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Receptor for Activated C-Kinase (RACK1) Homolog Cpc2 Facilitates the General Amino Acid Control Response through Gcn2 Kinase in Fission Yeast*[S]

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Background: General amino acid control (GAAC) is important for cell survival under amino acid starvation.
Results: Absence of fission yeast Cpc2, a homolog of mammalian RACK1, causes defects in eIF2α phosphorylation, induction of amino acid biosynthesis genes, and Gcn2 autophosphorylation.
Conclusion: Cpc2 stimulates the GAAC response by facilitating Gcn2 activation.
Significance: This study provides evidence that RACK1 homolog promotes the GAAC response.

General amino acid control (GAAC) is crucial for sensing and adaptation to nutrient availability. Amino acid starvation activates protein kinase Gcn2, which plays a central role in the GAAC response by phosphorylating the α-subunit of eukaryotic initiation factor 2 (eIF2α), leading to the translational switch to stimulate selective expression of stress-responsive genes. We report here that in fission yeast *Schizosaccharomyces pombe*, Cpc2, a homolog of mammalian receptor for activated C-kinase (RACK1), is important for the GAAC response. Deletion of *S. pombe cpc2* impairs the amino acid starvation-induced phosphorylation of eIF2α and the expression of amino acid biosynthesis genes, thereby rendering cells severely sensitive to amino acid limitation. Unlike the *Saccharomyces cerevisiae* Cpc2 ortholog, which normally suppresses the GAAC response, our findings suggest that *S. pombe* Cpc2 promotes the GAAC response. We also found that *S. pombe* Cpc2 is required for starvation-induced Gcn2 autophosphorylation, which is essential for Gcn2 function. These results indicate that *S. pombe* Cpc2 facilitates the GAAC response through the regulation of Gcn2 activation and provide a novel insight for the regulatory function of RACK1 on Gcn2-mediated GAAC response.

Translational control contributes to stress-induced fine-tuning of gene expression patterns (1, 2). Phosphorylation of the α-subunit of eukaryotic translation initiation factor eIF2 (eIF2α) is an important event in translational regulation in response to stress (3). Its molecular mechanism has been extensively studied in the budding yeast *Saccharomyces cerevisiae* (4), and the phenomenon is known as the general amino acid control (GAAC)[3] response, in which depletion of a single amino acid stimulates the expression of many genes involved in the biosynthesis of all amino acids. Stress-induced eIF2α phosphorylation inhibits the formation of an active ternary complex consisting of eIF2, methionyl-tRNA, and GTP, leading to a decrease in global translation initiation efficiency (3, 4). At the same time, translation of a subset of stress-responsive genes, such as *S. cerevisiae GCN4* and mammalian *ATF4* transcriptional factors, is specifically promoted by a mechanism involving short upstream open reading frames in the 5′-untranslated region of these mRNAs (5–7). Gcn4 and ATF4 induce the expression of a number of genes required for amino acid biosynthesis and stress response (8, 9). Starvation-induced expression of amino acid biosynthesis genes is also observed in the fission yeast *Schizosaccharomyces pombe*, although the relevant transcriptional factor is not known (10). A single eIF2α kinase, Gcn2, regulates this response in *S. cerevisiae*, whereas four (GCN2, HRI, PKR, PERK) or three (Gcn2, Hri1, Hri2) eIF2α kinases regulate eIF2α phosphorylation depending on the types of stress in mammals or *S. pombe*, respectively (3, 11). Because eIF2α kinases are activated in response to diverse forms of stress, eIF2α phosphorylation functions to integrate various stress stimuli into translational controls (12).

Among the eIF2α kinases, Gcn2 (GCN2 in mammals) is a dominant regulator of the GAAC response. Gcn2 binds preferentially to non-aminoacylated (uncharged) tRNA *in vitro*, through the domain homologous to histidyl-tRNA synthetase (13, 14). Gcn2 forms a dimer, and autophosphorylation within its kinase domain is essential for full activation of Gcn2 (15–17). Moreover, Gcn2 binds to translating ribosomes (18). From these observations and structural analysis of Gcn2, it is predicted that uncharged tRNA binding activates Gcn2 by inducing a conformational change (19) and that ribosomal localization of Gcn2 facilitates the binding of uncharged tRNA in the ribosomal A-site or the interaction with its substrate eIF2α. In addition to tRNA binding, posttranslational modifications or

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[S] This article contains supplemental Figs. S1 and S2.

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[3] The abbreviations used are: GAAC, general amino acid control; RACK1, receptor for activated C-kinase; 3AT, 3-aminotriazole; EMM, Edinburgh minimal medium.

TABLE 1
***S. pombe* strains used in this study**

Strain		Genotype
JK317	h^-	*leu1-32 ura4-D18*
YT2307	h^-	*cpc2::ura4$^+$ leu1-32 ura4-D18*
YT2313	h^-	*cpc2:2HA6His:ura4$^+$ leu1-32 ura4-D18*
YT2453	h^-	*hri2::ura4$^+$ leu1-32 ura4-D18*
YT2459	h^-	*hri2::ura4$^+$ gcn2::Kan^r leu1-32 ura4-D18*
YT2465	h^-	*rpS3:Flag:ura4$^+$ leu1-32 ura4-D18*
YT2824	h^-	*cpc2::ura4$^+$ gcn2::Kan^r leu1-32 ura4-D18*
YT3033	h^-	*leu1-32*
YT3173	h^-	*hri2::ura4$^+$ cpc2::ura4$^+$ leu1-32 ura4-D18*
YT3360	h^-	*gcn2::ura4$^+$ leu1-32 ura4-D18*
YT3372	h^-	*5Flag:gcn2 leu1-32 ura4-D18*
YT3540	h^-	*cpc2::Kan^r leu1-32 ura4-D18*
YT3542	h^-	*cpc2_DE leu1-32 ura4-D18*
YT3559	h^-	*5Flag:gcn2 cpc2_DE:ura4$^+$ leu1-32 ura4-D18*
YT3598	h^-	*5Flag:gcn2 cpc2::ura4$^+$ leu1-32 ura4-D18*
YT3648	h^-	*5Flag:gcn2::ura4$^+$ leu1-32 ura4-D18*
YT3656	h^-	*5Flag:gcn2_K585R leu1-32 ura4-D18*
YT3657	h^-	*5Flag:gcn2_T818/823A leu1-32 ura4-D18*
YT4251	h^-	*atf1::ura4$^+$ leu1-32 ura4-D18*
YT4254	h^-	*5Flag:gcn2 atf1::ura4$^+$ leu1-32 ura4-D18*
YT4279	h^+/h^-	*5Flag:gcn2/12myc:gcn2 ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*
YT4280	h^+/h^-	*5Flag:gcn2/12myc:gcn2 cpc2::ura4$^+$/cpc2::ura4$^+$ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*
YT4281	h^+/h^-	*12myc:gcn2/gcn2$^+$ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*
YT4299	h^-	*cpc2_W43* leu1-32 ura4-D18*
YT4309	h^-	*5Flag:gcn2 cpc2_W43* leu1-32 ura4-D18*
YT4376	h^+/h^-	*5Flag:gcn2/gcn2$^+$ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*
YT4386	h^-	*Flag:cpc2 leu1-32 ura4-D18*
YT4388	h^-	*Flag:cpc2_W43* leu1-32 ura4-D18*
YT4389	h^-	*cpc2::Kan^r:2HA6His:ura4$^+$ leu1-32 ura4-D18*
YT4390	h^-	*cpc2_W43*:2HA6His:ura4$^+$ leu1-32 ura4-D18*

regulatory factors modulate Gcn2 activity (20–23), although the precise mechanism of regulation of Gcn2 activity by these additional inputs is not fully understood.

The receptor for activated C-kinase (RACK1) is a highly conserved protein among eukaryotes (homologs are known as Asc1 in *S. cerevisiae* (24) and Cpc2 in *S. pombe* (25)). RACK1 homolog is linked to diverse physiological processes through interactions with numerous signaling molecules (25–28). Also, recent studies have revealed that RACK1 is a stoichiometric component of ribosomes (29, 30). RACK1 homologs facilitate global and selective translation through various regulatory mechanisms (24, 29, 31, 32). Because RACK1 associates with several signaling factors, such as protein kinase C and Src, on ribosomes, it has been thought that RACK1 integrates cellular signals to translational regulation (32, 33).

RACK1 homologs are involved in control of the GAAC response. The absence of *S. cerevisiae ASC1* increased the Gcn4-mediated transcription of amino acid biosynthesis genes under nonstarvation conditions (34), presumably by the destabilization of translation initiation complexes on ribosomes (35). *ASC1* deletion suppressed the growth defect of *gcn2*Δ cells under limiting amino acid conditions, indicating that Asc1 negatively regulates the GAAC response in *S. cerevisiae*. On the other hand, in *Neurospora crassa*, *cpc-2*, encoding a RACK1 homolog, was originally isolated as a gene regulating cross-pathway control (36, 37), a similar phenomenon to the GAAC response. The *cpc-2 U142* allele impaired the induction of amino acid biosynthesis genes in response to amino acid limitation (36), suggesting a positive effector role of Cpc-2 in the GAAC response. However, the molecular mechanism of action of Cpc-2 in *N. crassa* has not been determined. Furthermore, it remains unclear how RACK1 homologs regulate the GAAC response in *S. pombe* and mammals.

Here, we analyzed the function of *S. pombe* Cpc2 in response to amino acid starvation. We found that *S. pombe* Cpc2 is required for phosphorylation of eIF2α and the expression of amino acid biosynthesis genes induced by histidine starvation. Moreover, *cpc2* deletion caused defects in the autophosphorylation of the eIF2α kinase Gcn2, indicating that *S. pombe* Cpc2 stimulates Gcn2 activation and the GAAC response.

EXPERIMENTAL PROCEDURES

Yeast Strains and General Techniques—S. pombe strains used in this study are listed in Table 1. Growth media and basic techniques for *S. pombe* have been described elsewhere (38). For 3-aminotriazole (3AT) treatment, cells were grown in Edinburgh minimal medium (EMM) liquid medium or on EMM plates supplemented with 10 mM 3AT. To construct an epitope-tagged Gcn2 plasmid, nucleotides 1–3,162 of *gcn2*$^+$ genomic DNA (numbers are relative to the translation start site) were cloned into the plasmid pT7Blue (Merck) to obtain pYTR193. A *ura4*$^+$ marker cassette was inserted into the EcoRV-HincII sites in pYTR193 to obtain pYTR194. The upstream region of the *gcn2*$^+$ gene (nucleotides −496 to −1) was cloned into the KpnI-NdeI sites in pYTR194 followed by 5× FLAG or 12× Myc tag insertion at the NdeI site to obtain pYTR198 or pYTR222, respectively. *cpc2* ORF was cloned in the plasmid pBlueScriptII SK(−) to obtain pYTR120. Point mutations of *gcn2* or *cpc2* were introduced into pYTR193 or pYTR120, respectively, using a QuikChange site-directed mutagenesis kit (Agilent Technologies). Fragments containing *gcn2* or *cpc2* mutations were transformed into *Flag:gcn2*Δ (YT3648) or *cpc2*Δ (YT2307) cells, respectively. To construct expression plasmids, cDNA of *S. pombe cpc2*, *S. cerevisiae asc1*, and human *RACK1* (*GNB2L1*) were cloned into the NdeI-SmaI site in the plasmid pREP2 (39).

TABLE 2
Primer sets used in quantitative RT-PCR

Gene	Forward primer	Reverse primer
his4	TAGATCAGGTGCCGACAAAG	CCGAAATAACAACTGCCTGA
SPAC56E4.03	GCTACATCCATGCCGATAAA	CTAGGGTCAACAACGAACCA
SPCC364.07	TCAACTCTCCATACGCCAAC	CACCTTGTTCCACTCACCAC
SPAC10F6.13c	TTTGCTGAATGGGAACAAGA	CAACAAGAGAATCGCGAAGA
arg3	GTCATCCCGAGGAAGTGTCT	GTCCATTTGCGGTTCTCTG
dld1	CTGAAGTTGCTTGGGTTGGT	GGCATCCATATTGGTCTTGG
lys3	TCTGGTTATTGGGGCTCTTG	TTAATGTCCCAGCGAAGAATG
leu3	TCTCTCCATCCCCATAACGA	CCAAACAAACAGCCCTCAA
SPBC19F5.04	GCAGCACAGATACCAAAGCA	AACCAAATCATGCACAGAACC
cdk9	CTCTTTGCGGTGCTATTTTG	TTGCTGGATATGGTGGTGTT
18 S rRNA	GGGAACCAGGACTTTTACCTTGA	AACTTGCCTGCTTTGAACACTCTA
cpc2	CTCTGACTGGGTTTCTTGTGTG	GCCATAGTGAGAAGTGCGAAG
snoU24b	TATTTGCTACTTCGGAGGCCTTA	GGTGATTTGTTTTGTCTCATCG

RT-PCR—Total RNA was prepared as described previously (40). cDNA was synthesized using an RNA PCR kit (Takara) with random 9-mer primers. Gene expression levels were analyzed by quantitative PCR using a StepOnePlus real-time PCR system (Invitrogen). Information about the primers used in this study is provided in Table 2. The primers for 18 S rRNA are the same as those used previously (41).

Immunoblotting—For preparation of whole cell extracts, harvested cells were suspended in alkaline lysis buffer (1.85 M NaOH, 7.4% 2-mercaptoethanol) and incubated for 10 min on ice. Samples were combined with an equal volume of 50% trichloroacetic acid, incubated for 10 min, and centrifuged. Precipitated proteins were suspended in 2× sample buffer. For detection of Gcn2-phospho-Thr-818, cell lysates were extracted with zirconia beads and lysis buffer A (25 mM MOPS, pH 7.2, 15 mM EGTA, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 10% glycerol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, Complete protease inhibitor (Roche Applied Science), PhosSTOP (Roche Applied Science)) using a Multi-beads shocker (Yasui Kikai). FLAG-tagged Gcn2 was immunoprecipitated using anti-FLAG M2-agarose resin (Sigma). Anti-Gcn2-phospho-Thr-818 antibody was raised against the following peptide, ADEDL(P)TTGVGC (Medical & Biological Laboratories). Anti-Cpc2 antibody was raised against a recombinant Cpc2 protein. The other antibodies used in this study were anti-phosphorylated eIF2α (44-728G; Invitrogen), anti-FLAG M2 (F-3165; Sigma), anti-c-Myc (Sc-40; Santa Cruz Biotechnology), and anti-Cdc2 (PSTAIRE) (Sc-53; Santa Cruz Biotechnology) antibodies.

Co-immunoprecipitation Assay—Cell lysates were extracted in lysis buffer A as described above. FLAG-tagged Gcn2 was immunoprecipitated using Dynabeads anti-mouse IgG (Dynal Biotech) preconjugated with anti-FLAG M2 antibody in lysis buffer A. After incubation for 2 h, the beads were washed four times with lysis buffer A containing 300 mM NaCl and suspended in 2× sample buffer for immunoblotting.

Sucrose Gradient and Polysome Fractionation—Sucrose gradients were performed as described previously with modifications (29). 100 μg/ml cycloheximide was added prior to harvesting cells. Cells were washed and resuspended in TSM buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 30 mM $MgCl_2$, 50 μg/ml cycloheximide, 200 μg/ml heparin, 1 mM phenylmethylsulfonyl fluoride, Complete protease inhibitor). After breakage with zirconia beads using a Multi-beads shocker, cell lysates were clarified by centrifugation. Supernatants were loaded on 15–50% (w/v) sucrose gradients in Gradient buffer (7.5 mM Tris-HCl, pH 7.4, 70 mM NH_4Cl, 3.9 mM MgOAc) and ultracentrifuged for 3 h at 40,000 rpm in a Beckman SW41 rotor. Samples were fractionated using a gradient station (Biocomp Instruments), and polysome profiles were obtained by monitoring the absorbance at 260 nm along the gradient.

In Vitro Kinase Assay—Cells were cultured to log phase. Cell lysates were extracted with zirconia beads and lysis buffer B (50 mM Tris-HCl, pH 8.0, 5 mM EGTA, 150 mM NaCl, 0.1% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, Complete protease inhibitor) using a Multi-beads shocker. Immunoprecipitation was performed using anti-FLAG M2-agarose resin. After washing twice with lysis buffer B and twice with kinase buffer (20 mM Hepes-KOH, pH 7.5, 20 mM $MgCl_2$, 5 mM EGTA, 2 mM dithiothreitol), the resin was suspended in 30 μl of kinase buffer containing 8.5 μM ATP and 0.2 μl of [γ-^{32}P]ATP (10 mCi/ml) and then incubated for 30 min at 30 °C. Samples were resolved by SDS-PAGE, and autoradiography was performed using a Typhoon 9400 imager and ImageQuant software (GE Healthcare).

RESULTS

Cpc2 Plays an Important Role in the Induction of Amino Acid Biosynthesis Genes in S. pombe—To investigate whether S. pombe Cpc2 is involved in the response to amino acid starvation, wild-type, *gcn2*Δ and *cpc2*Δ cells were exposed to 3AT, which inhibits histidine biosynthesis and induces the GAAC response in *S. pombe*. A recent analysis revealed that 3AT treatment induces expression of about 40% of the genes that are predicted to function in amino acid biosynthesis in *S. pombe* (10). We selected nine genes from those genes as the GAAC genes (*his4*$^+$, *SPAC56E4.03*, *SPCC364.07*, *SPAC10F6.13c*, *arg3*$^+$, *dld1*$^+$, *lys3*$^+$, *leu3*$^+$, *SPBC19F5.04*) and checked their expression levels by quantitative RT-PCR. The addition of 3AT significantly increased the expression of all nine genes in wild-type cells, but not in *gcn2*Δ cells (Fig. 1), as reported in a previous microarray analysis (10). Furthermore, we found that deletion of *cpc2* abolished 3AT-induced expression of most of these amino acid biosynthesis genes (Fig. 1). The expression of some genes such as *SPAC10F6.13c* and *lys3* was detectable even in *cpc2*Δ cells. However, in the absence of *cpc2*, 3AT-induced upregulation of *SPAC10F6.13c* was not observed, and the fold increase of *lys3* expression upon 3AT addition was significantly

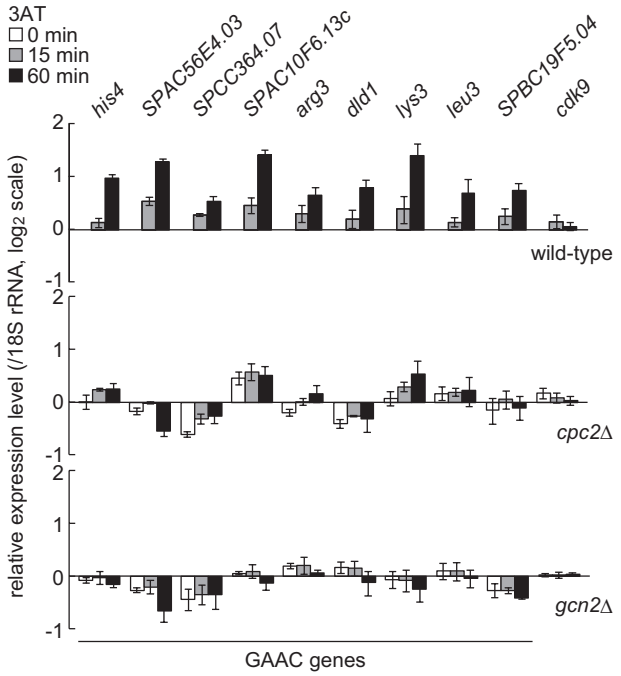

FIGURE 1. **Cpc2 is important for induction of amino acid biosynthesis genes.** Gene expression levels of nine GAAC genes and *cdk9*$^+$ (control) were analyzed by quantitative RT-PCR from wild-type (YT3033), *cpc2*Δ (YT2307), and *gcn2*Δ (YT3360) cells treated with 10 mM 3-aminotriazole (3AT) for 0 min (*white bar*), 15 min (*gray bar*), and 60 min (*black bar*). All data are normalized to the expression level of 18 S rRNA and shown as the relative fold to those at time 0 in wild-type cells with S.E. ($n = 3$). GAAC genes are: *his4*$^+$, imidazoleglycerol-phosphate synthase (predicted); *SPAC56E4.03*, aromatic aminotransferase (predicted); *SPCC364.07*, D-3 phosphoglycerate dehydrogenase (predicted); *SPAC10F6.13c*, aspartate aminotransferase (predicted); *arg3*$^+$, ornithine carbamoyltransferase; *dld1*$^+$, dihydrolipoamide dehydrogenase; *lys3*$^+$, saccharopine dehydrogenase; *leu3*$^+$, 2-isopropylmalate synthase; *SPBC19F5.04*, aspartate kinase (predicted).

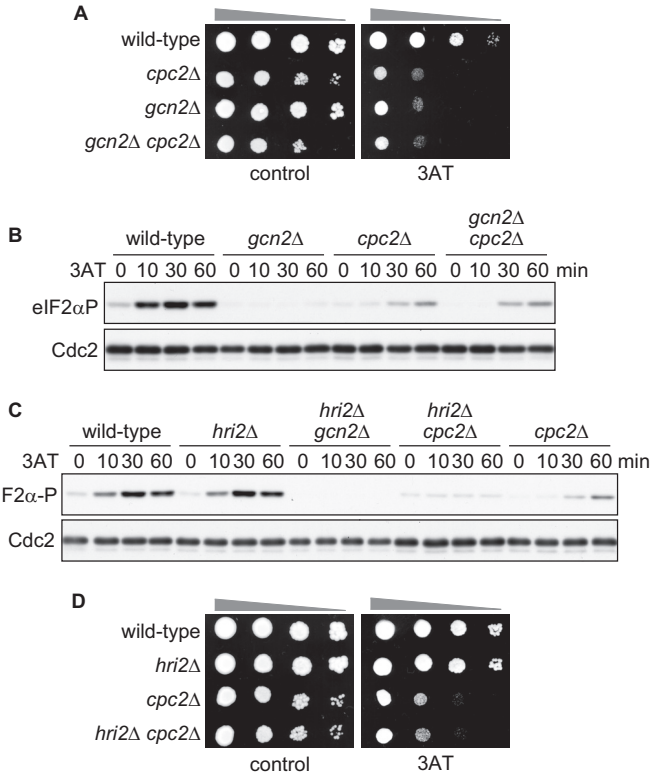

FIGURE 2. **Cpc2 is required for Gcn2-mediated eIF2α phosphorylation (eIF2α*P*) and survival under amino acid starvation.** *A*, 10-fold serial dilutions of wild-type (YT3033), *cpc2*Δ (YT2307), *gcn2*Δ (YT3360), and *gcn2*Δ *cpc2*Δ (YT2824) cells were spotted on EMM plates ± 10 mM 3AT. *B*, immunoblots of phosphorylated eIF2α and Cdc2 (control) from wild-type (YT3033), *cpc2*Δ (YT2307), *gcn2*Δ (YT3360), and *gcn2*Δ *cpc2*Δ (YT2824) cells treated with 10 mM 3AT. *C*, immunoblots of phosphorylated eIF2α and Cdc2 (control) from wild-type (YT3033), *hri2*Δ (YT2453), *hri2*Δ *gcn2*Δ (YT2459), *hri2*Δ *cpc2*Δ (YT3173), and *cpc2*Δ (YT2307) cells treated with 10 mM 3AT. *D*, 10-fold serial dilutions of wild-type (YT3033), *hri2*Δ (YT2453), *cpc2*Δ (YT2307), and *hri2*Δ *cpc2*Δ (YT3173) cells were spotted on EMM plates ± 10 mM 3AT.

smaller than that observed in wild-type cells. These data indicate that *S. pombe* Cpc2 is important for the precise regulation of gene expression in the GAAC response.

Cpc2 Is Required for Gcn2-mediated eIF2α Phosphorylation and Survival under Amino Acid Starvation—Mutant *cpc2*Δ cells exhibited a severe growth defect in the presence of 3AT (Fig. 2*A*), which is consistent with a defect in induction of amino acid biosynthesis genes, as described above. Moreover, 3AT sensitivity was not significantly different between *cpc2*Δ and *gcn2*Δ *cpc2*Δ double-mutant cells (Fig. 2*A*). These data suggest that *S. pombe* Cpc2 plays a role in the GAAC response through a common mechanism with Gcn2. Treatment of wild-type cells with 3AT induces phosphorylation of eIF2α in a Gcn2-dependent manner (Fig. 2*B*), which is a key step in the GAAC response. To examine whether Cpc2 is required for eIF2α phosphorylation, we checked the phosphorylation pattern after the addition of 3AT in *cpc2*Δ cells. Loss of *cpc2* resulted in a marked decrease in the level of eIF2α phosphorylation when compared with wild-type cells, indicating that Cpc2 is important for eIF2α phosphorylation under amino acid starvation (Fig. 2*B*, wild-type and *cpc2*Δ).

We observed a relatively weak and delayed eIF2α phosphorylation signal in *cpc2*Δ cells at 60 min after 3AT addition (Fig. 2*B*, wild-type and *cpc2*Δ). This signal pattern was similar in *gcn2*Δ *cpc2*Δ double-mutant cells (Fig. 2*B*), supporting the idea that Gcn2-dependent eIF2α phosphorylation is abrogated in *cpc2*Δ cells. On the other hand, these results raise the possibility that the delayed eIF2α phosphorylation in *cpc2*Δ cells is independent of Gcn2. Among three eIF2α kinases (Gcn2, Hri1, and Hri2) in *S. pombe*, Gcn2 plays a major role in phosphorylation of eIF2α under amino acid starvation (10, 11), as shown in the data for *gcn2*Δ cells (Fig. 2*B*, *gcn2*Δ). To test the possibility that other eIF2α kinases are responsible for the delayed eIF2α phosphorylation in *cpc2*Δ cells, the effects of deletions of other eIF2α kinases were examined. We found that although the eIF2α phosphorylation kinetics in *hri2*Δ cells were comparable with wild-type cells, the *hri2* deletion in a *cpc2*Δ background caused the loss of delayed eIF2α phosphorylation (Fig. 2*C*). These data suggest that prolonged 3AT treatment in *cpc2*Δ cells activates Hri2 and leads to Hri2-mediated delayed eIF2α phosphorylation. However, the 3AT sensitivity of *hri2*Δ *cpc2*Δ double-mutant cells was comparable with *cpc2*Δ cells (Fig. 2*D*), suggesting that delayed phosphorylation does not contribute substantially to cell viability under this condition. Collectively, these results indicate that *S. pombe* Cpc2 facilitates survival under amino acid starvation, presumably by promoting Gcn2-mediated eIF2α phosphorylation.

Cpc2 Is Dispensable for Ribosomal Association and Dimerization of Gcn2—Cpc2 and Gcn2 exhibited similar defects in the amino acid starvation response in *S. pombe* (Figs. 1 and 2). These observations raised the possibility that *S. pombe* Cpc2 modulates the Gcn2-mediated stress response. *S. cerevisiae* Gcn2 associates with the ribosome via its carboxyl-terminal domain, and appropriate ribosomal localization potentiates Gcn2 function in translational control (18). This carboxyl-terminal domain is also important for dimerization, which is essential for *S. cerevisiae* Gcn2 function *in vivo* (15, 16). Because Cpc2 is also a ribosomal binding protein (29), we first asked whether Cpc2 affects ribosomal binding of Gcn2 in *S. pombe*. To analyze the association of Gcn2 with ribosomes, we constructed cells in which both Gcn2 and rpS3, a component of the 40 S ribosomal subunit, were tagged with FLAG. Fractionation of whole cell extracts using sucrose gradient centrifugation yields ribosomes distributed along the gradient depending on their sedimentation coefficient (Fig. 3*A*). We detected a significant amount of Gcn2 in ribosomal fractions (fraction numbers 6–19) from wild-type cells (Fig. 3*B*), consistent with the idea that *S. pombe* Gcn2 interacts with ribosomes, as observed in *S. cerevisiae*. Ribosomal association of Gcn2 remained largely unchanged in *cpc2*Δ cells (Fig. 3*C*), suggesting that Gcn2 does not require Cpc2 to interact with ribosomes. We next examined the effect of Cpc2 on Gcn2 dimerization using heterozygous diploid strains expressing Gcn2 tagged with FLAG or Myc from each *gcn2* allele. Immunoprecipitation assays revealed that FLAG-tagged Gcn2 associates with Myc-tagged Gcn2 (Fig. 3*D*), suggesting that *S. pombe* Gcn2 formed a dimer, as is the case in *S. cerevisiae*. This interaction was independent of Cpc2 (Fig. 3*D*). These results indicate that *S. pombe* Cpc2 does not regulate ribosomal localization or dimerization of Gcn2.

Cpc2 Is Required for Gcn2 Autophosphorylation—To further investigate how Cpc2 regulates Gcn2-dependent eIF2α phosphorylation, we next focused on autophosphorylation of Gcn2. In *S. cerevisiae*, autophosphorylation of two threonine residues (Thr-882 and Thr-887) in Gcn2 protein is essential for its full kinase activity (17). To examine its relevance in *S. pombe*, we generated *gcn2_TA* cells carrying alanine substitutions at predicted autophosphorylation sites (Thr-818 and Thr-823) corresponding to Thr-882 and Thr-887 in *S. cerevisiae* Gcn2 (Fig. 4*A*). As a control, a kinase-dead mutant (*gcn2_KR*), in which a lysine residue (Lys-585) essential for kinase activity was replaced by arginine, was also created (Fig. 4*A*). We performed *in vitro* kinase assays using FLAG-tagged Gcn2 immunoprecipitated from wild-type and mutant *S. pombe* cells and observed a Gcn2 phosphorylation signal that was dependent on its kinase activity (Fig. 4*B*, wild-type and *gcn2_KR*). Moreover, this experiment revealed that the T818A and T823A mutations largely abolished the phosphorylation signal of Gcn2, suggesting that Thr-818 and/or Thr-823 of *S. pombe* Gcn2 are major target residues that are autophosphorylated *in vitro*, as shown in *S. cerevisiae* (17) (Fig. 4*B*, *gcn2_TA*). To assess the significance of the phosphorylation *in vivo*, we raised an anti-Gcn2-phospho-Thr-818 antibody. We detected an increased phosphorylation signal at Thr-818 of Gcn2 in wild-type cells upon 3AT treatment, whereas no phosphorylation was observed in *gcn2_TA* or *gcn2_KR* mutant cells (Fig. 4*C*), supporting the idea

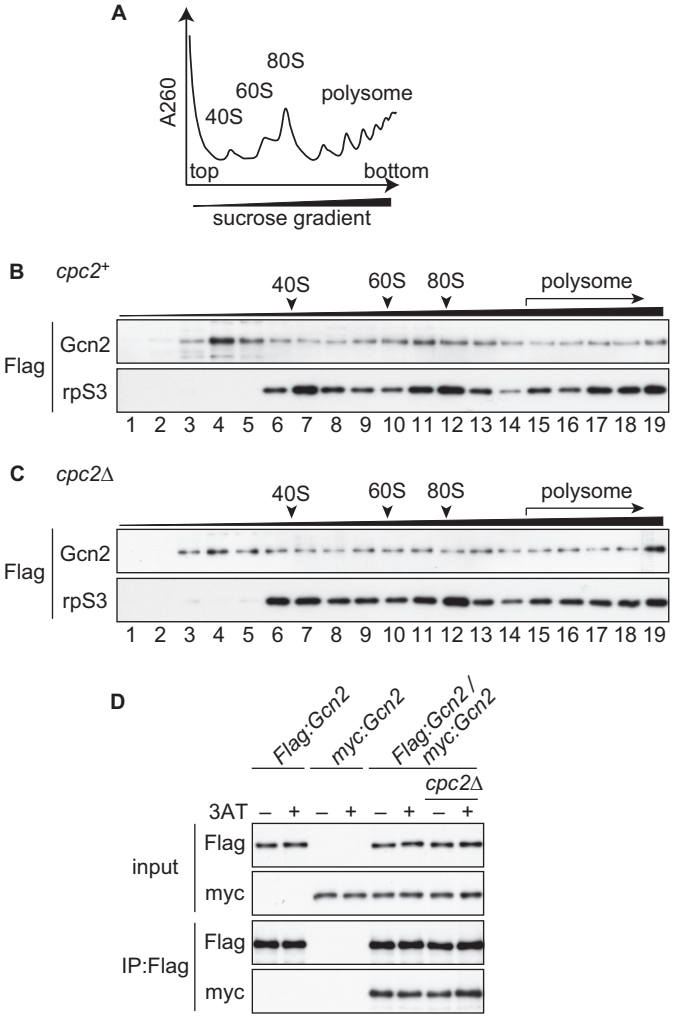

FIGURE 3. **Cpc2 is dispensable for ribosomal binding and dimerization of Gcn2.** *A*, schematic diagram of distribution of ribosomes after fractionation using sucrose gradient centrifugation. The predicted absorbance curve at 260 nm is shown along the gradient. *B* and *C*, immunoblots of gradient fractions to detect FLAG-Gcn2 and FLAG-rpS3. Whole cell extracts were prepared from *Flag:gcn2 rpS3:Flag* (YT3650) (*B*) or *Flag:gcn2 rpS3:Flag cpc2*Δ (YT3658) (*C*) cells, and both Gcn2 and rpS3 were detected using anti-FLAG antibody. *D*, co-immunoprecipitation (*IP*) assay of Gcn2 dimerization from *Flag:gcn2/gcn2*$^+$ diploid (YT4376), *myc:gcn2/gcn2*$^+$ diploid (YT4281), *Flag:gcn2/myc:gcn2* diploid (YT4279), and *Flag:gcn2/myc:gcn2 cpc2*Δ/*cpc2*Δ diploid (YT4280) cells treated with 10 mM 3AT 15 min (+) or not (−). Immunoblots were performed using anti-FLAG and anti-Myc antibodies.

that Thr-818 is an autophosphorylation site of *S. pombe* Gcn2. We also found that the increase in eIF2α phosphorylation under amino acid starvation was lost in *gcn2_TA* and *gcn2_KR* mutant cells (Fig. 5*A*). Concordantly, these mutant cells were highly sensitive to 3AT treatment (Fig. 5*B*). These results indicate that autophosphorylation is essential for Gcn2 activity and cell survival during amino acid starvation in *S. pombe*. To investigate whether Cpc2 is required for autophosphorylation of Gcn2, we examined Gcn2 Thr-818 phosphorylation in *cpc2*Δ cells. Intriguingly, the Gcn2 phospho-Thr-818 signal was virtually abrogated by the *cpc2* deletion (Fig. 5*C*), indicating that *S. pombe* Cpc2 is required for Gcn2 activation. Taken together, these results demonstrate that *S. pombe* Cpc2 positively regulates the GAAC response by facilitating Gcn2 autophosphorylation.

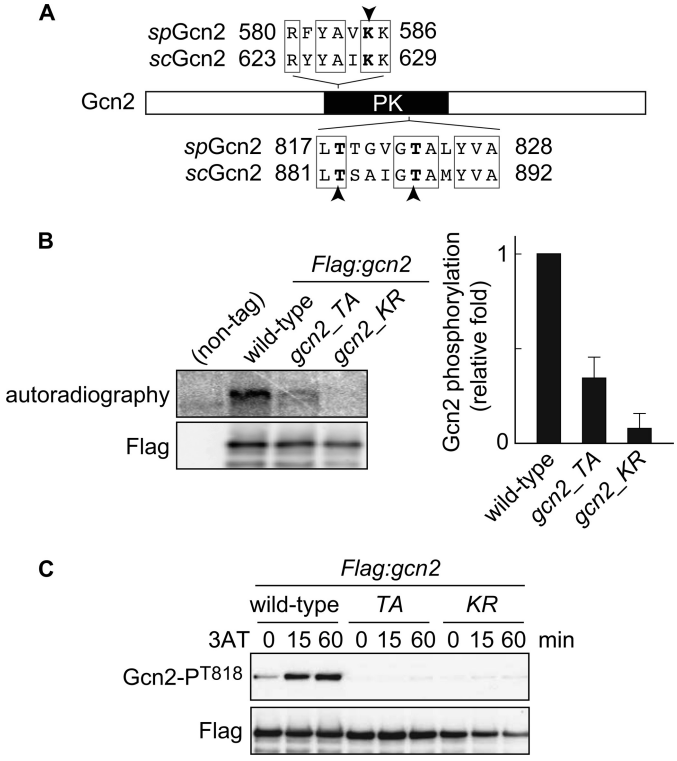

FIGURE 4. **Gcn2 is autophosphorylated *in vitro* and *in vivo*.** *A*, domain structure of Gcn2 protein. Lys-628 and threonines Thr-882 and Thr-887, indicated by *arrowheads*, in *S. cerevisiae* Gcn2 (*sc*Gcn2) are essential sites for kinase activity and autophosphorylation, respectively. The corresponding amino acids in *S. pombe* Gcn2 (*sp*Gcn2) are also shown. *PK*, protein kinase domain. *B*, *in vitro* kinase assay using FLAG-Gcn2 immunoprecipitated from cells harboring FLAG-tagged Gcn2 (*Flag:gcn2*, YT3372) and nontagged control cells (JK317). Autoradiography and immunoblot probed with anti-FLAG antibody are shown. *Flag:gcn2_TA* cells (YT3657) have alanine substitution at predicted autophosphorylation sites. *Flag:gcn2_KR* cells (YT3656) are kinase-dead mutants. Quantified Gcn2 phosphorylation signals (Gcn2P/Gcn2) are shown as the relative fold (S.E. ($n = 3$)) with respect to the level of *Flag:gcn2* cells. *C*, immunoblots of phosphorylated Gcn2 and FLAG (control) from *Flag:gcn2* (YT3372), *Flag:gcn2_TA* (YT3657), and *Flag:gcn2_KR* (YT3656) cells treated with 10 mM 3AT. FLAG-Gcn2 was immunoprecipitated before immunoblots.

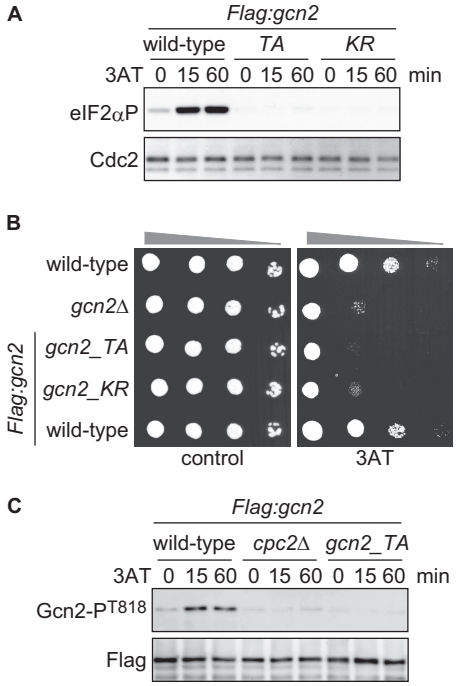

FIGURE 5. **Cpc2 is required for starvation-induced Gcn2 activation.** *A*, immunoblots of phosphorylated eIF2α and Cdc2 (control) from *Flag:gcn2* (YT3372), *Flag:gcn2_TA* (YT3657), and *Flag:gcn2_KR* (YT3656) cells treated with 10 mM 3AT. *B*, wild-type (JK317), *gcn2*Δ (YT3360), *Flag:gcn2* (YT3372), *Flag:gcn2_TA* (YT3657), and *Flag:gcn2_KR* (YT3656) cells were 10-fold serially diluted and spotted on EMM plates ± 10 mM 3AT. *C*, immunoblots of anti-FLAG-Gcn2 immunoprecipitates from *Flag:gcn2* (YT3372), *Flag:gcn2 cpc2*Δ (YT3598), and *Flag:gcn2_TA* (YT3657) cells treated with 10 mM 3AT, probed with anti-phospho-Gcn2 and anti-FLAG (control) antibodies.

Cpc2, but Not snoU24b, Is Important for the GAAC Response—An intron of the *S. pombe cpc2* gene contains *snoU24b*, encoding a C/D box U24 small nucleolar RNA (Fig. 6*A*). The particular *cpc2*Δ strain that we used was one in which the *cpc2* gene was disrupted by replacement of the first exon with a marker gene (supplemental Fig. S1*A*). As expected, this strain did not express Cpc2 protein (supplemental Fig. S1, *A* and *B*), but it also lost *snoU24b* expression (Fig. 6*B*, *cpc2*Δ). Although most previous studies have not taken this point into account, it has been recently reported that in *S. cerevisiae*, the ribosome assembly defect (half-mer polysomes) observed in the cells deleted within the *asc1*-containing genomic region is ascribed to the loss of a small nucleolar RNA carried in an intron of the *ASC1* gene (35), underlining the importance of testing whether defective *snoU24b* is responsible for phenotypes observed in *cpc2*Δ cells. To that end, we generated cells (*cpc2_W43**) harboring a nonsense mutation in place of Trp43 in Cpc2 (Fig. 6*A*). No Cpc2 protein was detected in *cpc2_W43** cells (supplemental Fig. S1, *A–C*). As expected, the expression level of *snoU24b* in *cpc2_W43** cells was comparable with that in wild-type cells (Fig. 6*B*). A decrease in *cpc2* mRNA abundance is presumably caused by nonsense-mediated mRNA decay. *cpc2_W43** cells exhibited a severe 3AT sensitivity similar to that in *cpc2*Δ cells (Fig. 6*C*). Moreover, 3AT-induced Gcn2 autophosphorylation was abrogated in *cpc2_W43** cells (Fig. 6*D*), indicating that Cpc2, but not snoU24b, contributes to regulation of Gcn2 and the GAAC response.

Ribosome-free Cpc2 Has a Role in Regulating Gcn2—Although Cpc2 does not affect the ribosomal association of Gcn2 (Fig. 3), the fact that both Cpc2 and Gcn2 bind to ribosomes prompted us to investigate whether the association of Cpc2 with ribosomes is a prerequisite for Gcn2 activation. To further examine this point, we prepared ribosome-unbound *cpc2_DE* mutant cells, in which two amino acids (Arg-36 and Lys-38) responsible for ribosome binding were substituted by aspartic acid and glutamic acid, respectively (30, 42). As reported previously (42), distribution of Cpc2_DE was observed in ribosome-free fractions after fractionation using sucrose gradient centrifugation (Fig. 7*A*). We examined whether eIF2α phosphorylation and Gcn2 autophosphorylation were abrogated in *cpc2_DE* mutant cells and found no significant difference in the kinetics or intensities of the phosphorylation signals between wild-type and *cpc2_DE* mutant cells (Fig. 7, *B* and *C*). These results suggest that Cpc2 retains an ability to regulate Gcn2 without ribosomal association. Moreover, *cpc2_DE* mutant cells exhibited no sensitivity to 3AT treatment (Fig. 7*D*), supporting the notion that ribosome-unbound Cpc2 is functional in Gcn2 regulation and the GAAC response.

RACK1 Homologs Compensate for the Defects in the Regulation of Gcn2 in S. pombe cpc2 Mutant Cells—As mentioned above, *S. pombe* Cpc2 facilitates the Gcn2-mediated GAAC

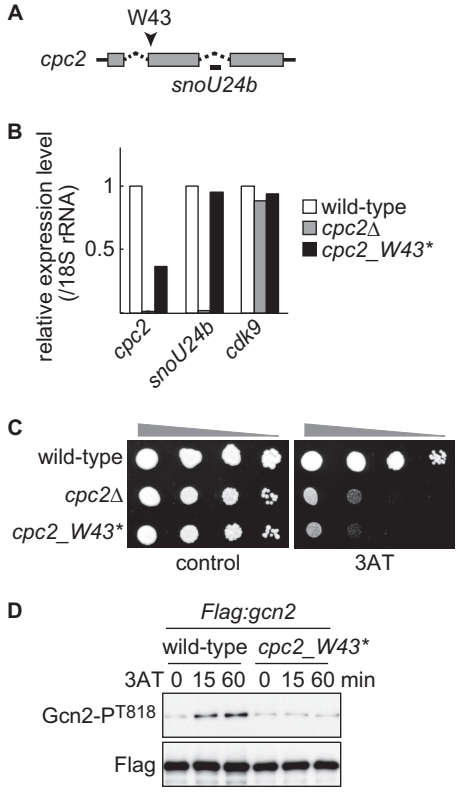

FIGURE 6. **Cpc2, but not snoU24b RNA, is important for Gcn2 regulation.** *A*, small nucleolar RNA U24b is encoded within the intron of *cpc2* mRNA. *Gray box*, coding sequence; *dashed line*, intron; *solid line*, untranslated region. The position encoding Trp43 is indicated by an *arrowhead*. *B*, gene expression levels of *cpc2*, *snoU24b*, and *cdk9*$^+$ (control) were analyzed by quantitative RT-PCR from wild-type (YT3033), *cpc2*Δ (YT2307), and *cpc2_W43** (YT4299) cells. All data are normalized to the expression level of 18 S rRNA, and the average expression level of two independent experiments is shown as the relative fold to those in wild-type cells. *C*, 10-fold serial dilutions of wild-type (JK317), *cpc2*Δ (YT3540), and *cpc2_W43** (YT4299) cells were spotted on EMM plates ± 10 mM 3AT. *D*, immunoblots of anti-FLAG-Gcn2 immunoprecipitates from *Flag:gcn2* (YT3372) and *Flag:gcn2 cpc2_W43** (YT4309) cells treated with 10 mM 3AT, probed with anti-phospho-Gcn2 and anti-FLAG (control) antibodies.

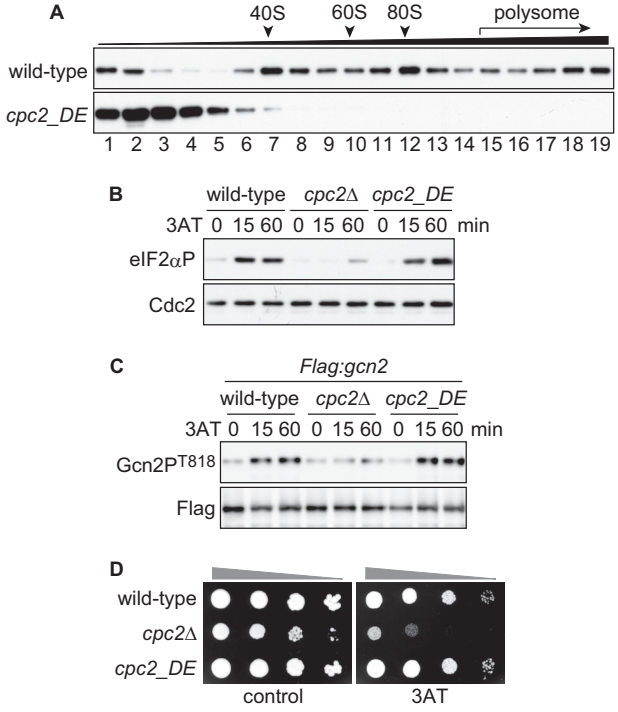

FIGURE 7. **Ribosome-free Cpc2 is able to regulate Gcn2.** *A*, immunoblots of Cpc2 from fractionated samples prepared from *Flag:gcn2* (YT3372) and *Flag:gcn2 cpc2_DE* (YT3559) cells. *B*, immunoblots of phosphorylated eIF2α and Cdc2 (control) from wild-type (JK317), *cpc2*Δ (YT3540), and *cpc2_DE* (YT3542) cells treated with 10 mM 3AT. *C*, immunoblots of anti-FLAG-Gcn2 immunoprecipitates from *Flag:gcn2* (YT3372), *Flag:gcn2 cpc2*Δ (YT3598), and *Flag:gcn2 cpc2_DE* (YT3559) cells treated with 10 mM 3AT, probed with anti-phospho-Gcn2 and anti-FLAG (control) antibodies. *D*, 10-fold serial dilutions of wild-type (JK317), *cpc2*Δ (YT3540), and *cpc2_DE* (YT3542) cells were spotted on EMM plates ± 10 mM 3AT.

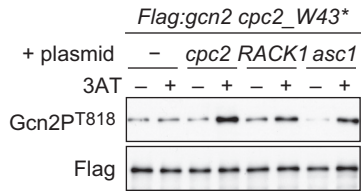

FIGURE 8. **RACK1 homologs compensate for the defects in the regulation of Gcn2.** *Flag:gcn2 cpc2_W43** (YT4309) cells harboring an empty plasmid (−) or the expression plasmids (*cpc2*, *RACK1*, or *asc1*) were treated with 10 mM 3AT 60 min (+) or not (−). Immunoblots of anti-FLAG-Gcn2 immunoprecipitates were probed with anti-phospho-Gcn2 and anti-FLAG (control) antibodies.

response. By contrast, deletion of *S. cerevisiae* Asc1 promotes the GAAC response (34). To address this discrepancy between the two species, we investigated whether *S. cerevisiae* Asc1 and human RACK1 rescue the Gcn2 defects in *S. pombe cpc2*Δ cells. We cloned cDNA of *cpc2*, *asc1*, and *RACK1* genes in an expression plasmid and introduced them individually into *cpc2* mutant cells. The expression levels of Asc1 and RACK1 proteins were similar to the level of Cpc2 (supplemental Fig. S2). Immunoblotting of the phosphorylated Gcn2 in the cells harboring the expression plasmids revealed that either *asc1* or *RACK1* expression restored 3AT-induced Gcn2 phosphorylation in *cpc2* mutant cells (Fig. 8), indicating that Asc1 and RACK1 play the same role as Cpc2 in the regulation Gcn2 in *S. pombe*. These observations suggest that the phenotypic difference between *S. pombe* and *S. cerevisiae* lies in other components in the system regulating the GAAC response.

DISCUSSION

It has been reported that RACK1 homologs are involved in the GAAC response in *S. cerevisiae* and *N. crassa*. However, the molecular mechanisms of action of the RACK1 homologs in the GAAC response have not been fully elucidated. In this study, we have focused on *S. pombe* Cpc2 in the GAAC response. Our findings demonstrate that deletion of *S. pombe cpc2* impairs 3AT-induced eIF2α phosphorylation and subsequent expression of amino acid biosynthesis genes and that *cpc2*Δ cells are highly sensitive to 3AT treatment. Moreover, *S. pombe* Cpc2 is required for autophosphorylation of Gcn2. Thus, *S. pombe* Cpc2 positively contributes to the GAAC response through the activation of eIF2α kinase Gcn2.

Amino acid sequences among RACK1 homologs (mammalian RACK1, *S. pombe* Cpc2, *S. cerevisiae* Asc1, and *N. crassa* Cpc-2) are highly conserved, and they show >70% similarity (25, 34). Indeed, ectopic expression of mammalian RACK1 compensated several defects observed in *S. pombe cpc2*Δ cells (25) and *S. cerevisiae asc1*Δ cells (43). Moreover, both *S. pombe* and *N. crassa* devoid of *cpc2* (*cpc-2*) exhibit defects in fertility

(25, 37), supporting the idea that RACK1 homologs phylogenetically share a similar function. On the other hand, the requirement of RACK1 homologs in the GAAC response seems to differ among species. In *S. pombe*, Cpc2 promotes the GAAC response as shown in this work. By contrast, *S. cerevisiae* Asc1 has an apparently opposite effect on the GAAC response as Asc1 physically interacts with the eIF3 complex to facilitate efficient translation initiation, which suppresses the GAAC response (35). In *N. crassa*, the *cpc-2 U142* mutation causes defects in amino acid deprivation-induced gene expression (36), suggesting that *N. crassa* Cpc-2 contributes to the GAAC response in a similar fashion to *S. pombe* Cpc2. However, because ectopic expression of the *N. crassa cpc-2 U142* allele in *S. cerevisiae asc1*Δ cells causes the defect in the GAAC response (34), one cannot exclude the possibility that *cpc-2 U142* produces an allele-specific effect. Our rescue experiment revealed that the ectopic expression of *S. cerevisiae asc1* and human *RACK1* restored 3AT-induced Gcn2 phosphorylation in *cpc2* mutant cells. This observation suggests that the discrepancy between the function of RACK1 homologs in *S. cerevisiae* and *S. pombe* is not due to different structures intrinsic to each of the RACK1 homologs, but rather to Gcn2 or other upstream factors mediating the activation signal of Gcn2. Thus, although RACK1 homologs have a conserved function, additional factor(s) may modify their function in the GAAC response in different species.

In *S. pombe cpc2*Δ cells, but not in *gcn2*Δ cells, Hri2-dependent delayed phosphorylation was observed. Hri2 is activated in response to oxidative stress (11). Prolonged 3AT treatment causes the accumulation of reactive oxygen species in the cells, and Gcn2 counters this by inducing the expression of antioxidant genes (44). Moreover, it is known that Cpc2 is also involved in the defense against oxidative stress by regulating the amount of Atf1 protein (42), which is an important transcriptional factor for stress-activated MAP kinase-dependent induction of stress-responsive genes. These results raised the possibility that defects in both Gcn2-mediated and Atf1-mediated responses in *cpc2*Δ cells cause oxidative stress in the cells, thence leading to the activation of Hri2. However, the delayed phosphorylation was not observed in *gcn2*Δ *atf1*Δ double-mutant cells,[4] suggesting that Cpc2 may suppress the delayed eIF2α phosphorylation through yet unknown mechanisms.

RACK1 homologs across many eukaryotic species generally locate at the head of the 40 S ribosomal subunit near the mRNA exit site on 80 S ribosomes (30). Ribosome-localized RACK1 promotes translational initiation (31, 35) or mediates translation of specific mRNAs through the interaction with RNA-binding protein (33). Indeed, *S. pombe cpc2* deletion causes the reduction of steady-state levels of several proteins, some of which are regulated at the translational level by ribosome-bound Cpc2 (29, 42). On the other hand, RACK1 homologs also have ribosome localization-independent roles (33, 45). In our study of *cpc2_DE* mutant cells, ribosomal association of Cpc2 was dispensable for regulation of Gcn2 and survival under amino acid starvation. This result suggests that the defect in Gcn2-mediated responses observed in *cpc2*Δ cells is not simply caused by the reduction of steady-state translation. Rather, it is conceivable that Cpc2 modulates an activity or interaction with regulatory proteins of Gcn2 to promote Gcn2 activation under stress conditions.

The observation that ribosome-free Cpc2 is functional for regulation of Gcn2 is intriguing because most of the Cpc2 and Gcn2 in the cell interact with ribosomes, as shown in our experiments. There are two plausible explanations for Gcn2 regulation by ribosome-unbound Cpc2. In one scenario, free Cpc2 may modulate the activity of Gcn2 at extra-ribosomal sites prior to ribosomal loading of Gcn2. Alternatively, Cpc2 may regulate Gcn2 on ribosomes. It has been thought that Gcn2 associates with the 60 S ribosomal subunit near the decoding A-site, which is distant from the Cpc2-binding site. If Cpc2 interacts with Gcn2, it seems likely that Cpc2 transiently dissociates from its binding site to access Gcn2 on ribosomes. Post-translational modifications modulate Gcn2 activity. TOR (target of rapamycin), an important kinase for nutrient response, indirectly regulates phosphorylation of Ser-577 of *S. cerevisiae* Gcn2, which inhibits Gcn2 activation (20, 46). Moreover, Snf1, an ortholog of mammalian AMP-activated kinase, promotes Gcn2 activation under amino acid starvation by an unknown mechanism in *S. cerevisiae* (23). These observations indicate that, besides uncharged tRNA, other signals including post-translational modifications modulate Gcn2 activity. RACK1 homologs function as a scaffold in signal transduction (26, 32). Thus, although it is unknown whether Cpc2 and Gcn2 interact directly in *S. pombe*, Cpc2 may transmit an activating signal from an upstream factor to Gcn2 in ribosomal or extra-ribosomal contexts. Precisely how and where Cpc2 regulates Gcn2 in the GAAC response will be explored in future analyses.

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[4] Y. Tarumoto, J. Kanoh, and F. Ishikawa, unpublished data.

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