* encoding a receptor of activated protein kinase C (33). *CPC2* is transcriptionally co-regulated with other ribosomal protein-encoding genes by the transcription factors Fhl1p and Ifh1p (34). A *CPC2* deletion suppresses the growth deficiency of a *gcn2*Δ yeast mutant under amino acid starvation conditions (28), whereas a mutated allele of *cpc-2* in *N. crassa* coding for a truncated protein results in sensitivity to amino acid starvation (31, 32).

Here we describe *CPC2* as a gene required for *FLO11* expression, adhesive growth, dimorphism, and cell wall integrity. Cpc2 protein levels were increased during amino acid starvation, and the presence of Cpc2p was required for a decreased phosphorylation status of translation initiation factors at non-starvation conditions. The absence of Cpc2p in a *cpc2*Δ strain resulted in increased amounts of phosphoprotein of these translational regulators.

TABLE II
Plasmids used in this work

Plasmid	Description	Ref.
pHVX2-MUC1	^{prom}*PGK1-FLO11-*^{term}*PGK1*	56
pME2071	2.5-kb *GAL1*(p)::*TEC1* fusion in pRS316	11
pRS316	*URA3*-marked centromere vector	83
pRS314	*TRP1*-marked centromere vector	83

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Yeast strains are derivatives of *S. cerevisiae* Σ1278b (Table I). Plasmids used is this work are listed in Table II. Deletion of *CPC2* was achieved by using the *cpc2* deletion plasmid pRSBH1-14 (28) or the Euroscarf *cpc2-kanR* deletion cassette. Transformations were carried out using the lithium acetate method (35). For non-starvation conditions, strains were cultivated in liquid synthetic minimal medium (YNB) containing respective supplements at 30 °C overnight, diluted, and cultivated to midlog phase before isolation of protein extracts or total RNAs. For amino acid starvation conditions, 10 mM histidine analog 3-aminotriazole (3AT) was added to yeast cultures grown to midlog phase, and cells were incubated for 1, 6, or 10 h at 30 °C, respectively. For adhesive growth tests strains were grown on solid (2% agar) YNB medium containing respective supplements and 10 mM 3AT to induce amino acid starvation. For haploid invasive growth tests cells were grown on solid (2% agar) yeast extract-peptone-dextrose (YPD) medium or on solid (2% agar) synthetic minimal medium (YNB) containing 2% glucose or 2% galactose. Qualitative pseudohyphal growth was assayed on SLAD plates (3). Cell wall integrity was tested by supplementation of the indicated amounts of calcofluor white (Fluorescent Brightener 28, Sigma) to solid YPD medium.

2D DIGE Analysis and 2D Phosphoproteome—Two serial 10-ml YNB precultures, the first incubated overnight and the second incubated for 2 h, were used to inoculate 100 ml of YNB medium. These main cultures were grown to an A_{600} of ~0.8 before harvesting cells. Amino acid starvation was induced at an A_{600} of 0.3 by adding 3AT to a final concentration of 10 mM. Cells were disrupted with Y-PER®-S reagent (Pierce). Protein concentrations were determined with the Pierce BCA kit according to the supplier's manual. Recommended amounts of cell extracts were purified by methanol-chloroform extraction (36) and differentially labeled with the fluorescent dyes Cy2, Cy3, or Cy5 according to the supplier's protocol (GE Healthcare,

product number 25-8010-65). The "minimal" labeling technique was used, linking the CyDye DIGE fluors covalently to the ε amino group of lysine of proteins via an amide linkage. The protein standard, which was labeled with Cy2, consists of a protein pool of equal aliquots of each extract used for the analysis. This Cy2-labeled protein standard and the extracts of cultures with or without amino acid starvation, labeled with Cy3 or Cy5, respectively, were combined for IEF and PAGE. The differentially labeled proteins of the 2D gels were serially imaged by means of laser scanning with a Typhoon scanner (GE Healthcare). Images were normalized against the protein standard and quantitatively analyzed by using the DeCyder™ software from GE Healthcare (37). Student's *t* test was performed with the DeCyder BVA (biological variation analysis) module to validate the significance of the detected differences between spot intensities of the starved (1, 6, or 10 h) and non-starved cells (*t* test *p* values required for spot consideration were set at $\leq$0.001 for 10-h starvation *versus* non-starvation).

The 2D phosphoproteome of methanol-chloroform-extracted (36) cell lysates was analyzed in triplicate (independent cultures) according to the protocol of Ray and Haystead (38). The resulting autoradiographs were quantified with the imaging software PDQuest™ (Bio-Rad).

LC-MS/MS Protein Identification—Excised polyacrylamide gel pieces of stained protein bands were digested with trypsin according to Shevchenko *et al.* (39). Tryptic peptides extracted from each gel slice were injected onto a reversed-phase liquid chromatographic column (Dionex NAN75-15-03-C18 PM) by using the *ultimate* HPLC system (Dionex, Amsterdam, Netherlands) to further reduce sample complexity prior to mass analyses with an LCQ DecaXP mass spectrometer (Thermo Electron Corp., San Jose, CA) equipped with a nanoelectrospray ion source. Cycles of MS spectra with *m/z* ratios of peptides and four data-dependent MS2 spectra were recorded by mass spectrometry. The "peak list" was created with extractms provided by the Xcalibur software package (BioworksBrowser 3.1). The MS2 spectra with a total ion current higher than 10,000 were used to search for matches against a yeast genome protein sequence database from the National Center for Biotechnology Information (NCBI) *Saccharomyces* Genome Database (Stanford, CA, 6882 sequences, March 2005, plus 180 sequences of the most commonly appearing contaminants, *e.g.* keratins and proteases, provided with the BioworksBrowser package) using the TurboSEQUEST algorithm (40) of the Bioworks software (Version 3.1, Thermo Electron Corp). The search parameters included based on the TurboSEQUEST algorithm were: (i) precursor ion mass tolerance less than 1.4 amu, (ii) fragment ion mass tolerance less than 1.0 amu, (iii) up to three missed tryptic cleavages allowed, and (iv) fixed cysteine modification by carboxyamidomethylation (plus 57.05 amu) and variable modification by methionine oxidation (plus 15.99 amu) and phosphorylation of serine, threonine, or tyrosine (plus 79.97 amu). In accordance with the criteria described by Link *et al.* (41) matched peptide sequences of identified proteins had to pass the following: (i) the cross-correlation scores (Xcorr) of matches must be greater than 2.0, 2.5, and 3.0 for peptide ions of charge state 1, 2, and 3, respectively; (ii) ΔCn values of the best peptide matches must be at least 0.4; and (iii) the primary scores (Sp) must be at least 600. Protein identification required at least two different peptides matching these criteria. The degree of completeness of the b- and y-ion series for each TurboSEQUEST result was manually checked for every protein identified. Peptides of identified proteins were individually blasted against the *Saccharomyces* Genome Database (BLASTP at seq.yeastgenome.org/cgi-bin/blast-sgd.pl against the dataset *Protein Encoding Genes*) to ensure their unambiguous assignment to the TurboSEQUEST-specified protein. Only in the case of the identification of eIF4A, encoded by the two identical ORFs *TIF1* and *TIF2*, was discrimination between Tif1p (gi|6322912|) and Tif2p (gi|6322323|) not feasible. See also Supplemental Tables 1 and 2 and Supplemental Fig. 1 for detailed TurboSEQUEST identifications and phosphorylation site determination.

Adhesive Growth Assays and Microscopy—Amino acid starvation-induced adhesive growth tests on solid YNB medium were performed as described previously (14). Strains were pregrown on solid YNB medium containing respective supplements for 20 h. Cells were patched on fresh YNB containing supplements and 10 mM 3AT to induce amino acid starvation. After incubation for 1 day at 30 °C, plates were photographed and then carefully washed under a stream of water. The plates were photographed again to document adhesive growing cells. Haploid invasive growth was assayed as described previously (1). After 5 days of growth on solid YPD medium or solid YNB medium containing 2% galactose, cells were washed by water and photographed to document haploid invasive cells. For qualitative diploid pseudohyphal development assays cells were grown on solid SLAD medium for 5 days. Pseudohyphal colonies were viewed with an Axiovert microscope (Carl Zeiss, Jena, Germany) and photographed using a Xillix microimager digital camera with the Improvision Openlab software (Improvision, Coventry, UK). For electron microscopy chemical fixation, dehydration and resin embedding was performed as described by Spurr (42). Poststaining of ultrathin sections was performed with uranyl acetate or lead citrate according to Venable and Coggeshall (43) and Hoppert and Holzenburg (44).

Zymolyase Assay—Sensitivity of yeast cells to Zymolyase 100T was assayed by incubating 1 ml of a cell suspension (a 50-μl aliquot of cell suspension in 1 ml water giving an A_{600} of $\sim$2.4) with 100 μl of 10 mg/ml Zymolyase 100T. Subsequent transfer of 50-μl aliquots to 1 ml of 1% SDS in a time course of 24 min and following measurement of the A_{600} (caused by increasing cell lysis) gave the relative extent of cell wall digestion of different strains.

Northern Analysis—Total RNAs from yeast were isolated following the protocol described by Cross and Tinkelenberg (45). RNAs were separated on a 1.4% agarose gel containing 3% formaldehyde and transferred onto nylon membranes by capillary blotting. Gene-specific probes were ^{32}P-radiolabeled with the MBI Fermentas HexaLabel™ DNA labeling kit. Hybridizing signals were quantified using a BAS-1500 phosphorimaging scanner (Fuji, Tokyo, Japan).

Western Hybridization Analysis—Cell extracts were taken up in SDS loading dye and heated at 65 °C for 15 min. Samples were subjected to one-dimensional PAGE or 2D PAGE followed by transfer to nitrocellulose membranes. eIF2, eIF2α-P, and Cpc2p were detected using ECL technology (Amersham Biosciences). Primary antibodies used were polyclonal rabbit anti-eIF2 (21) or anti-eIF2α-P (BIOSOURCE, Nivelles, Belgium) and anti-Cpc2p (30). Peroxidase-coupled goat anti-rabbit IgG was used as secondary antibody (Dianova, Hamburg, Germany).

β-Galactosidase Assay—Assays were performed with extracts grown in liquid medium. Specific β-galactosidase activities were normalized to the total protein (46) in each extract and equalized ($A_{415} \times 1.7$)/(0.0045 $\times$ protein concentration $\times$ extract volume $\times$ time) (47). Assays were performed for at least three independent cultures.

RESULTS

2D DIGE Reveals Seven Highly Up-regulated Protein Spots of Adherent Yeasts during Amino Acid Starvation—2D DIGE experiments of amino acid-starved yeast cells were performed to identify protein spots significantly increased during adhesive growth. The experimental setup is illustrated in Fig. 1*A*. Amino acid starvation was induced by the addition of the histidine analog 3AT to the cultures. The diploid wild type strain (RH2656) was incubated in the absence or in the presence of 10 mM 3AT for various time periods. Protein extracts

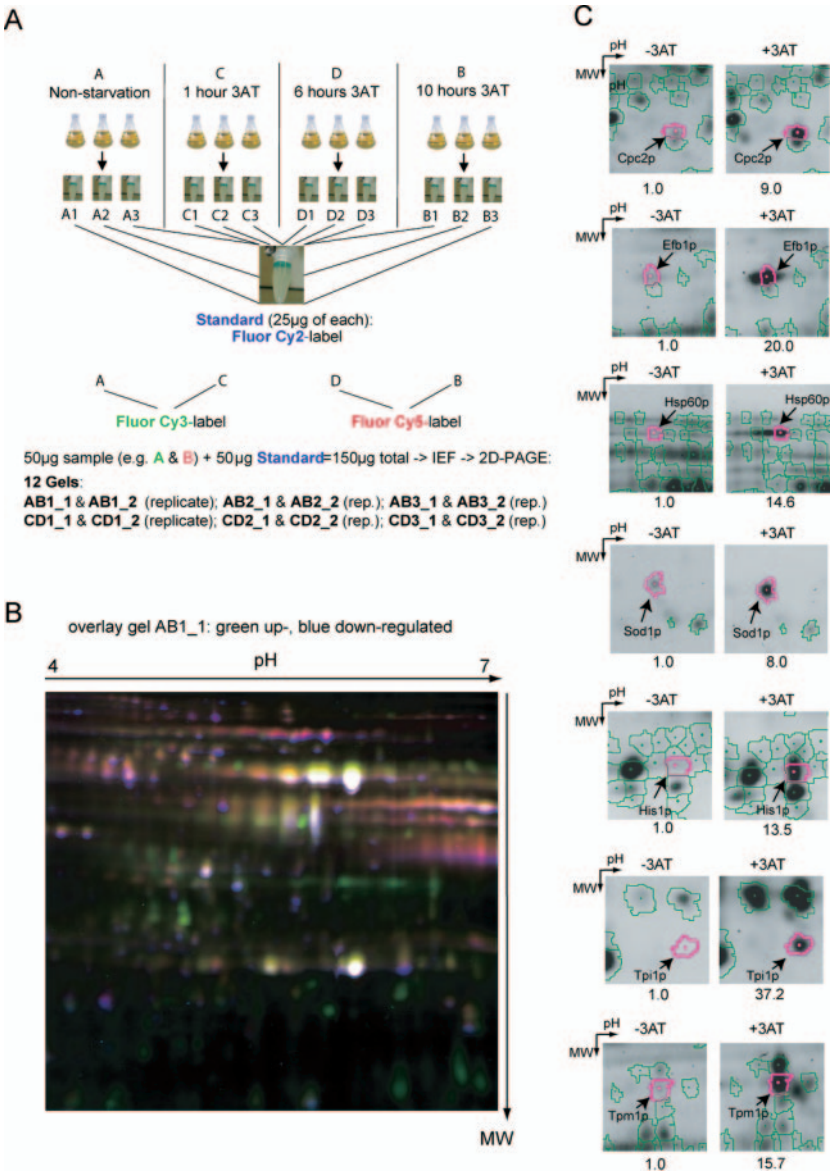

FIG. 1. **2D DIGE analysis of *S. cerevisiae* cells starving for amino acids.** *A*, experimental setup. Strain RH2656 (Σ1278b) was cultivated in YNB medium in the absence or presence of the histidine analog 3AT for 1, 6, or 10 h at 30 °C. Three independent cultures of each time point were used for protein extraction. The extract for the internal standard consists of aliquots of each culture extract and was labeled with Cy2; *extracts A* and *C* were labeled with Cy3, and *extracts D* and *B* were labeled with Cy5. Duplicates of three AB gel pairs and three CD gel pairs together with the standard resulted in 12 independent gels, each consisting of a Cy2/Cy3/Cy5 triplet. *B*, Cy3-Cy5 color overlay of one exemplary gel triplet, *AB1_1*, normalized against Cy2. *C*, protein spot pairs with at least 8-fold increased intensity at amino acid starvation. Indicated proteins were identified by nano-LC-MS/MS2.

of cells of the exponential growth phase were isolated and labeled with the fluorescent dyes Cy3 or Cy5. To normalize the various 2D DIGEs, an internal standard pool consisting of equal aliquots of all individual extracts was labeled with Cy2. This resulted in two groups of 2D DIGE, namely 0 *versus* 10 h (AB gels) and 1 *versus* 6 h (CD gels) with all gels also comprising the same amount of Cy2-standard extract. Fig. 1*B* shows an example of an overlay of one gel triplet. After group analysis with the DeCyder imaging software (GE Healthcare), protein spots that displayed at least an 8-fold increase and a statistical *t* test significance of at least 0.001 (10 h) were defined as highly derepressed or activated. Protein spots appearing in less than 27 of all 33 valid gel images were excluded (six physical AB and five physical CD gels; replicate gel CD2_2 was excluded from the analysis because of poor quality, resulting in 33 valid gel images). Seven protein spots matched these criteria and are illustrated in Fig. 1*C* and specified in Table III (for mass spectrometry data see Supplemental Table 1).

Two protein spots that were up-regulated include translation factors. The protein Cpc2p is a ribosomal Gβ-like tryptophan-aspartate repeats-containing (WD) protein assumed to be involved in translational regulation (30). The protein spot was 9-fold up-regulated after 10 h of amino acid starvation. In contrast, microarray experiments (18), which were confirmed by Northern hybridizations (Fig. 2*B*), did not reveal any transcriptional induction for *CPC2* under amino acid starvation. Efb1p is part of the translation elongation factor eEF1. Efb1p is the GDP-GTP exchange factor that regenerates eEF1-α-GTP for subsequent elongation cycles. The protein spot showed a 20-fold intensity increase after 10 h of amino acid starvation. Again no transcriptional regulation of the *EFB1*

TABLE III

Highly up-regulated protein spots after 3AT treatment in S. cerevisiae

DeCyder spot numbers are as follows: His1p, 402; Tpm1p, 424; Sod1p, 558; Hsp60p, 69; Efb1p, 435; Tpi1p, 550; Cpc2p, 443. For mass spectrometry data see Supplemental Table 1.

Protein	Factor (*t* test)			Number of spot appearances (total number of gels)
	1-h amino acid starvation	6-h amino acid starvation	10-h amino acid starvation	
His1p	2.3 (0.0046)	1.7 (0.0049)	13.5 (0.00019)	33 (33)
Tpm1p[a]	3.4 (0.054)	3.6 (0.015)	15.7 (2.9*e*−006)	33 (33)
Sod1p	1.5 (0.3)	2.0 (0.014)	8.0 (0.00013)	27 (33)
Hsp60p[a]	3.4 (0.15)	2.3 (0.16)	14.6 (6.1*e*−005)	30 (33)
Efb1p[a]	3.0 (0.097)	3.1 (0.026)	20.0 (0.0011)	33 (33)
Tpi1p[a]	3.5 (0.44)	3.6 (0.17)	37.2 (5.4*e*−005)	30 (33)
Cpc2p[a]	4.7 (0.011)	4.9 (0.006)	9.0 (8.3*e*−005)	33 (33)

[a] Proteins not transcriptionally up-regulated according to Refs. 17 and 18.

mRNA has been detected at the transcriptional profiling of yeast cells under amino acid starvation (18).

In addition, specific protein spots identified as the chaperone Hsp60p and the oxidative stress response protein Sod1p were up-regulated after 10 h by a factor of 14 and 8, respectively. The conserved heat shock factor Hsp60p is an essential tetrameric mitochondrial chaperone required for the folding of precursor polypeptides and complex assembly (48, 49). The Sod1p superoxide dismutase protects yeast cells against reactive oxygen species. The transcription profile of *HSP60* was reduced to half, and transcription of *SOD1* was only weakly induced by a factor of 1.4 (18).

His1p and Tpi1p are metabolic proteins exhibiting protein spots of increased intensities. A His1p spot was increased by a factor of 13.5 after 10 h of amino acid starvation. Transcription of the *HIS1* gene in non-adherent S288c cells had been determined to increase ~5-fold (17) compared with only 1.6-fold in Σ1278b cells (18). Tpi1p is a triose-phosphate isomerase that catalyzes the aldose-ketose isomerization of two triose phosphates from the glycolytic pathway (50). A Tpi1p spot was 37-fold induced, although no transcriptional regulation has been reported for the *TPI1* gene upon amino acid starvation.

A protein spot identified as Tpm1p showed an increased intensity by a factor of 15. Tropomyosin isoform 1, Tpm1p, is the major isoform of tropomyosin, an actin-binding protein that stabilizes actin filaments (51). However, the amount of *TPM1* mRNA has been determined to undergo a 3-fold reduction under conditions of amino acid starvation (18).

In summary, these data suggest that six of the seven most up-regulated protein spots in adherent yeast cells upon amino acid starvation are not due to transcriptional activation of their encoding genes. Because modified versions and isoforms of one protein might appear at different positions in 2D gels, the increased spot intensities might either reflect an increase of the total amount of a given protein (*e.g.* by translational control) or an increase of only a modified version of the protein (*e.g.* by increased phosphorylation or acetylation). Although this can only be discriminated by further experimentation, for some of the presented candidates, as *e.g.* Tpi1p, it has been shown earlier that a phosphorylated version and an unphosphorylated version migrate to different positions within the 2D gel (52). For this protein the detected increase might therefore rather be due to protein modification than to total protein abundance at amino acid starvation conditions. Some of the post-transcriptionally regulated proteins found by the 2D DIGE might rather fall in the category of a general stress response than in the specific amino acid starvation response. This might be in analogy to the stereotypical environmental stress response (53) or the common environmental response (54), both acting on the level of gene transcription upon different nutritional limitations and a variety of environmental changes.

We focused in the following experiments on the role of Cpc2p at amino acid starvation. A 2D Western hybridization using an anti-Cpc2p antibody was performed to clarify which of the possibilities mentioned above apply to this protein. The result showed that the total protein abundance of Cpc2p was increased (almost 8-fold) upon 2 h of amino acid starvation (Fig. 2*A*).

FLO11-dependent Adhesive Growth and Filament-like Pseudohyphal Development Are Cpc2/Asc1p-dependent—The *CPC2* gene has previously been related to the general control system of amino acid biosyntheses because its additional deletion in a *gcn2*Δ strain background suppresses the growth defect at amino acid starvation conditions caused by the *GCN2* deletion (28). Because the key regulators of the general control system of amino acid biosyntheses, Gcn2p and Gcn4p, also control adhesive growth upon amino acid starvation (14), the impact of depleted Cpc2p on adhesive growth and pseudohyphal development was further investigated. The haploid *cpc2*Δ deletion strain and the diploid *cpc2*Δ *cpc2*Δ deletion strain were compared with wild type and *flo11*Δ and *sfl1*Δ deletion strains, respectively, to investigate adhesive growth and pseudohyphae formation. The *flo11*Δ control is constitutively non-adhesive, whereas the *sfl1*Δ control results in a hyperadhesive phenotype because it encodes a repressor of *FLO11* (6). In contrast to *sfl1*Δ, all other tested strains were non-adhesive under non-starvation conditions as expected (Fig. 3*A*, *YNB 1d*). Growth at amino acid starvation for 1 day induced adhesive growth of the *CPC2* wild type strain. However, similar to the *flo11*Δ negative

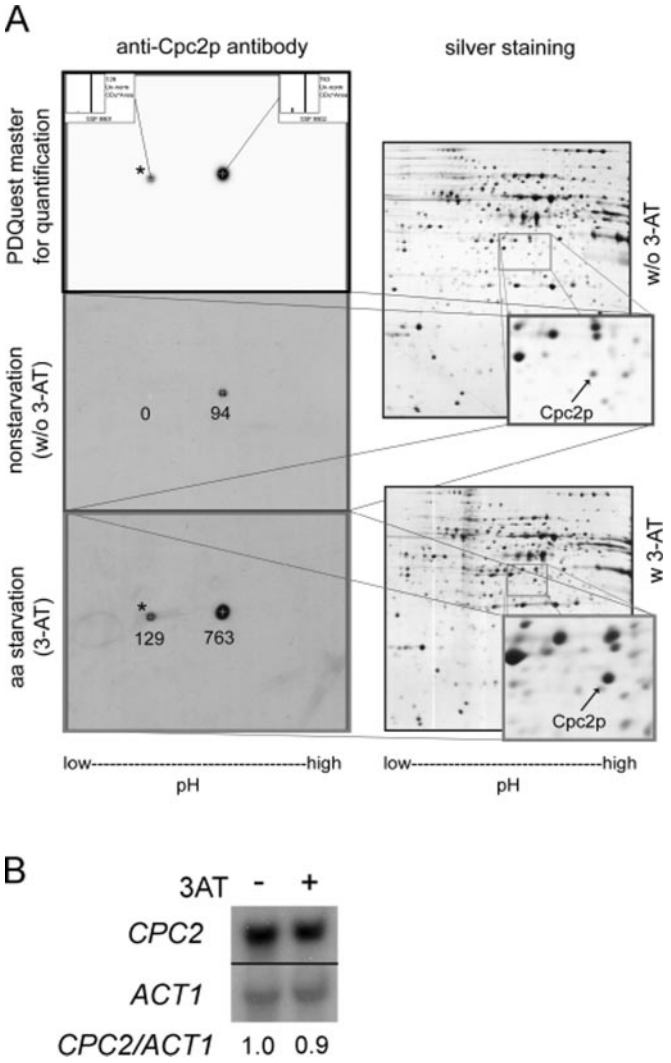

FIG. 2. **CPC2/Cpc2p expression in response to amino acid starvation.** *A*, immunodetection and quantification of the Cpc2 protein after 2D separation using an anti-Cpc2p antibody. The gels on the *right* were stained with silver and serve as a loading control. 2D separation and gel loading for the following membrane blotting and immunodetection were identical. Quantification was performed with the imaging software PDQuest (Bio-Rad). The *numbers* given are spot intensities after background subtraction. The spot marked with an *asterisk* (3AT) was not exclusively found for starvation but also appeared in replicates of non-starved cells. It might reflect carbamylation of Cpc2p during sample preparation (81); phosphatase treatment did not remove this additional spot (not shown). *B*, Northern hybridizations against *CPC2* mRNA quantified and normalized against *ACT1* mRNA levels as internal standard. *w 3AT*, with 3AT; *w/o 3AT*, without 3AT; *aa*, amino acid.

control, the haploid *cpc2*Δ deletion strain did not grow adhesively under amino acid limitation (Fig. 3*A*, *YNB 3AT 1d*). Loss of adhesive growth at amino acid starvation was also observed for a diploid *cpc2*Δ *cpc2*Δ strain (data not shown) and further supports that the deletion of the *CPC2* gene results in an adhesion-deficient phenotype of amino acid-starved *S. cerevisiae* cells. Northern hybridization experiments and

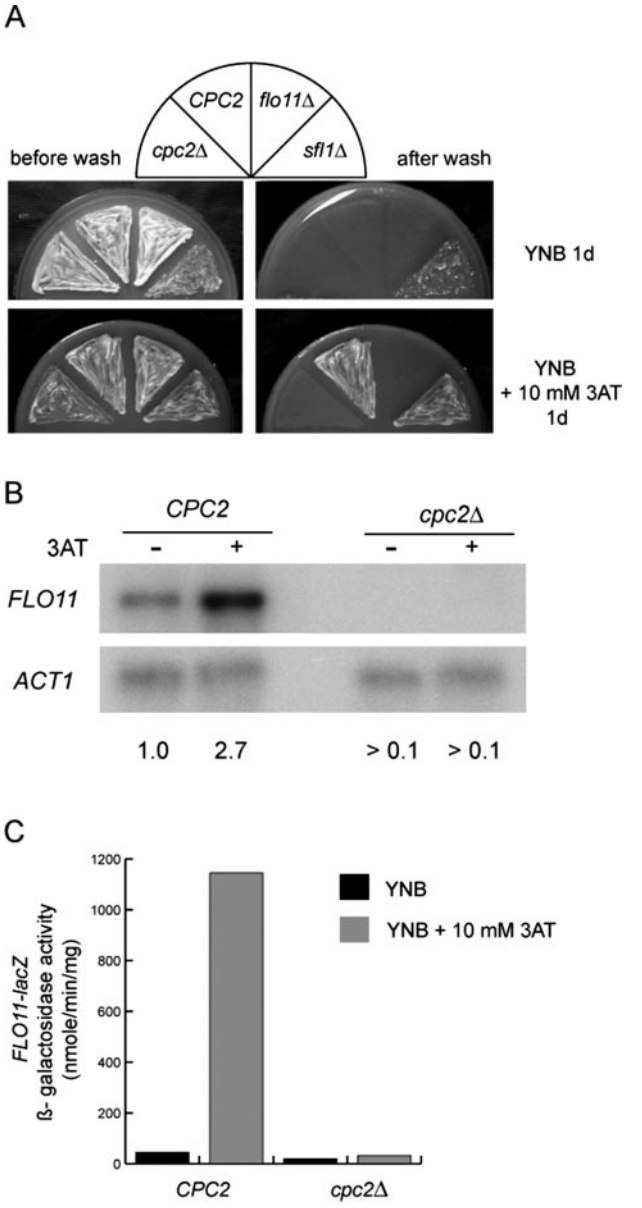

FIG. 3. **Amino acid starvation-induced adhesive growth and *FLO11* expression are *CPC2*-dependent.** *A*, the haploid Σ1278b yeast strains RH2817 (*CPC2*), RH3220 (*sfl1*Δ), RH2662 (*flo11*Δ), and RH3263 (*cpc2*Δ) were patched on solid YNB medium (+ uracil and Trp) and on identical medium supplemented with 10 mM 3AT, respectively. After 1 day of incubation at 30 °C, plates were photographed prior (total growth) and after washing (adhesive growth) with a stream of water. Non-adhesive growing cells were washed off the agar surface. *B*, 20 μg of total RNA of the haploid yeast strains RH2817 (*CPC2*) and RH3263 (*cpc2*Δ) cultivated in liquid YNB medium (+ uracil and Trp) in the absence (−) or presence (+) of 10 mM 3AT were used for Northern analysis. *Numbers* indicate transcript levels of *FLO11* relative to the internal standard *ACT1*. *C*, the same yeast strains each carrying a *FLO11-lacZ* reporter were grown to logarithmic phase in YNB (+Trp) in the absence (*black bars*) or in the presence (*gray bars*) of 10 mM 3AT before specific β-galactosidase activities were assayed. Units of specific β-galactosidase activities are shown in nanomoles per minute per milligram. *Bars* depict means of at least three independent measurements with a standard deviation not exceeding 20%. *d*, day(s).

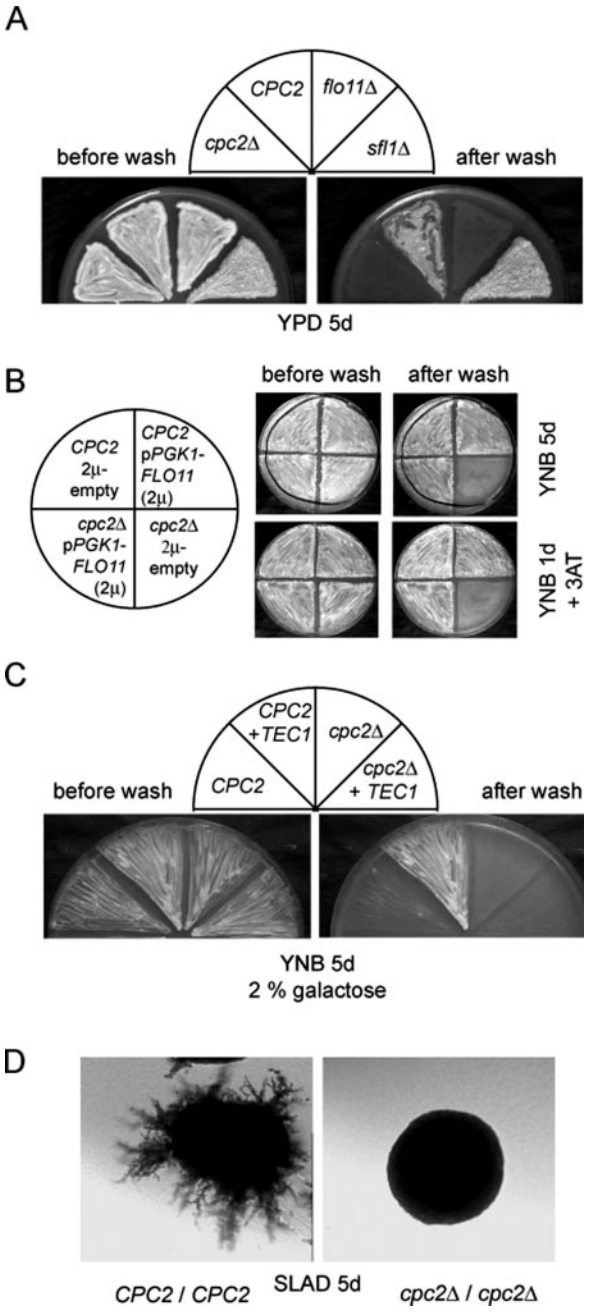

FIG. 4. **Glucose starvation-induced haploid invasive growth and nitrogen starvation-induced diploid pseudohyphal development are *CPC2*-dependent.** *A*, the haploid Σ1278b strains RH2817 (*CPC2*), RH3220 (*sfl1*Δ), RH2662 (*flo11*Δ), and RH3263 (*cpc2*Δ) were patched on solid YPD medium for 5 days at 30 °C. Cells were photographed before (total growth) and after washing (invasive growth) with water. Non-invasive growing yeast cells were washed off the agar surface. *B*, strains RH2819 (*CPC2*) and RH3328 (*cpc2*Δ) were transformed with plasmid pHVX2-*MUC1* (56) carrying a ^{prom}*PGK1-FLO11* allele (Table II), and invasiveness was tested after 1 day of growth. The identical strains with an empty vector served as negative controls. *C*, the haploid strains RH2817 (*CPC2*) and RH3263 (*cpc2*Δ) were transformed with plasmids pME2071 (^{prom}*GAL1-TEC1*) and empty pRS316 (Table II), respectively. Strains were cultivated on solid YNB (+Trp) medium with 2% galactose for 5 days at 30 °C. Plates were assayed for total and invasive growth. *D*, the yeast strains RH2656 (*CPC2*/*CPC2*) and RH3264 (*cpc2*Δ/*cpc2*Δ), with plasmids pRS316 and pRS314 to complement uracil or tryptophan deficiency, were cultivated on nitrogen starvation plates (SLAD) for 5 days at 30 °C to stimulate diploid pseudohyphal development. *d*, day(s).

measurements of ^{prom}*flo11-lacZ*-encoded β-galactosidase activities (plasmid B3782 2 μm (12)) demonstrated that *FLO11* expression was abolished in *cpc2*Δ cells under all tested conditions (Fig. 3, *B* and *C*).

It was then examined whether Cpc2p is specifically required for amino acid induced adhesive growth or whether the absence of Cpc2p also prevents adhesion upon glucose limitation, another signal leading to *FLO11* activation and adhesive growth (8, 11, 12, 55). Consumption of glucose by extended growth on solid medium resulted for the *cpc2*Δ strain in a non-adhesive behavior similar to that of the *flo11*Δ negative control (Fig. 4*A*). Therefore, the Cpc2 protein seems to be essential for the expression of Flo11p independently of the input signal triggering *FLO11* expression and subsequent adhesion.

To test whether the Cpc2p requirement for *FLO11* expression was dependent on the promoter region, a *FLO11* allele with its ORF fused to the *PGK1* promoter (56) was analyzed in the *cpc2*Δ strain. The expression of this recombinant *FLO11* allele restored adhesiveness (Fig. 4*B*). The transcription factor Tec1p acts in complex with Ste12p on the *FLO11* promoter and is activated by the Kss1p/mitogen-activated protein kinase (MAPK) signaling pathway (8, 11, 55). Overexpression of Tec1p induced adhesive growth in wild type but not in *cpc2*Δ cells (Fig. 4*C*) and therefore cannot restore the *FLO11* expression defect of *cpc2*Δ cells. In addition, the overexpression of Tpk2p, the kinase subunit of PKA that phosphorylates and activates the transcription factor Flo8p (57), from a high copy number plasmid did not restore adhesive growth (not shown). The restored adhesiveness by the exchange of the *FLO11* promoter by the *PGK1* promoter suggests that the *FLO11* upstream region specifically requires Cpc2p for efficient expression.

Filament-like pseudohyphae formation is another *FLO11*-dependent growth mode induced upon nitrogen limitation in diploid cells. In contrast to wild type cells, the diploid *cpc2*Δ *cpc2*Δ cells were unable to develop any filament-like structures. They remained in a smooth-border round colony even at nitrogen limitation (Fig. 4D).

Taken together, the deletion of the *CPC2* gene resulted in abolishment of *FLO11* expression and as a consequence impaired *FLO11*-dependent morphological developments of *S. cerevisiae*. This suggests that Cpc2p is essential for *FLO11* expression.

Deletion of CPC2/ASC1 Results in Increased Cell Size and Increased Sensitivity to Zymolyase and Calcofluor White—A comparison of the cells by microscopic analysis revealed an increased cell size for the *cpc2*Δ deletion strain in comparison with the wild type strain (Fig. 5*A*). At non-starvation the largest

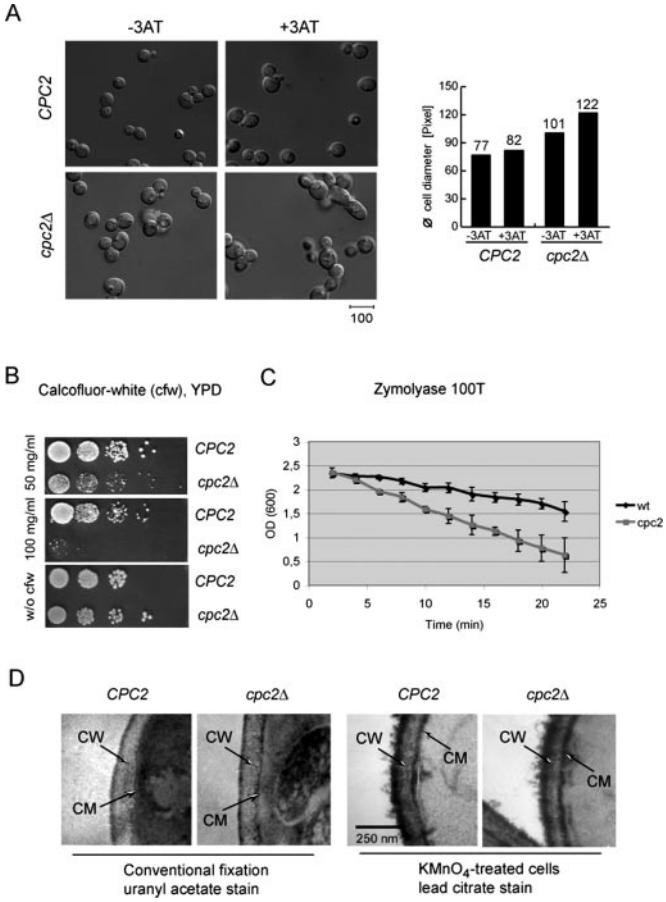

FIG. 5. **Yeast cell wall integrity and cell size are regulated by *CPC2*.** Cell cultures were grown to an A_{600} of 0.8. *A*, cell diameter comparison of haploid strains RH2817 (*wt*) and RH3263 (*cpc2*Δ) by light microscopy. The cell cultures were grown in the presence or absence of 3AT. *B*, sensitivity of wild type (RH2817) and *cpc2*Δ (RH3263) strains to the cell wall drug calcofluor white (*cfw*) was assayed by growth on solid medium supplemented with calcofluor white at two different concentrations and in its absence (*w/o cfw*). *C*, glucanase sensitivity of the same strains recorded as A_{600} of cells in 1% SDS after incubation with Zymolyase 100T. *Error bars* represent the 2-fold standard deviation from mean values calculated from 9 individual measurements from three independent cultures. *D*, electron micrographs of thin sections of wild type and *cpc2*Δ cells. The images show the cell periphery with cell wall (*CW*) and cytoplasmic membrane (*CM*) after uranyl acetate staining of sections and permanganate-treated cells after subsequent lead citrate staining of sections as indicated.

cells of the *cpc2*Δ strain showed an ~2.5-fold increase in volume in comparison with the largest wild type cells. This size effect was even more pronounced when cells were cultured at amino acid starvation, which led to *cpc2*Δ cells of about 3.3-fold the volume of wild type cells.

The glycosylphosphatidylinositol-anchored Flo11 protein is one of many cell wall proteins interacting with the extracellular environment. RACK1, which is the mammalian counterpart of yeast Cpc2p, was identified as receptor of activated C kinase, Pkc1p (33). Pkc1p is the protein kinase C of *S. cerevisiae* and is located upstream of the yeast cell wall integrity MAP kinase pathway. Direct interaction of Cpc2p and Pkc1p in *S. cerevisiae* has not yet been described. However, phosphorylation of Slt2p, which is the MAP kinase of this pathway, is increased in a *cpc2*Δ background (58). Therefore a *cpc2*Δ strain was analyzed for defects in cell wall integrity. The deletion of *CPC2* resulted in significantly increased sensitivity to the chitin-binding cell wall drug calcofluor white. Similarly treatment with the glucanase Zymolyase 100T indicated cell wall changes beyond the presence or absence of the adhesin Flo11p (Fig. 5, *B* and *C*). A close inspection of the cell wall by electron microscopy did not reveal obvious differences between wild type and *cpc2*Δ cells in size and morphology suggesting that the Cpc2p-caused changes are subtle (Fig. 5*D*).

The conclusion is that Cpc2p is essential for the cell wall-mediated adhesive growth on surfaces as a player with a more general role in the maintenance of the cell wall integrity. The change in cell size observed in the *cpc2*Δ mutant strain might be the consequence of an impaired cell wall integrity control.

Deletion of Cpc2p Increases the Phosphorylation of Elongation Initiation Factors—Amino acid starvation resulted in phosphorylation of the translation initiation factor eIF2α in both wild type and *cpc2*Δ cells (Fig. 6*A*). Surprisingly even in the absence of amino acid starvation, *cpc2*Δ cells showed elevated eIF2α phosphorylation. The eIF2α phosphorylation was abolished in the *gcn2*Δ background in *cpc2*Δ and wild type cells demonstrating that eIF2 phosphorylation is Gcn2p-dependent (Fig. 6*A*). This hints to a Cpc2p function as part of the mechanism preventing eIF2α phosphorylation in wild type cells during non-starvation conditions.

Because the phosphorylation status of the MAP kinase Slt2p has also been described to increase in the absence of Cpc2p (42), Cpc2p might affect the phosphorylation of additional proteins besides Slt2p and eIF2. Thereby Cpc2p might affect several kinases because the mammalian homolog RACK1 was not only shown to function as receptor of various isoforms of activated kinase C (33, 59, 60) but also of the Src kinase (61). 2D phosphoproteome analyses were performed to find further putative phosphoproteins under the control of Cpc2p. Cultures of wild type and *cpc2*Δ cells were precultivated in minimal YNB medium and then shifted for metabolic labeling and further growth to a low phosphate YNB medium supplemented with [^{32}P]phosphoric acid. Chloroform-methanol-purified cell extracts were separated by two-dimensional gel electrophoresis. Proteins were silver-stained, and phosphate incorporation was visualized by autoradiography.

Analysis of the phosphoprotein spots by the PDQuest (Bio-Rad) imaging analysis software tools revealed more than 100 phosphoproteins. Several of these phosphoproteins showed different spot intensities in wild type and *cpc2*Δ cells (Fig. 6*B*, *circled spots*). Two of the regulated phosphoprotein spots are presented here (for mass spectrometry data see Supplemental Table 2).

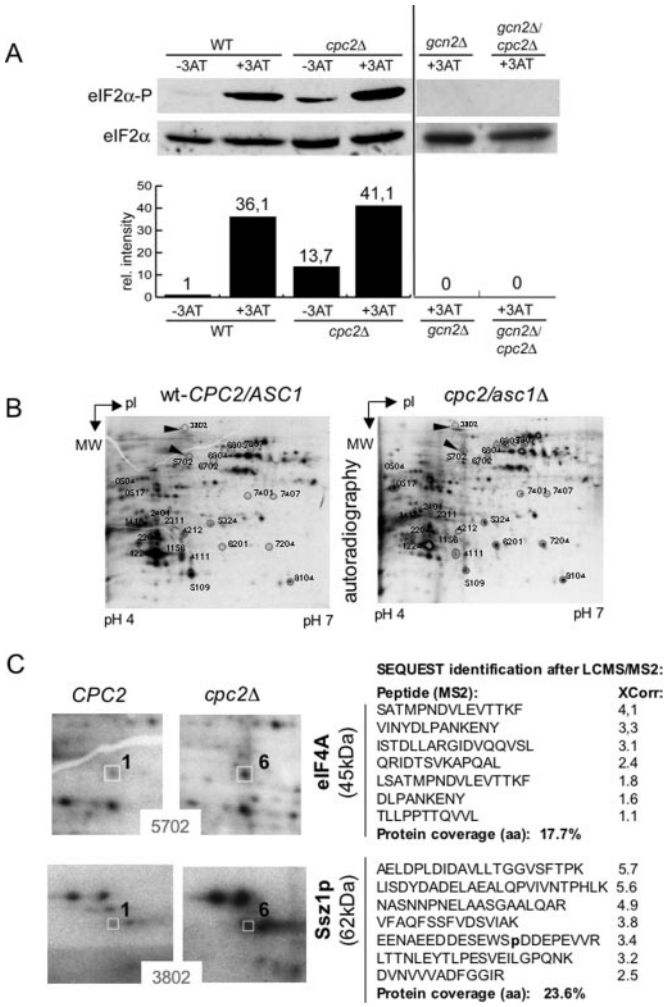

FIG. 6. **Cpc2p reduces phosphorylation of translation initiation factors.** *A*, immunodetection of phosphorylated eIF2 with anti-eIF2α-P antibody quantified and normalized against total eIF2 levels detected with anti-eIF2 antibody. Wild type (RH2817) and *cpc2*Δ cells (RH3263) were cultivated in the absence (−) or presence (+) of 3AT for 1 h. *B*, autoradiography of a 2D phosphoproteome comparison of metabolically [^{32}P]orthophosphate-labeled cell extracts of wild type and *cpc2*Δ cells. *Circled spots* were found to be increased in intensity by a factor of 2 or more in *cpc2*Δ cells and are assigned with PDQuest (Bio-Rad) standard spot numbers (SSP). *C*, the intensity of spot SSP5702 is 6-fold increased in *cpc2*Δ cells, and this protein was identified by TurboSEQUEST analysis of its LC-MS/MS2 data as translation initiation factor eIF4A. TurboSEQUEST cross-correlation values (Xcorr) for the identified tryptic peptides are given. *WT*, wild type; *rel.*, relative; *aa*, amino acid.

The translation initiation factor eIF4A could be identified as one of the proteins significantly up-regulated in its phosphorylation in *cpc2*Δ cells (Fig. 6, *B* and *C*, spot number 5702, 6-fold intensity increase). The eIF4A protein is the prototype of DEAD box RNA helicases and a subunit of the eukaryotic cap-binding complex eIF4F. In addition to eIF2, this is the second translation initiation factor found in this study whose phosphorylation increased upon deletion of *CPC2*. Because the modification was not located within the 17.7% coverage of the identified protein, the phosphorylated residue could not be identified. Phosphorylation of eIF4A at the same N-terminal serine of the protein where it has been demonstrated for the pheromone signaling pathway (62) is possible.

Another phosphoprotein of increased intensity in *cpc2*Δ cells was identified as Ssz1p (Fig. 6, *B* and *C*, spot number 3802, 6-fold intensity increase). Ssz1p is an HSP70 protein together with Zuo1p forming the ribosome-associated complex RAC. The phosphorylation site could be mapped to serine 514 (2-fold charged peptide with Xcorr of 3.4; MS2 spectrum is in Supplemental Fig. 1), a site that has been shown earlier to be phosphorylated in response to the pheromone signaling pathway (62). Ssz1p, eIF4A, eIF2α, and Cpc2p all bind to the "multiadaptor" protein eIF4G (63) suggesting that the presence of Cpc2p is necessary to prevent phosphorylation of the other proteins when cells are sated to allow adjusted initiation of translation.

DISCUSSION

Mammalian RACK1 physically interacts with activated protein kinase C and Src kinase, which are soluble signal transduction proteins (33, 64). In addition, RACK1 also interacts with the cytosolic domains of membrane-spanning receptors (65, 66) and controls processes that involve contacts with the extracellular matrix during cell spreading, the establishment of focal adhesions, and cell-cell contacts (60, 67, 68). RACK1 therefore presumably serves as a versatile platform for signal transduction pathways, which change the expression of distinct groups of genes (68). Here it is shown that yeast Cpc2p has roles similar to that of its equivalent RACK1 because it is also required for extracellular interactions. In yeast these roles are the cell-cell and the cell-surface interactions mediated by Flo11p, and Cpc2p is necessary for the expression of the *FLO11* gene. Cpc2p is also required for the cell wall integrity, although the exact relationship between Cpc2p and the *S. cerevisiae* protein kinase C is yet elusive (69). Cpc2/Asc1p orthologous proteins of other fungi like *Aspergillus nidulans* and *Schizosaccharomyces pombe* (70, 71) and even of plants (56) also seem to be involved in cellular differentiation processes. RACK1 homologs might also play a prominent role in pathogenesis. In the pathogen *Cryptococcus neoformans*, a novel Gβ-like/RACK1 homolog seems to be associated to cAMP signaling and virulence (72). For *Trypanosoma brucei*, the causative agent of sleeping sickness, a RACK1 homolog has been shown to be required for the onset and progression of cytokinesis (73).

The interaction of ribosomal RACK1 in mammals to activated protein kinase C results in the phosphorylation of the initiation factor eIF6. Phosphorylated eIF6 dissociates from the large 60 S ribosomal subunit thereby enabling assembly with the smaller 40 S subunit to promote translation (74). Protein kinase C in addition phosphorylates a serine of the cap-binding protein eIF4E *in vivo* (75).

Our data with the yeast counterpart of RACK1, Cpc2p/Asc1p, corroborate the importance of this protein for the

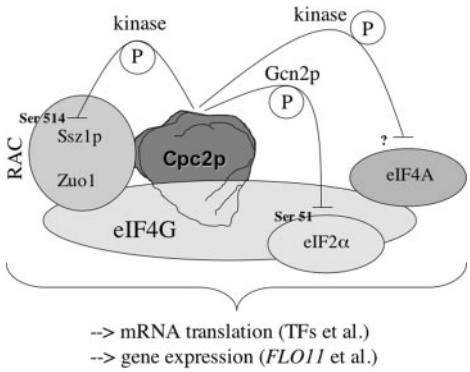

FIG. 7. **Model for the Cpc2p function in translational control.** Phosphorylations are marked by *circled Ps* reflecting phosphate groups. Phosphorylation sites are indicated if known (eIF2α and Ssz1p) and marked with a *question mark* if not (eIF4A). *TFs*, transcription factors.

phosphorylation of even more initiation factors of translation. A physical interaction of the *S. cerevisiae* sensor kinase Gcn2p has not been found with Cpc2/Asc1p and might be transient. However, deletion of *CPC2/ASC1* results in constitutive phosphorylation of the translation initiation factor eIF2, hence reducing translation initiation by reducing the levels of the ternary complex eIF2-GTP-Met-tRNA$_i^{Met}$. Cpc2p/Asc1p is also necessary to prevent phosphorylation of the translation initiation factor eIF4A and of the HSP70 protein Ssz1p in sated cells. The initiation factor eIF4A is a DEA(D/H) box RNA helicase that presumably unwinds secondary RNA structures near the mRNA cap to allow translation initiation (76). A pheromone-induced cell cycle arrest correlates with phosphorylation of eIF4A at the very N-terminal serine of the protein (62). eIF4A phosphorylation also seems to be part of developmental processes in other organisms including pollen tube germination in tobacco (77).

Interestingly eIF2α, eIF4A, eIF4E, the RAC subunit Ssz1p, and Cpc2p are all interacting partners of eIF4G (63), the multiadaptor translation initiation factor. Cpc2p might have a role in coordinating the phosphorylation of these translation factors at eIF4G that might result in distinct differences in mRNA translation. Changes in the expression of mRNA transcripts for transcription factors might result in overall changes in gene expression of a variety of genes as *e.g.* the *FLO11* gene (Fig. 7). The loss of the *FLO11*-dependent adhesive and pseudohyphal growth is obviously not the only cell wall phenotype caused by the *CPC2/ASC1* deletion. Increased sensitivity to cell wall-affecting drugs hint to an important role of Cpc2p/Asc1p in appropriate cell wall biosynthesis. Profiling the translation efficiency of selected transcription factor mRNAs by polysome fractionation and subsequent mRNA probing in *cpc2*Δ compared with wild type cells might provide an informative basis for comprehending the extent of the role of Cpc2p in translation and gene expression.

The hypothesis that Cpc2p could be a link between eIF4G-associated proteins and kinases to coordinate gene expression finds some support by the finding that the translational derepression of the signaling pathway-responsive transcription factor Ste12p during filamentous growth is regulated by eIF4E-binding proteins. These include Caf20p and Eap1p, the decapping activator Dhh1p, and the RNA helicase eIF4A (78, 79). It is tempting to speculate that the eIF4G-interacting protein Cpc2p might have a similar role in derepressing translation of specific transcripts and that the underlying mechanism requires various kinases and eIF2α, eIF4A, and the RAC subunit Ssz1p as targets for these kinases.

Cpc2/Asc1p has lately been identified as the Gβ-subunit of Gα protein Gpa2 (80) suggesting that there is a link between signal transduction and translational regulation on ribosomes. Therefore a significant impact of Cpc2/Asc1p on a variety of protein phosphorylations by signal transduction pathways seems to be possible. Its dual role as receptor-coupled G-protein subunit and as ribosomal protein imposes questions about the nature of the connectivity of both functions for signal transduction and the regulation of gene expression on the level of mRNA translation.

In conclusion, the ribosomal Cpc2/Asc1p is a post-transcriptionally regulated protein controlling ribosomal settings for the initiation of mRNA translation. It might thereby mediate the translational adaptation to environmental stimuli and/or nutritional conditions that are initially transferred by signaling receptors and transduction pathways and finally lead to the modification of translation initiation factors. The expression or co-/post-translational modification of transcription factors might then affect the transcription of target genes of signaling pathways, such as the *S. cerevisiae* dimorphism gene *FLO11*. The presented model is that Cpc2/Asc1p represents a ribosomal interface for signal transduction, translation initiation, and transcription factor formation.

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‡ Both authors contributed equally to this work.

§ Present address: Dept. of Heart and Circulatory Physiology, Georg August University, 37077 Göttingen, Germany.

¶ Present address: Dept. of Cardiology and Pneumology, Georg August University, 37077 Göttingen, Germany.

‖ Present address: Dept. of Human and Animal Cell Cultures, DSMZ-German Collection of Microorganisms and Cell Cultures, 38124 Braunschweig, Germany.

** To whom correspondence should be addressed: Inst. of Microbiology and Genetics, Georg August University, Grisebachstrasse 8, D-37077 Göttingen, Germany. Tel.: 49-551-39-37-71; Fax: 49-551-39-38-20; E-mail: gbraus@gwdg.de.

REFERENCES

1. Roberts, R. L., and Fink, G. R. (1994) Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes Dev.* **8,** 2974–2985
2. Guo, B., Styles, C. A., Feng, Q., and Fink, G. R. (2000) A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 12158–12163
3. Gimeno, C. J., Ljungdahl, P. O., Styles, C. A., and Fink, G. R. (1992) Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* **68,** 1077–1090
4. Mösch, H. U., and Fink, G. R. (1997) Dissection of filamentous growth by transposon mutagenesis in *Saccharomyces cerevisiae*. *Genetics* **145,** 671–684
5. Cullen, P. J., and Sprague, G. F., Jr. (2000) Glucose depletion causes haploid invasive growth in yeast. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 13619–13624
6. Robertson, L. S., and Fink, G. R. (1998) The three yeast A kinases have specific signaling functions in pseudohyphal growth. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13783–13787
7. Pan, X., and Heitman, J. (1999) Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **19,** 4874–4887
8. Lo, W. S., and Dranginis, A. M. (1998) The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **9,** 161–171
9. Lengeler, K. B., Davidson, R. C., D'Souza, C., Harashima, T., Shen, W. C., Wang, P., Pan, X., Waugh, M., and Heitman, J. (2000) Signal transduction cascades regulating fungal development and virulence. *Microbiol. Mol. Biol. Rev.* **64,** 746–785
10. Elion, E. A., Qi, M., and Chen, W. (2005) Signal transduction. Signaling specificity in yeast. *Science* **307,** 687–688
11. Mösch, H. U., Kübler, E., Krappmann, S., Fink, G. R., and Braus, G. H. (1999) Crosstalk between the Ras2p-controlled mitogen-activated protein kinase and cAMP pathways during invasive growth of *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **10,** 1325–1335
12. Rupp, S., Summers, E., Lo, H. J., Madhani, H., and Fink, G. (1999) MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast *FLO11* gene. *EMBO J.* **18,** 1257–1269
13. van Dyk, D., Pretorius, I. S., and Bauer, F. F. (2005) Mss11p is a central element of the regulatory network that controls FLO11 expression and invasive growth in Saccharomyces cerevisiae. *Genetics* **169,** 91–106
14. Braus, G. H., Grundmann, O., Brückner, S., and Mösch, H. U. (2003) Amino acid starvation and Gcn4p regulate adhesive growth and *FLO11* gene expression in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **14,** 4272–4284
15. Hinnebusch, A. G., and Natarajan, K. (2002) Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot. Cell* **1,** 22–32
16. Dever, T. E., and Hinnebusch, A. G. (2005) GCN2 whets the appetite for amino acids. *Mol. Cell* **18,** 141–142
17. Natarajan, K., Meyer, M. R., Jackson, B. M., Slade, D., Roberts, C., Hinnebusch, A. G., and Marton, M. J. (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol. Cell. Biol.* **21,** 4347–4368
18. Kleinschmidt, M., Grundmann, O., Blüthgen, N., Mösch, H. U., and Braus, G. H. (2005) Transcriptional profiling of *Saccharomyces cerevisiae* cells under adhesion-inducing conditions. *Mol. Genet. Genomics* **273,** 382–393
19. Yin, Z., Stead, D., Selway, L., Walker, J., Riba-Garcia, I., McLnerney, T., Gaskell, S., Oliver, S. G., Cash, P., and Brown, A. J. (2004) Proteomic response to amino acid starvation in *Candida albicans* and *Saccharomyces cerevisiae*. *Proteomics* **4,** 2425–2436
20. Wek, S. A., Zhu, S., and Wek, R. C. (1995) The histidyl-tRNA synthetase-related sequence in the eIF-2α protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. *Mol. Cell. Biol.* **15,** 4497–4506
21. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992) Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of *GCN4* in yeast. *Cell* **68,** 585–596
22. Zhu, S., Sobolev, A. Y., and Wek, R. C. (1996) Histidyl-tRNA synthetase-related sequences in GCN2 protein kinase regulate in vitro phosphorylation of eIF-2. *J. Biol. Chem.* **271,** 24989–24994
23. Hao, S., Sharp, J. W., Ross-Inta, C. M., McDaniel, B. J., Anthony, T. G., Wek, R. C., Cavener, D. R., McGrath, B. C., Rudell, J. B., Koehnle, T. J., and Gietzen, D. W. (2005) Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* **307,** 1776–1778
24. Hinnebusch, A. G. (1997) Translational regulation of yeast *GCN4*. A window on factors that control initiator-tRNA binding to the ribosome. *J. Biol. Chem.* **272,** 21661–21664
25. Kornitzer, D., Raboy, B., Kulka, R. G., and Fink, G. R. (1994) Regulated degradation of the transcription factor Gcn4. *EMBO J.* **13,** 6021–6030
26. Pries, R., Bömeke, K., Irniger, S., Grundmann, O., and Braus, G. H. (2002) Amino acid-dependent Gcn4p stability regulation occurs exclusively in the yeast nucleus. *Eukaryot. Cell* **1,** 663–672
27. Bömeke, K., Pries, R., Korte, V., Scholz, E., Herzog, B., Schulze, F., and Braus, G. H. (2006) Yeast Gcn4p stabilization is initiated by the dissociation of the nuclear Pho85p/Pcl5p complex. *Mol. Biol. Cell* **17,** 2952–2962
28. Hoffmann, B., Mösch, H. U., Sattlegger, E., Barthelmess, I. B., Hinnebusch, A., and Braus, G. H. (1999) The WD protein Cpc2p is required for repression of Gcn4 protein activity in yeast in the absence of amino-acid starvation. *Mol. Microbiol.* **31,** 807–822
29. Chantrel, Y., Gaisne, M., Lions, C., and Verdiere, J. (1998) The transcriptional regulator Hap1p (Cyp1p) is essential for anaerobic or heme-deficient growth of *Saccharomyces cerevisiae:* Genetic and molecular characterization of an extragenic suppressor that encodes a WD repeat protein. *Genetics* **148,** 559–569
30. Gerbasi, V. R., Weaver, C. M., Hill, S., Friedman, D. B., and Link, A. J. (2004) Yeast Asc1p and mammalian RACK1 are functionally orthologous core 40S ribosomal proteins that repress gene expression. *Mol. Cell. Biol.* **24,** 8276–8287
31. Krüger, D., Koch, J., and Barthelmess, I. B. (1990) *cpc-2*, a new locus involved in general control of amino acid synthetic enzymes in *Neurospora crassa*. *Curr. Genet.* **18,** 211–215
32. Müller, F., Kruger, D., Sattlegger, E., Hoffmann, B., Ballario, P., Kanaan, M., and Barthelmess, I. B. (1995) The *cpc-2* gene of *Neurospora crassa* encodes a protein entirely composed of WD-repeat segments that is involved in general amino acid control and female fertility. *Mol. Gen. Genet.* **248,** 162–173
33. Ron, D., Chen, C. H., Caldwell, J., Jamieson, L., Orr, E., and Mochly-Rosen, D. (1994) Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G proteins. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 839–843
34. Kleinschmidt, M., Schulz, R., and Braus, G. H. (2006) The yeast *CPC2/ASC1* gene is regulated by the transcription factors Fhl1p and Ifh1p. *Curr. Genet.* **49,** 218–228
35. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153,** 163–168
36. Wessel, D., and Flügge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138,** 141–143
37. Yan, J. X., Devenish, A. T., Wait, R., Stone, T., Lewis, S., and Fowler, S. (2002) Fluorescence two-dimensional difference gel electrophoresis and mass spectrometry based proteomic analysis of *Escherichia coli*. *Proteomics* **2,** 1682–1698
38. Ray, R., and Haystead, T. A. (2003) Phosphoproteome analysis in yeast. *Methods Enzymol.* **366,** 95–103
39. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68,** 850–858
40. Eng, J. K., McCormack, A. L., and Yates, J. R., III (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5,** 976–989
41. Link, A. J., Eng, J., Schieltz, D. M., Carmack, E., Mize, G. J., Morris, D. R., Garvik, B. M., and Yates, J. R., III (1999) Direct analysis of protein

complexes using mass spectrometry. *Nat. Biotechnol.* **17,** 676–682
42. Spurr, A. R. (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26,** 31–43
43. Venable, J. H., and Coggeshall, R. (1965) A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25,** 407–408
44. Hoppert, M., and Holzenburg, A. (2003) *Electron Microscopy in Microbiology*, pp. 11–18, Springer, New York
45. Cross, F. R., and Tinkelenberg, A. H. (1991) A potential positive feedback loop controlling *CLN1* and *CLN2* gene expression at the start of the yeast cell cycle. *Cell* **65,** 875–883
46. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254
47. Rose, M., and Botstein, D. (1983) Construction and use of gene fusions to *lacZ* (β-galactosidase) that are expressed in yeast. *Methods Enzymol.* **101,** 167–180
48. Cheng, M. Y., Hartl, F. U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L., and Horwich, A. L. (1989) Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature* **337,** 620–625
49. Johnson, R. B., Fearon, K., Mason, T., and Jindal, S. (1989) Cloning and characterization of the yeast chaperonin *HSP60* gene. *Gene* (*Amst.*) **84,** 295–302
50. Krietsch, W. K. (1975) Triosephosphate isomerase from yeast. *Methods Enzymol.* **41,** 434–438
51. Liu, H. P., and Bretscher, A. (1989) Disruption of the single tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. *Cell* **57,** 233–242
52. Futcher, B., Latter, G. I., Monardo, P., McLaughlin, C. S., and Garrels, J. I. (1999) A sampling of the yeast proteome. *Mol. Cell. Biol.* **19,** 7357–7368
53. Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11,** 4241–4257
54. Causton, H. C., Ren, B., Koh, S. S., Harbison, C. T., Kanin, E., Jennings, E. G., Lee, T. I., True, H. L., Lander, E. S., and Young, R. A. (2001) Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell* **12,** 323–337
55. Brückner, S., Köhler, T., Braus, G. H., Heise, B., Bolte, M., and Mösch, H. U. (2004) Differential regulation of Tec1 by Fus3 and Kss1 confers signaling specificity in yeast development. *Curr. Genet.* **46,** 331–342
56. Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F., Subden, R. E., Young, R. A., Lonvaud, A., Denayrolles, M., and van Vuuren, H. J. (1997) Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. *Nat. Biotechnol.* **15,** 253–257
57. Pan, X., and Heitman, J. (2002) Protein kinase A operates a molecular switch that governs yeast pseudohyphal differentiation. *Mol. Cell. Biol.* **22,** 3981–3993
58. Chasse, S. A., Flanary, P., Parnell, S. C., Hao, N., Cha, J. Y., Siderovski, D. P., and Dohlman, H. G. (2006) Genome-scale analysis reveals Sst2 as the principal regulator of mating pheromone signaling in the yeast *Saccharomyces cerevisiae*. *Eukaryot. Cell* **5,** 330–346
59. Rosdahl, J. A., Mourton, T. L., and Brady-Kalnay, S. M. (2002) Protein kinase C δ (PKCδ) is required for protein tyrosine phosphatase μ (PTPμ)-dependent neurite outgrowth. *Mol. Cell. Neurosci.* **19,** 292–306
60. Hermanto, U., Zong, C. S., Li, W., and Wang, L. H. (2002) RACK1, an insulin-like growth factor I (IGF-I) receptor-interacting protein, modulates IGF-I-dependent integrin signaling and promotes cell spreading and contact with extracellular matrix. *Mol. Cell. Biol.* **22,** 2345–2365
61. Chang, B. Y., Conroy, K. B., Machleder, E. M., and Cartwright, C. A. (1998) RACK1, a receptor for activated C kinase and a homolog of the β subunit of G proteins, inhibits activity of src tyrosine kinases and growth of NIH 3T3 cells. *Mol. Cell. Biol.* **18,** 3245–3256
62. Gruhler, A., Olsen, J. V., Mohammed, S., Mortensen, P., Faergeman, N. J., Mann, M., and Jensen, O. N. (2005) Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. *Mol. Cell. Proteomics* **4,** 310–327
63. Gavin, A. C., Aloy, P., Grandi, P., Krause, R., Boesche, M., Marzioch, M., Rau, C., Jensen, L. J., Bastuck, S., Dumpelfeld, B., Edelmann, A., Heurtier, M. A., Hoffman, V., Hoefert, C., Klein, K., Hudak, M., Michon, A. M., Schelder, M., Schirle, M., Remor, M., Rudi, T., Hooper, S., Bauer, A., Bouwmeester, T., Casari, G., Drewes, G., Neubauer, G., Rick, J. M., Kuster, B., Bork, P., Russell, R. B., and Superti-Furga, G. (2006) Proteome survey reveals modularity of the yeast cell machinery. *Nature* **440,** 631–636
64. Chang, B. Y., Harte, R. A., and Cartwright, C. A. (2002) RACK1: a novel substrate for the Src protein-tyrosine kinase. *Oncogene* **21,** 7619–7629
65. Liliental, J., and Chang, D. D. (1998) Rack1, a receptor for activated protein kinase C, interacts with integrin β subunit. *J. Biol. Chem.* **273,** 2379–2383
66. Buensuceso, C. S., Woodside, D., Huff, J. L., Plopper, G. E., and O'Toole, T. E. (2001) The WD protein Rack1 mediates protein kinase C and integrin-dependent cell migration. *J. Cell Sci.* **114,** 1691–1698
67. McCahill, A., Warwicker, J., Bolger, G. B., Houslay, M. D., and Yarwood, S. J. (2002) The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. *Mol. Pharmacol.* **62,** 1261–1273
68. Nilsson, J., Sengupta, J., Frank, J., and Nissen, P. (2004) Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. *EMBO Rep.* **5,** 1137–1141
69. Levin, D. E., and Bartlett-Heubusch, E. (1992) Mutants in the *S. cerevisiae PKC1* gene display a cell cycle-specific osmotic stability defect. *J. Cell Biol.* **116,** 1221–1229
70. Hoffmann, B., Wanke, C., Lapaglia, S. K., and Braus, G. H. (2000) c-Jun and RACK1 homologues regulate a control point for sexual development in *Aspergillus nidulans*. *Mol. Microbiol.* **37,** 28–41
71. Jeong, H. T., Oowatari, Y., Abe, M., Tanaka, K., Matsuda, H., and Kawamukai, M. (2004) Interaction between a negative regulator (Msa2/Nrd1) and a positive regulator (Cpc2) of sexual differentiation in *Schizosaccharomyces pombe*. *Biosci. Biotechnol. Biochem.* **68,** 1621–1626
72. Palmer, D. A., Thompson, J. K., Li, L., Prat, A., and Wang, P. (2006) Gib2, a novel Gβ-like/RACK1 homolog, functions as a Gβ subunit in cAMP signaling and is essential in *Cryptococcus neoformans*. *J. Biol. Chem.* **281,** 32596–32605
73. Rothberg, K. G., Burdette, D. L., Pfannstiel, J., Jetton, N., Singh, R., and Ruben, L. (2006) The RACK1 homologue from Trypanosoma brucei is required for the onset and progression of cytokinesis. *J. Biol. Chem.* **281,** 9781–9790
74. Ceci, M., Gaviraghi, C., Gorrini, C., Sala, L. A., Offenhauser, N., Marchisio, P. C., and Biffo, S. (2003) Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. *Nature* **426,** 579–584
75. Smith, M. R., Jaramillo, M., Tuazon, P. T., Traugh, J. A., Liu, Y. L., Sonenberg, N., and Kung, H. F. (1991) Modulation of the mitogenic activity of eukaryotic translation initiation factor-4E by protein kinase C. *New Biol.* **3,** 601–607
76. Linder, P. (2003) Yeast RNA helicases of the DEAD-box family involved in translation initiation. *Biol. Cell* **95,** 157–167
77. op den Camp, R. G., and Kuhlemeier, C. (1998) Phosphorylation of tobacco eukaryotic translation initiation factor 4A upon pollen tube germination. *Nucleic. Acids Res.* **26,** 2058–2062
78. Ibrahimo, S., Holmes, L. E., and Ashe, M. P. (2006) Regulation of translation initiation by the yeast eIF4E binding proteins is required for the pseudohyphal response. *Yeast* **23,** 1075–1088
79. Park, Y. U., Hur, H., Ka, M., and Kim, J. (2006) Identification of translational regulation target genes during filamentous growth in Saccharomyces cerevisiae: regulatory role of Caf20 and Dhh1. *Eukaryot. Cell* **5,** 2120–2127
80. Zeller, C. E., Parnell, S. C., and Dohlman, H. G. (2007) The RACK1 ortholog Asc1 functions as a G protein β subunit coupled to glucose responsiveness in yeast. *J. Biol. Chem.* **282,** 25168–25176
81. McCarthy, J., Hopwood, F., Oxley, D., Laver, M., Castagna, A., Righetti, P. G., Williams, K., and Herbert, B. (2003) Carbamylation of proteins in 2-D electrophoresis—myth or reality? *J. Proteome Res.* **2,** 239–242
82. Strittmatter, A. W., Fischer, C., Kleinschmidt, M., and Braus, G. H. (2006) *FLO11* mediated filamentous growth of the yeast *Saccharomyces cerevisiae* depends on the expression of the ribosomal *RPS26* genes. *Mol. Genet. Genomics* **276,** 113–125
83. Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122,** 19–27