Nonoptimal Codon Usage Is Critical for Protein Structure and Function of the Master General Amino Acid Control Regulator CPC-1

Xueliang Lyu,a,b Yi Liu a

aDepartment of Physiology, The University of Texas Southwestern Medical Center, Dallas, Texas, USA
bState Key Laboratory of Agricultural Microbiology, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, China

ABSTRACT Under amino acid starvation conditions, eukaryotic organisms activate a general amino acid control response. In Neurospora crassa, Cross Pathway Control Protein 1 (CPC-1), the ortholog of the Saccharomyces cerevisiae bZIP transcription factor GCN4, functions as the master regulator of the general amino acid control response. Codon usage biases are a universal feature of eukaryotic genomes and are critical for regulation of gene expression. Although codon usage has also been implicated in the regulation of protein structure and function, genetic evidence supporting this conclusion is very limited. Here, we show that Neurospora cpc-1 has a nonoptimal NNU-rich codon usage profile that contrasts with the strong NNC codon preference in the genome. Although substitution of the cpc-1 NNU codons with synonymous NNC codons elevated CPC-1 expression in Neurospora, it altered the CPC-1 degradation rate and abolished its amino acid starvation-induced protein stabilization. The codon-manipulated CPC-1 protein also exhibited different sensitivity to limited protease digestion. Furthermore, CPC-1 functions in rescuing the cell growth of the cpc-1 deletion mutant and activation of the expression of its target genes were impaired by the synonymous codon changes. Together, these results reveal the critical role of codon usage in regulation of CPC-1 expression and function and establish a genetic example of the importance of codon usage in protein folding.

IMPORTANCE The general amino acid control response is critical for adaptation of organisms to amino acid starvation conditions. The preference to use certain synonymous codons is a universal feature of all genomes. Synonymous codon changes were previously thought to be silent mutations. In this study, we showed that the Neurospora cpc-1 gene has an unusual codon usage profile compared to other genes in the genome. We found that codon optimization of the cpc-1 gene without changing its amino acid sequence resulted in elevated CPC-1 expression, an altered protein degradation rate, and impaired protein functions due to changes in protein structure. Together, these results reveal the critical role of synonymous codon usage in regulation of CPC-1 expression and function and establish a genetic example of the importance of codon usage in protein structure.

KEYWORDS codon usage, CPC-1, Neurospora, cross-pathway control, GCN4, cotranslational protein folding, translation elongation

Transcriptional regulation allows organisms to respond to changes in environmental conditions. Under amino acid starvation conditions, fungi activate a general amino acid control response that induces expression of genes involved in amino acid biosynthesis (1–4). The signal transduction pathways that mediate these responses are similar in eukaryotic cells from yeast to mammals. In the budding yeast Saccharomyces cerevisiae and the filamentous fungus Neurospora crassa, bZIP

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Address correspondence to Yi Liu, Yi.Liu@UTsouthwestern.edu.

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transcription factors GCN4 and Cross-Pathway Control Protein 1 (CPC-1), respectively, are the master transcriptional regulators that activate amino acid biosynthetic genes in response to amino acid limiting conditions (1–6). Like GCN4, CPC-1 binds to the 5’-TGA(C/G)TCA-3’ motifs in target gene promoters to activate transcription (1–3, 5).

A mechanism involving upstream open reading frames (uORFs) in GCN4 modulates GCN4 protein production (1, 2). Under normal conditions, the translation of the uORFs prevents translation initiation from the GCN4 ORF, resulting in the suppression of GCN4 expression. Under amino acid starvation conditions, however, the scanning 40S ribosomes bypass the uORFs and initiate translation at the downstream GCN4 ORF, resulting in the induction of GCN4 protein expression. This type of uORF-mediated mechanism is conserved in general amino acid control responses from fungi to mammals: translational induction of CPC-1 in Neurospora and of ATF4 in mammals is controlled by this mechanism (7, 8). We recently showed that impaired tRNA I34 modification also triggers an amino acid starvation-like response (9). Ribosome profiling experiments, which were used to monitor ribosome occupancy on translating mRNAs, showed that there were many more ribosomes bypassing the two cpc-1 uORFs and translating the downstream open reading frame region when tRNA I34 modification was suppressed (9).

Posttranslational regulation of GCN4 stability is another mechanism that contributes to its upregulation in response to amino acid starvation (1). GCN4 is very unstable when yeast cells are cultured in rich medium, with a half-life of several minutes, but its degradation becomes much slower under amino acid starvation conditions (10, 11). The degradation of GCN4 is mediated by the proteasome ubiquitination pathway and is dependent on its phosphorylation by cyclin-dependent kinases (11–13). It is not clear whether amino acid availability also regulates CPC-1 stability in Neurospora.

Due to the degeneracy of genetic code, most amino acids are encoded by two to six synonymous codons. Codon usage bias, the preference for certain synonymous codons for almost all amino acids, has been found in all genomes examined (14–17). Codon usage bias is an important determinant of gene expression levels in both eukaryotes and prokaryotes (18–21). We and other groups previously showed that codon usage regulates translation elongation speed: common codons enhance the local rate of translation elongation, whereas rare codons slow translation elongation (22–25). Rare codons preferentially cause ribosome stalling on an mRNA during translation, and this can result in premature translation termination and reduce translation efficiency (22, 24, 26). Furthermore, codon usage bias can regulate gene expression by affecting transcription (27–31).

In addition to the effect of codon usage on gene expression (32), accumulating biochemical and genetic evidence suggests that codon usage can also influence the cotranslational protein folding process through its effects on translation elongation speed, which influences the time available for cotranslational folding (19, 22, 26, 28, 33–46). It was previously shown in Escherichia coli that codon usage can affect the protein activity and structures of some overexpressed proteins (37, 38, 40, 43, 47). In eukaryotes, a synonymous single-nucleotide polymorphism of the human MDR1 gene was previously shown to cause altered protein activity of the MDR1 protein transiently overexpressed in human cells, suggesting the involvement of codon usage in eukaryotic protein folding (39). More recently, codon usage was also shown to influence protein activity and/or structures of several other human proteins (28, 45, 48–50). However, those previous studies relied on protein overexpression, which could also influence protein folding in cells, and the degree of impact of codon usage on protein structure/function was often modest.

By studying the circadian clock genes in Neurospora and Drosophila, we previously demonstrated that the codon usage of circadian clock gene frq in Neurospora and Per in Drosophila plays a major role in determining the protein structure and function in vivo (19, 36). Importantly, those studies did not use protein overexpression and the functional impacts of codon usage on protein function and structure were very robust.
in these genetic systems, thus confirming the physiological role of codon usage in protein folding in eukaryotic systems. Furthermore, genome-wide correlations between gene codon usage and predicted protein structures have been observed in prokaryotes and eukaryotes, suggesting that codon usage functions as a universal code to broadly modulate protein folding (33, 51–53). However, there are currently only very few genetic examples that demonstrate the robust physiological influence of codon usage on protein folding and function (19, 36, 38).

*Neurospora crassa* has a strong codon usage bias for C/G at wobble positions (33, 54), but we observed that the *cpc-1* gene has an abnormal NNU-rich codon usage bias. Amino acid starvation triggers the stabilization of yeast GCN4, and we observed a similar starvation-induced stabilization of CPC-1 protein. By changing the NNU codons of *cpc-1* to synonymous NNC codons, we demonstrated that the codon usage of *cpc-1* is required for CPC-1 stabilization in response to amino acid starvation, and that it is critical for the CPC-1 structure and function in vivo. Together, our results demonstrate the role of codon usage in controlling CPC-1 expression and function and establish another genetic example of the importance of codon usage in protein folding.

**RESULTS**

**Abnormal codon usage profile of *cpc-1***. Examination of the *N. crassa cpc-1* gene revealed that it has an unusual codon usage profile. The *Neurospora* genome has a strong preference for NNC codons in every ADAT-related codon family (the A34 positions of their corresponding tRNAs can be converted to I34 by adenosine deaminases acting on tRNAs, known as ADATs) and for NNC/NNG codons in other codon families (33, 54). In contrast, for *cpc-1*, NNU codons are the most preferred codons for five (Ala, Pro, Arg, Ser, and Val) of the eight ADAT-related codon families (Fig. 1A). For Leu codons of *cpc-1*, the normally preferred CUC codon is one of the least used codons; it has a lower usage frequency than CUU. The usage frequency of ACU, which codes for Thr, is also higher in *cpc-1* than the genome average (Fig. 1A). Interestingly, the genome-preferred NNC codons are also not the preferred codons for the majority of the ADAT-related codon families in homologous *cpc-1* genes in *Neurospora tetrasperma*, *Sordaria macrospora*, and *Aspergillus nidulans* (see Fig. S1 in the supplemental material), suggesting that nonoptimal *cpc-1* codon usage is conserved. These results raised the possibility that the nonoptimal nature of the *cpc-1* codon usage profile is functionally important.

**Regulation of CPC-1 expression by *cpc-1* codon usage**. The unusual NNU-rich codon usage profile of *cpc-1* suggests that it may play a biological role in regulating CPC-1 expression or function. To test this hypothesis, we created two versions of the *cpc-1* ORF: *cpc-1*(WT) (*cpc-1* wild type), in which all native codons were maintained, and *cpc-1*(T→C), in which all eight ADAT-related NNU codons (except for the codons for the N-terminal 16 amino acids) were substituted synonymously with the genome-preferred NNC codons without altering the amino acid sequence (Fig. S2). Thus, *cpc-1*(T→C) has more optimal codons than the wild-type (WT) *cpc-1* gene. We expressed 5’ epitope-tagged versions of *cpc-1*(WT) and *cpc-1*(T→C) ORFs under the control of the *ccg-1* promoter and the *ccg-1* 5’ untranslated region (5’ UTR) to exclude the effect of the uORFs and the 5’ UTR of *cpc-1* on translation. To minimize the potential impact of codon usage on translation initiation, the N-terminal regions (which include 3×Flag, an 8×Gly linker, and the codons for the initial 16 N-terminal amino acids of CPC-1) of the two versions of *cpc-1* were identical (Fig. 1B). Constructs containing the *cpc-1* transgenes were individually transformed into *Neurospora* strain 87-3 at the targeted *csr-1* locus. Homokaryotic transformant strains were cultured in 2% glucose medium with or without 3-aminotriazole (3-AT). 3-AT treatment results in amino acid starvation in *Neurospora* because it is a competitive inhibitor of the product of *his-3* gene, which is an enzyme required for histidine biosynthesis (6, 55–57). Because of the cross-pathway control in *Neurospora*, depletion of one amino acid leads to a general amino acid starvation response (3).
FIG 1 The ADAT-related NNU-rich codon usage of cpc-1 contributes to the regulation of CPC-1 production. (A) The codon usage of cpc-1 represented by the relative codon contents in each codon family compared to the genome-wide average codon usage. (B) Graphical representation of the constructs used for the expression of cpc-1(WT) and optimized cpc-1(T→C). To avoid the influences of the cpc-1 uORFs and 5' UTR, the ccg-1 promoter (P_{ccg-1}) and its 5' UTR were used to drive the expression of cpc-1. The constructs were integrated into the csr-1 locus in the N. crassa genome by homologous recombination. To avoid the influence of codon optimization on translation initiation, the first 50 codons (including the codons of 3×Flag and 8×Gly and 16 codons at the N terminus of cpc-1) were kept the same in both constructs. In cpc-1(T→C), 71 of the 270 ADAT-related NNU codons were changed to the most preferred NNC codons of N. crassa genome. For details, see Fig. S2. (C) The relative mRNA levels of cpc-1(WT) and cpc-1(T→C) detected by qRT-PCR in the host strain cultured in 2% glucose medium with or without 5 mM 3-aminotriazole (3-AT). The cpc-1 mRNA levels were normalized to that of the β-tubulin gene (NCU04054). Primers used for qRT-PCR were designed to correlate to the 5' region of the transcript, which is common to the two constructs (as shown in panel B) to ensure the same amplification efficiency. The cpc-1(WT) transcript level was set as 1.0. (D) (Upper panel) Western blot analysis of CPC-1 expressed in the cpc-1(WT) and cpc-1(T→C) strains cultured in 2% glucose medium with or without 5 mM 3-aminotriazole (3-AT). A nonspecific constitutive band detected by the anti-Flag antibody was used as the control. (Lower panel) Densitometric analyses of the CPC-1 levels from three independent experiments. The CPC-1 protein level produced from cpc-1(WT) was set as 1.0. Data in panels C and D are means ± standard deviations (SD) (n = 3). *, P < 0.05, as determined by Student's two-tailed t test.
Gene codon optimization usually results in increased mRNA and protein levels in *Neurospora* (27, 58, 59). As expected, the mRNA levels of *cpc-1* (T→C) were significantly higher than those of *cpc-1* (WT) when the genes were expressed in the host strain cultured in 2% glucose medium with or without 3-AT (Fig. 1C). Similarly, the CPC-1 protein levels were also upregulated in the *cpc-1* (T→C) strain (Fig. 1D). These results suggest that the NNU-rich codon usage profile of *cpc-1* suppresses CPC-1 expression.

Codon usage of *cpc-1* and culture conditions affect CPC-1 protein stability. GCN4, the ortholog of CPC-1 in *S. cerevisiae*, is rapidly degraded under rich nutrient conditions but is stabilized under amino acid starvation conditions, a response that contributes to GCN4 upregulation after amino acid starvation (1, 10, 11, 60). To determine whether CPC-1 protein stability is affected by amino acid starvation and codon usage, we compared the CPC-1 turnover rates measured after the addition of the protein synthesis inhibitor cycloheximide (CHX) in the *cpc-1* (WT) and *cpc-1* (T→C) strains grown in 2% glucose medium with and without 3-AT. 3-AT treatment resulted in marked stabilization of CPC-1 in the *cpc-1* (WT) strain (Fig. 2), suggesting that, as with GCN4 in yeast (1), protein stability was altered under amino acid starvation conditions. However, CPC-1 was more stable in the *cpc-1* (T→C) strain than in the *cpc-1* (WT) strain when the strains were grown in 2% glucose medium without 3-AT. Furthermore, the stabilization of CPC-1 observed in the *cpc-1* (WT) strain upon 3-AT treatment was not observed in the *cpc-1* (T→C) strain (Fig. 2). These results indicate that the NNU-biased *cpc-1* codon usage plays an important role in regulating CPC-1 protein stability under amino acid starvation conditions.

*cpc-1* codon usage affects CPC-1 structure and function. The observed effect of *cpc-1* codon usage on CPC-1 protein stability raised the possibility that proper cotranslational folding of CPC-1 depends on codon usage as observed for other *Neurospora* proteins (19, 22, 24). To examine this possibility, we performed a limited trypsin digestion assay to probe the structure differences of CPC-1 proteins in the *cpc-1* (WT) and *cpc-1* (T→C) strains grown in 2% glucose medium with and without 3-AT. 3-AT treatment resulted in marked stabilization of CPC-1 in the *cpc-1* (WT) strain (Fig. 2), suggesting that as, with GCN4 in yeast (1), protein stability was altered under amino acid starvation conditions. However, CPC-1 was more stable in the *cpc-1* (T→C) strain than in the *cpc-1* (WT) strain when the strains were grown in 2% glucose medium without 3-AT. Furthermore, the stabilization of CPC-1 observed in the *cpc-1* (WT) strain upon 3-AT treatment was not observed in the *cpc-1* (T→C) strain (Fig. 2). These results indicate that the NNU-biased *cpc-1* codon usage plays an important role in regulating CPC-1 protein stability under amino acid starvation conditions.

**FIG 2** Codon usage optimization alters CPC-1 stability in response to amino acid starvation. (Upper panels) Representative Western blots showing the CPC-1 protein levels in *cpc-1* (WT) and *cpc-1* (T→C) strains grown in 2% glucose medium with or without 5 mM 3-AT. Cycloheximide (CHX, 10 μg ml⁻¹) was added at time zero, and cultures were harvested at the indicated time points. (Lower panels) Densitometric analyses of the Western blot experiments described in the upper panels. Data are means ± SD (n = 3). *, P < 0.05, as determined by Student’s two-tailed t test. Min, minutes.
From the endogenous cpc-1 level in a WT strain (Fig. S3). Thus, the effect of codon usage on CPC-1 structure is not due to its overexpression.

To determine whether the structural differences caused by codon usage result in changes in protein function, we introduced the cpc-1(WT) and cpc-1(T→C) constructs individually into the cpc-1/H9004 strain. We then compared the abilities of these two constructs to rescue the growth defect of the cpc-1/H9004 mutant under amino acid starvation conditions. Under normal growth conditions, the WT and cpc-1/H9004 strains had similar growth rates but the cpc-1/H9004 strains expressing the cpc-1(WT) or cpc-1(T→C) had a slightly but significantly lower growth rate in constant light at room temperature (Fig. 4A). In constant light, cpc-1(WT) and cpc-1(T→C) are constitutively expressed, and

![Graph showing protein expression levels](image)

**FIG 3** Codon usage affects CPC-1 cotranslational folding. (Top) Western blots of CPC-1 expression in the cpc-1(WT) and cpc-1(T→C) strains cultured in 2% glucose medium in the absence or presence of 5 mM 3-AT. Trypsin (0.25 μg/ml) was added into the freshly isolated protein extracts, and protein samples were analyzed at the indicated time points. (Bottom) Densitometric analyses of the full-length CPC-1 levels from the experiments described above. Data are means ± SD (n = 3). *, P < 0.05, as determined by Student’s two-tailed t test.

![Graph showing growth rates](image)

**FIG 4** Optimization of cpc-1 codon usage impairs CPC-1 biological functions. (A) Growth rates of the WT strain, the cpc-1Δ strain, and the cpc-1Δ strains expressing cpc-1(WT) or cpc-1(C→T) after 48 and 72 h as determined in race tube assays performed without 3-AT. *, P < 0.05; NS, not significant; as determined by Student’s two-tailed t test. (B) Growth rates of the cpc-1Δ strain and the cpc-1Δ strains expressing cpc-1(WT) or cpc-1(C→T) after 48, 72, and 96 h as determined in race tube assays performed with 5 mM 3-AT. (C) The relative mRNA levels of selected CPC-1 target genes in the indicated strains. The relative mRNA level of each gene was determined by qRT-PCR, and the expression levels of the genes were normalized to that of the β-tubulin gene (NCU04054). The mRNA level of each gene in the cpc-1Δ strain was set as 1.0. Data in panels are means ± SD (n = 3).
cpc-1 translation is not regulated by the uORFs due to the use of the ccg-1 5′ UTR in the transgene strains. The reduced growth rate in these strains is consistent with the known role of GCN4 as a repressor of protein synthesis (61). In the presence of 3-AT, the growth rate of the cpc-1Δ strain was dramatically reduced in a race tube assay (Fig. 4B). The growth phenotype was drastically improved in the cpc-1(WT) strain, indicating a functional rescue of the cpc-1Δ strain by the cpc-1(WT) transgene. The growth rate of the cpc-1Δ, cpc-1(T→C) strain, however, was much lower than that of the cpc-1Δ, cpc-1(WT) strain in the presence of 3-AT (Fig. 4B), indicating that the T→C codon usage profile changes impaired the CPC-1 protein function even though they increased the CPC-1 protein level (Fig. 1D).

To further confirm this conclusion, we compared the mRNA levels of 14 genes regulated by CPC-1 that are involved in amino acid biosynthesis (3, 9). The mRNA levels of these CPC-1 target genes in the cpc-1Δ, cpc-1Δ, cpc-1(WT); and cpc-1Δ, cpc-1(T→C) strains treated with 3-AT were determined. The mRNA levels of all the 14 genes were dramatically upregulated in the cpc-1Δ, cpc-1(WT) strain compared to those in the cpc-1Δ strain (Fig. 4C). As expected, the mRNA level induction of these genes was reduced in the cpc-1Δ, cpc-1(T→C) strain (Fig. 4C). Together, these results demonstrate that the NNU-biased codon usage profile of cpc-1 plays an important role in determining CPC-1 protein structure and function in vivo.

DISCUSSION

CPC-1 is the master transcription regulator of gene expression in Neurospora in response to amino acid starvation (3, 7, 55, 62). Like the situation of its yeast ortholog GCN4, expression of CPC-1 is also translationally regulated by a mechanism involving uORFs (7, 9). The translation of cpc-1 can be translationally activated by bypassing its uORFs under amino acid starvation conditions. As reported previously for GCN4 (10, 11), here, we showed that 3-AT treatment triggers CPC-1 stabilization, which also contributes to CPC-1 accumulation under amino acid starvation conditions. Although the mechanism of CPC-1 degradation is not known, it is possible that, as with GCN4, amino acid starvation regulates posttranslational modification of CPC-1, which affects its degradation by the ubiquitin-proteasome pathway (11–13).

In this study, we demonstrated that the nonoptimal codon usage profile of cpc-1 has a major impact on the structure and function of CPC-1. By changing the cpc-1 codon usage from the NNU-rich profile to the NNC-rich profile typical of the Neurospora genome, we showed that codon usage is critical for CPC-1 protein structure and function. This conclusion was supported by several lines of evidence. First, the codon manipulation altered the CPC-1 protein degradation rate and abolished amino acid starvation-induced CPC-1 stabilization (Fig. 2), suggesting that codon usage can affect CPC-1 structure. Second, the codon optimization altered the sensitivity of CPC-1 to limited trypsin digestion, indicating that codon optimization affected protein structure (Fig. 3). Third, in the presence of 3-AT, CPC-1(T→C) was less stable and more sensitive to trypsin digestion than CPC-1 (WT) was (Fig. 2; see also Fig. 3), suggesting that codon usage-mediated structure changes of CPC-1 affected its ability to be regulated by potential posttranslational mechanisms triggered by amino acid starvation conditions. Fourth, despite the upregulation of CPC-1 protein levels in Neurospora upon codon optimization, expression of cpc-1(T→C) did not rescue the growth defects of the cpc-1Δ strain under amino acid starvation conditions as effectively as that of cpc-1(WT) did. Finally, the impaired CPC-1 function of the cpc-1(T→C) strain was further indicated by the reduced induction of CPC-1 target genes in response to amino acid starvation. Note that codon optimization of cpc-1 did not cause its overexpression (see Fig. S3 in the supplemental material). Thus, our study established a critical physiological role of codon usage in regulating CPC-1 structure and function.

In addition, our analyses established another in vivo example of the influence of codon usage on protein structure. Due to the role of codon usage in regulating the rate of translation elongation, codon usage was previously proposed to influence the cotranslational protein folding process (19, 22, 26, 28, 33–38). However, genetic evi-
dence in support of such a role of codon usage is quite limited. By studying the codon usage function of the circadian clock genes frequency in Neurospora and Period in Drosophila, we previously showed that codon usage plays an important role in affecting the structures and, therefore, the functions of these two proteins in vivo (19, 36). Similarly to the frequency and Period genes, cpc-1 is enriched in nonoptimal codons. Additionally, as with FRQ and PER proteins, most regions of the CPC-1 protein are predicted unstructured domains in a genome-wide manner in Neurospora and other organisms (33, 52). The structure of the DNA binding domain of the yeast GCN4 was previously shown to be flexible (63–65). GCN4 exhibits a concentration-dependent α-helical transition: the transition of the GCN4 basic region from an unfolded to a folded conformation depends on its accessibility to DNA binding sites (65). Such properties may make it more sensitive to the cotranslational folding process.

Taken together, our results suggest that the unusual codon profile of cpc-1 represents another example of evolutionary adaption that results in its optimal protein structure and function in response to environmental changes.

MATERIALS AND METHODS

Strains and growth conditions. N. crassa strain 87-3 (bd, q) was used as the control and was further used as the host strain for the expression of various versions of cpc-1 unless otherwise specified. For the growth rate assay, the FGSC 4200 (a, WT) strain was used as the control. The cpc-1Δ strain was obtained from the Neurospora knockout library (66). Liquid cultures were grown in 2% glucose medium (1× Vogel’s, 2% glucose) or in 0.1% glucose medium (1× Vogel’s, 0.1% glucose, 0.17% arginine). Race tube medium contained 1× Vogel’s, 0.1% glucose, 0.17% arginine, 50 ng ml⁻¹ biotin, and 1.5% agar. All the strains were cultured on slants containing 1× Vogel’s, 2% sucrose, and 1.5% agar before various experiments were performed. All the strains were cultured under constant light at room temperature.

Plasmid constructs. For gene expression at the csr-1 locus in N. crassa, a hygromycin B resistance gene (hph) was inserted downstream of the ccg-1 promoter of a parental plasmid, Pcsr1, to create a new plasmid, Pcsr1-hyg. Pcsr1-hyg is a csr-1-targeting expression vector with an expression cassette in which Pcsr1 and hph flank the gene of interest, and this cassette is flanked by two csr-1-related fragments that serve as the double recombination sites (67). When this plasmid was transfected into N. crassa cells, it was integrated into the csr-1 gene locus by replacing csr-1 with the expression cassette by double homologous recombination. The resulting transformants were screened for both hygromycin B (200 μg ml⁻¹) resistance and cyclosporine (5 μg ml⁻¹) resistance conferred by the presence of hph and the absence of csr-1, respectively. The levels of efficiency and accuracy of this approach were very high (>90% positive transformants). In this study, two versions of cpc-1(WT) and cpc-1(T→C) with a 3×Flag tag and an 8×Gly linker at the N termini were separately introduced into the Pcsr1-hyg construct. The resulting constructs were transformed into host strains by electroporation. Homokaryon strains were obtained by microconidium purification.

Protein stability and limited trypsin digestion assays. For protein stability assay, the cyclolheximide (CHX) working concentration and experimental procedures were the same as those previously described (19). For culture conditions, fresh conidia (1 week postinoculation on slants) of the host strains were cultured in 50 ml 2% glucose medium at room temperature for 2 days. The cultures were cut into small discs with a diameter of 1 cm, and then the discs were transferred into flasks with the same liquid medium and were grown with orbital shaking (200 rpm) for one more day before addition of CHX (final concentration, 10 μg ml⁻¹). For CHX concentrations of 3-AT, the culture discs in 2% glucose medium were treated with 5 mM 3-AT for 8 h before sample collection. Cells were collected at the indicated time points after addition of CHX. For the limited trypsin digestion assay, the culture conditions and sample collection procedures were the same as those described above except for the addition of CHX. The working concentration of trypsin was 0.25 μg/ml. Protein extraction and Western blot analyses were performed as previously described (68). Equal amounts of total proteins (100 μg) were loaded into all lanes of 7.5% SDS-PAGE gels containing 37:5:1 acrylamide/bisacrylamide. The primary and secondary antibodies used for detecting the 3×Flag were monoclonal anti-Flag M2 antibody produced in mouse (Sigma-Aldrich, catalog no. F3165) and goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP) conjugate (Bio-Rad, catalog no. 170-6516), respectively. Densitometry was performed using Image J.

Quantitative reverse transcription-PCR (qRT-PCR) and mRNA-seq. For qRT-PCR, the sample collection procedures were the same as those described for the protein stability assay except that CHX was not added. For cultures treated with 3-AT as indicated in the figures, the liquid cultures were treated with 5 mM 3-AT for 8 h before sample collection. RNA extraction and qRT-PCR were performed as previously described (69). β-tubulin (NCU04054) was quantified as an internal control. Primers used for qRT-PCR are listed in Table S1 in the supplemental material. The relative mRNA levels of cpc-1 in the WT,
cpc-1Δ, and cpc-1Δ, cpc-1WT) strains under amino acid starvation conditions were measured by determination of their RPKM (reads per kilobase per million) values from our high-throughput mRNA sequencing (mRNA-seq) data. The mRNA sequencing libraries used in this study were generated from cultures maintained in 2% glucose medium with 5 mM 3-AT treatment for 8 h before sample collection. The sample collection procedures were the same as those described for the protein stability assay except that CHX was not added. Total RNAs were extracted using TRIzol reagents (Invitrogen) and treated with DNase (Turbo DNase; Ambion). The libraries were prepared using NEBNext Ultra kits for RNA and sequenced by an Illumina HiSeq 2000 instrument. mRNA-seq experiments were performed by Joint Genome Institute (JGI) on an Illumina NovaSeq platform.

**Codon manipulation and data collection from databases.** The codons of cpc-1 were optimized based on *N. crassa* codon usage frequency data from the Codon Usage Database (https://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=5141). The mutated sites for the optimized cpc-1(T→C) are shown in FIG S2 in the supplemental material.

**Data availability.** The raw and processed sequencing data have been submitted to the NCBI Gene Expression Omnibus under accession number GSE150287.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, PDF file, 0.3 MB.
**FIG S2**, PDF file, 0.04 MB.
**FIG S3**, PDF file, 0.4 MB.
**TABLE S1**, PDF file, 0.03 MB.

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