

Impaired Ribosome Biogenesis Disrupts the Integration between Morphogenesis and Nuclear Duplication during the Germination of *Aspergillus fumigatus*[∇]

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***Aspergillus fumigatus* is an important opportunistic fungal pathogen that is responsible for high mortality rates in the immunosuppressed population. CgrA, the *A. fumigatus* ortholog of a *Saccharomyces cerevisiae* nucleolar protein involved in ribosome biogenesis, contributes to the virulence of this fungus by supporting rapid growth at 37°C. To determine how CgrA affects ribosome biogenesis in *A. fumigatus*, polysome profile and ribosomal subunit analyses were performed on both wild-type *A. fumigatus* and a $\Delta cgrA$ mutant. The loss of CgrA was associated with a reduction in the level of 80S monosomes as well as an imbalance in the 60S:40S subunit ratio and the appearance of half-mer ribosomes. The gene expression profile in the $\Delta cgrA$ mutant revealed increased abundance of a subset of translational machinery mRNAs relative to the wild type, suggesting a potential compensatory response to CgrA deficiency. Although $\Delta cgrA$ conidia germinated normally at 22°C, they swelled excessively when incubated at 37°C and accumulated abnormally high numbers of nuclei. This hypernucleated phenotype could be replicated pharmacologically by germinating wild-type conidia under conditions of reductive stress. These findings indicate that the germination process is particularly vulnerable to global disruption of protein synthesis and suggest that CgrA is involved in both ribosome biogenesis and polarized cell growth in *A. fumigatus*.**

Aspergillus fumigatus is an opportunistic fungal pathogen that has become the predominant mold species responsible for infections in the immunocompromised population (27). Despite the best treatment with recently approved antifungal drugs, invasive aspergillosis continues to have a poor outcome (19, 31, 34, 35, 38, 41), resulting in the highest hospitalization costs among the systemic mycoses (10, 57). The infection is generally acquired through the inhalation of conidia that, in the absence of adequate host defenses, develop into invasive hyphae that cause severe tissue damage (13, 29). Upon entering the lung, the conidia must transition from a metabolically dormant state at ambient temperature to filamentous growth at 37°C. *A. fumigatus* germinates very rapidly at 37°C (2), and analysis of growth rate variability among clinical isolates has revealed that faster growth correlates with increased virulence in animal models (42). This high rate of growth places considerable demand on the translational machinery, requiring an increase in ribosome production in proportion to the demand for new proteins.

Ribosome biogenesis begins in the nucleolus, a specialized nuclear compartment that is responsible for the transcription of the ribosomal DNA (rDNA) genes, processing of pre-

rRNA, and coordinated assembly of pre-rRNAs with ribosomal proteins (17, 18, 53). We previously showed that the ability of *A. fumigatus* to grow optimally at temperatures above 25°C requires CgrA, the ortholog of a nucleolar protein involved in *Saccharomyces cerevisiae* ribosome biogenesis (4, 39). CgrA is necessary for wild-type (wt) virulence of *A. fumigatus* in a mouse model of invasive aspergillosis but is dispensable for virulence in a *Drosophila* infection model at 25°C. This suggests that CgrA contributes to pathogenesis by providing an adequate pool of ribosomes to sustain rapid growth at mammalian body temperature (4).

Although ribosome biogenesis has been intensively studied in *S. cerevisiae*, much less is known about the process in filamentous fungi. In this study, polysome profiling and ribosome subunit analyses were performed to determine how CgrA deficiency affects ribosome biogenesis in *A. fumigatus*. Here we demonstrate that the loss of CgrA creates an imbalance in ribosome subunit stoichiometry and the accumulation of half-mer ribosomes, irrespective of growth temperature. This was associated with an increased abundance of a subset of mRNAs involved in the translational machinery, suggesting that these mRNAs are part of a compensatory response to the ribosome biogenesis defect. Although the $\Delta cgrA$ conidia germinated normally when incubated at 22°C, when incubated at 37°C, they showed a delay in polarized growth, excessive isotropic growth, and the accumulation of large numbers of nuclei. Similar observations were made when wt conidia were germinated in the presence of dithiothreitol (DTT), a reducing agent that unfolds

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proteins and disrupts ribosomes (45). Together, these findings establish a role for CgrA in ribosome biogenesis in *A. fumigatus* and provide evidence that CgrA contributes to polarized growth during germination at 37°C.

MATERIALS AND METHODS

Strains and culture conditions. The wt strain used in this study is a clinical isolate designated H237. The $\Delta cgrA$ strain was generated by gene disruption following the insertion of a phleomycin resistance cassette (4). This strain was reconstituted to wt (r-wt) by restoring a single copy of the *cgrA* gene adjacent to the original gene disruption as previously described (4). All conidia were harvested from strains grown on *Aspergillus* minimal medium (9). For experiments involving extraction of polysomes, conidia were inoculated into liquid cultures of YG (0.5% yeast extract, 2% glucose).

The germination of *A. fumigatus* conidia involves a series of morphological changes, beginning with a short period of isotropic growth (swelling) that is followed by the establishment of an axis of polarity and the extension of the first germ tube (37). Continued growth of the germling allows the establishment of interconnections between neighboring hyphae, resulting in an asynchronous population of hyphal compartments (54). Thus, all incubation times in this study were adjusted in order to obtain a homogeneous population of young germlings, defined here as conidia with germ tubes approximately 30 μm in length (see Fig. 2A). This allowed a comparison between equivalent growth stages, with the caveat that nuclear abundance differed between the wt and the $\Delta cgrA$ mutant. The alternative approach of normalizing to nuclear number was not employed because any comparison between the wt and the slower growing $\Delta cgrA$ strain would be confounded by stage-specific effects (7).

Polysome profiles and RNA analysis. For polysome analysis, 1×10^8 conidia from the wt and the $\Delta cgrA$ strain were inoculated into 100 ml of YG medium and incubated at 200 rpm. Due to the lower growth rate of the $\Delta cgrA$ mutant at higher temperatures, incubation times were adjusted so that each culture was harvested when the conidia had elaborated a germ tube that was approximately 30 μm in length. The wt strain was incubated for 44 h at 22°C and for 11 h at 37°C, whereas the $\Delta cgrA$ strain was incubated for 48 h at 22°C and for 24 h at 37°C. For polysome profile analysis, cultures were first treated with cycloheximide (0.1 mg/ml) to halt translation and prevent ribosomes from running off the mRNA. The germlings were then disrupted by crushing in liquid nitrogen and resuspended in a high- Mg^{2+} lysis buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 30 mM MgCl_2 , 0.1 mg/ml cycloheximide, and 0.2 mg/ml heparin) to preserve ribosome subunit associations. For ribosome subunit analysis, cycloheximide was omitted to allow polysome runoff. In addition, cultures were treated with 1 mM NaN_3 for 15 min before harvesting, and germling extracts were prepared by crushing in liquid nitrogen and resuspending in a low- Mg^{2+} lysis buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, and 1 mM DTT) to disrupt subunit associations. Each lysis buffer was cleared by centrifugation at $12,000 \times g$ at 4°C for 15 min followed by a second clearing spin for 10 min at the same speed. The supernatant was removed, and the RNA content was quantified at 260 nm. Equal numbers of A_{260} units were loaded onto a 10-ml linear sucrose gradient (7 to 47%). The composition of the buffer used for the high- Mg^{2+} gradient was 50 mM Tris-acetate, 50 mM NH_4Cl , 12 mM MgCl_2 , 1 mM DTT, and 0.1% diethyl pyrocarbonate. The buffer for low- Mg^{2+} gradient was prepared with 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM DTT. The gradients were centrifuged in an Sorvall SW 41Ti rotor at $200,000 \times g$ for 2.5 h at 4°C. Gradient analysis was performed with an ISCO gradient collector with continuous monitoring at 254 nm.

For Northern blot analysis, samples of equal volume were collected from the fractionated ribosome gradient using an ISCO fraction collector system. Total RNA was extracted using TRI reagent LS (Molecular Research Center, Cincinnati, OH), fractionated by formaldehyde gel electrophoresis (9), and visualized by SYBR green staining. The RNA was transferred to BioBond nylon membranes (Sigma) and hybridized to a ^{32}P -labeled DNA probe for *A. fumigatus* β -actin. The β -actin fragment was PCR amplified from *A. fumigatus* genomic DNA using the following primers: forward (5'-ATGTCAGTGTGAGATTGT C-3') and reverse (5'-TTAGAAGCACTTGCGGTGAA-3').

Transcriptional profiling. A whole-genome microarray was performed to identify genes that show differential expression in the absence of *cgrA* during thermal stress, using a strategy that was previously used for the genome reference strain Af293 (40). A total of 5×10^6 conidia from the wt, r-wt, and $\Delta cgrA$ strains were cultured at 30°C (minimal thermal stress for the mutant) until they had become germlings (16 h for the wt and r-wt strains and 22 h for the $\Delta cgrA$ mutant) in 50 ml of complete minimal medium (*Aspergillus* minimal medium

supplemented with 0.1% yeast extract, 0.2% peptone, and 0.1% tryptone). RNA was extracted from the zero time point at 30°C, which served as the reference sample. The remaining cultures were shifted to 37°C, a temperature at which the $\Delta cgrA$ mutant is growth impaired. RNA was extracted after 20, 40, 60, 80, and 100 min of incubation at 37°C by crushing the mycelium in liquid nitrogen and extracting with Trizol reagent (Invitrogen).

The *A. fumigatus* Af293 DNA amplicon microarray containing 9,516 genes was used in this study (40). The protocols used for microbial RNA aminoallyl labeling (M007) and microbial hybridization of labeled probes (M008) are available at <http://pfgrc.tigr.org/protocols.shtml>. In order to identify genes exhibiting altered transcription after the temperature shift, the 0-min sample served as the reference upon which up- or down-regulation was measured. All the hybridizations were repeated in dye-swap sets. Hybridized slides were scanned using the Axon GenePix 4000B microarray scanner, and the TIFF images generated were analyzed using TIGR Spotfinder software to obtain relative transcript levels. Hybridization data was normalized using a local regression technique LOWESS (locally weighted scatterplot smoothing) using the software tool MIDAS. The resulting data were averaged from three gene spots on each array and from duplicate flip-dye arrays for each experiment equalizing six intensity data points for each gene. Differentially expressed genes at the 95% confidence level were determined using intensity-dependent Z scores (with $Z = 1.96$) as implemented in MIDAS for all experiments. To identify genes where expression is affected by the *cgrA* deletion following a shift from 30°C to 37°C, we identified all genes exhibiting differential expression in the wt, $\Delta cgrA$, and r-wt at each time point. Genes that were found to have significantly different expression in each set of experiments were then combined and organized based on similar expression vectors using Euclidean distance and hierarchical clustering with average linkage clustering method with TIGR TM4. All microarray analysis software is available at <http://pfgrc.tigr.org/tools.shtml>.

Real-time PCR. Validation of the microarray data was performed by real-time reverse transcriptase PCR (RT-PCR) on two selected ribosome biogenesis genes, *nop4* and *brx1* (6, 52). To obtain RNA, the wt and $\Delta cgrA$ strains were grown under the same conditions used for microarray analysis. RNA was extracted as described above, from cultures grown at 30°C, which served as the 0-min time point, and at 80 min following the shift to 37°C. Five micrograms of total RNA was converted to cDNA using Superscript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed on the cDNA using a SYBR GreenER quantitative PCR supermix universal kit (Invitrogen) using a Smart-Cycler II instrument (Cepheid, Sunnyvale, CA). The PCR protocol involved 40 cycles, with each cycle consisting of 95°C for 15 s, annealing for 3 s, and extension at 72°C for 30 s. The C_t (cycle threshold) value was determined by the PCR cycle number at which the fluorescent signal crossed the default threshold set at 30 fluorescent units. C_t values were calculated for the two target genes, *brx1* and *nop4* (6, 52) at the 0- and 80-min time points, as well as for the housekeeping gene *fks1* (3). The values for the 80-min time point were then normalized to the values for the 0-min sample and reference gene *fks* using the method described by Pfaffl (43). The data were presented as a relative expression ratio, using the following formula: ratio = $E_{\text{target}}^{-\Delta C_t(\text{control} - \text{sample})} / E_{\text{ref}}^{-\Delta C_t(\text{control} - \text{sample})}$ where E is the exponential amplification calculated by $10^{-1/\text{slope}}$ and E_{ref} is the E value for the reference sample. The following primer pairs were used for amplification of *fks*, *brx1*, and *nop4*: Fks-forward, 5'-GCCTGGTAGTGAAGC TGAGCGT-3'; Fks-reverse, 5'-CGGTGAATGTAGGCATGTTGTGCC-3'; Brx1-forward, 5'-GTTTACAGTCACAACTGCA-3'; Brx1-reverse, 5'-CAATCCGC GGTACTCGTTTC-3'; Nop4-forward, 5'-GAGGAGTACCGGAGGTCAAAGG-3'; and Nop4-reverse, 5'-GCGCATGTGCATGTGTGTAGTA-3'.

Confocal microscopy. To visualize nuclei in live cultures, strains were transfected with a NopA-green fluorescent protein (GFP) expression construct previously shown to localize to nucleoli in *A. fumigatus* (5). Conidia were inoculated into 5 ml of *Aspergillus* minimal medium in a 35-mm petri dish containing a 25-mm-diameter coverslip. The cultures were incubated at 22°C or 37°C without shaking until the conidia formed a germ tube that was approximately 30 μm in length. Fluorescent nuclei were observed with a Leica TCS SP2 laser-scanning confocal microscope using a 63 \times oil objective. For experiments involving nuclear staining with propidium iodide (PI), the germlings were fixed for 3 min at room temperature in a solution containing 3.7% formaldehyde, 0.2% Triton X-100, and 50 mM phosphate buffer, pH 7. The slides were then treated with a 10-mg/ml solution of RNase A for 1 h at 37°C, followed by nuclear staining with PI (12.5 $\mu\text{g/ml}$). The laser source was an Ar/Kr laser set for GFP detection (excitation, 488 nm; emission, 507 nm) or for PI detection (excitation, 530 nm; emission, 615 nm). Cell walls were stained by incubating in a solution of 0.4 $\mu\text{g/ml}$ calcofluor (fluorescent brightener 28; Sigma) for 5 min at room temperature and visualized by confocal microscopy using a UV laser set for 4',6'-diamidino-2-phenylindole (DAPI) detection (excitation, 372 nm; emission, 456 nm).

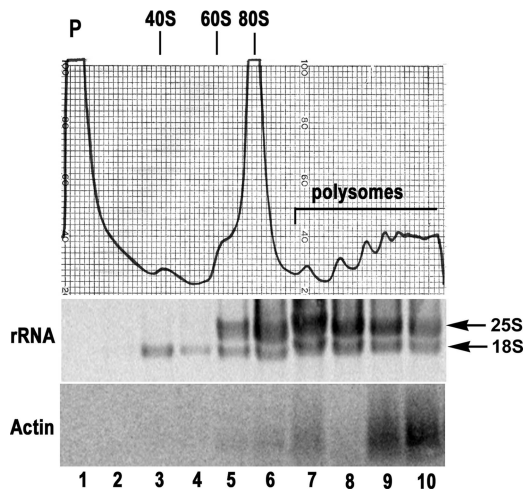


FIG. 1. Polysome profile analysis in wt *A. fumigatus*. (Top) Polysome profile of wt *A. fumigatus* germlings cultured in rich medium at 37°C. The profile shows a protein peak (P), followed by peaks representing free 40S and 60S subunits, the 80S monosome and polysomes representing two or more ribosomes per mRNA. (Middle) SYBR green staining of rRNA in each polysome fraction (10 fractions). (Bottom) Northern blot showing hybridization of RNA in each fraction to an *A. fumigatus* β -actin probe.

Microarray data accession number. The full data set for this study has been deposited with ArrayExpress at the EMBL European Bioinformatics Institute (<http://www.ebi.ac.uk/>) under the accession number E-MEXP-1324.

RESULTS

Polysome profiling in *A. fumigatus*. In order to develop techniques for the analysis of ribosome distributions in *A. fumigatus*, we adapted a protocol from *S. cerevisiae* (28) and optimized the conditions for analysis of *A. fumigatus* germlings as described in Materials and Methods. A typical profile showing the normal distribution of ribosome subunits in wt *A. fumigatus* germlings is shown in Fig. 1 (top panel). The wt profile showed the expected protein peak, followed by peaks representing free 40S subunits, free 60S subunits, the 80S monosomes and polysomes (polyribosomes) corresponding to two or more ribosomes per mRNA. Under these conditions, the 18S rRNA in the 40S subunit was found in fractions 3 and 4, the 60S subunit containing both rRNAs appeared in fraction 5, and the 80S monosome and polysomes were present in fractions 6 to 10 (Fig. 1). The presence of mRNA in the polysome fractions was demonstrated by extracting RNA from each gradient and hybridizing to an actin-specific probe by Northern blot analysis. As expected for actively growing germlings, the majority of the actin mRNA was present in the polysome fractions.

Effects of *CgrA* disruption on ribosome biogenesis in *A. fumigatus*. To determine how the loss of *CgrA* affects the steady-state distribution of ribosomes in *A. fumigatus*, the polysome profiles of wt and $\Delta cgrA$ germlings were compared (Fig. 2A). Monosome levels were reduced in the $\Delta cgrA$ mutant by about 40% relative to that in the wt, and half-mer ribosomes were apparent on the $\Delta cgrA$ monosome and polysome peaks (Fig. 2A). Comparable defects were previously observed in an *S. cerevisiae* $\Delta cgr1$ mutant, although the reduction in monosomes was somewhat greater in yeast (over 60%) (39). Half-

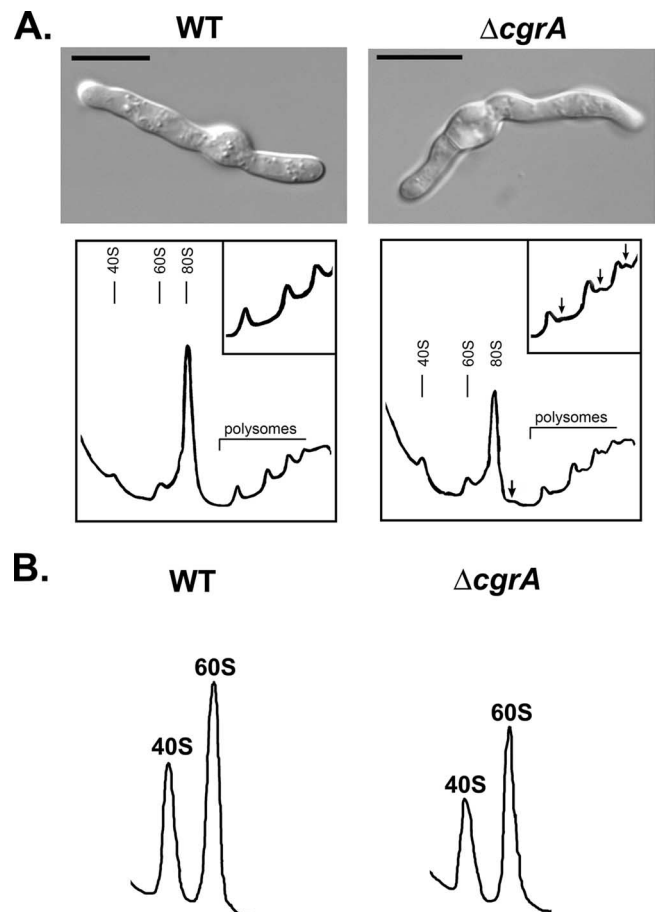


FIG. 2. The loss of *CgrA* impairs ribosome biogenesis. (A) Comparison of the polysome profiles of wt and $\Delta cgrA$ germlings. Strains were cultured in YG for 44 to 48 h at 22°C. Extracts were isolated under high-magnesium conditions and fractionated on a 7 to 47% linear sucrose gradient with continuous monitoring at 254 nm. Half-mer ribosomes on the monosome and polysome peaks in the $\Delta cgrA$ mutant are indicated by the arrows (insets in the top right corner of each profile show an enlargement of the polysome profiles). A differential interference contrast image showing the stage of growth used for analysis is shown at the top of the figure. Bars, 10 μ m. (B) Total subunit profiles from wt and $\Delta cgrA$ germlings isolated under low-magnesium conditions. Polysome and subunit profiles were performed at least three times with similar findings.

mers, evident as shoulders following the monosome and polysome peaks, represent 43S initiation complexes that are stalled at the AUG and await binding of 60S subunits to form the 80S monosome (30). The presence of half-mers suggest a defect in 60S subunit assembly as has been previously reported in a yeast *cgr1* mutant (39). To determine whether the half-mers observed in the $\Delta cgrA$ strain were due to an imbalance in subunit stoichiometry, ribosomes were extracted under low-Mg²⁺ conditions to disrupt subunit association. Total 40S and 60S subunits in the $\Delta cgrA$ mutant were approximately 25 to 30% lower than in the wt (Fig. 2B). The ratio of 60S:40S subunits was 1.82 in wt *A. fumigatus*, similar to wt ratios in *S. cerevisiae* (11, 12, 39). However, the corresponding ratio in the $\Delta cgrA$ strain was 1.66, suggesting imbalanced subunit stoichiometry. The same ribosome defects were evident in the $\Delta cgrA$ mutant grown at

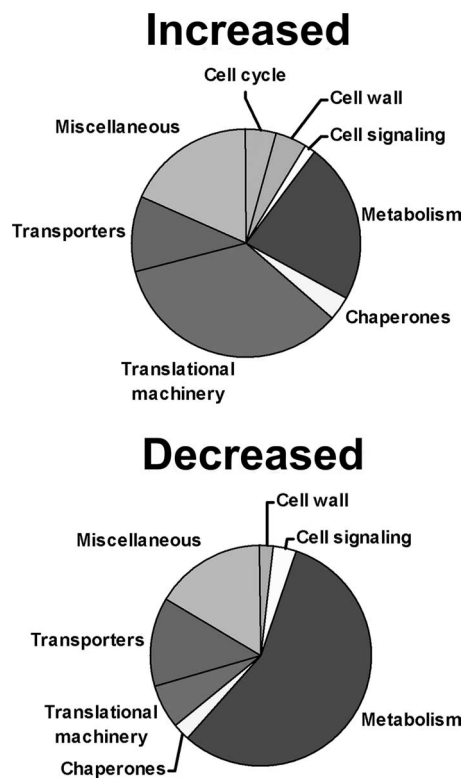


FIG. 3. Transcriptional response to CgrA deficiency. Summary of functional categories of mRNAs showing increased or decreased abundance in the $\Delta cgrA$ mutant relative to the wt, 80 min after a shift from 30°C to 37°C.

either 22°C or 37°C, despite the fact that the growth rate of the $\Delta cgrA$ mutant was indistinguishable from the growth rate of the wt at 22°C but 65% less than the growth rate of the wt at 37°C (4). This indicates that the loss of CgrA leads to a phenotypic growth defect that is enhanced by elevated temperature, rather than a ribosome biogenesis defect that is temperature dependent.

Transcriptional response to CgrA deficiency. Although the $\Delta cgrA$ mutant showed a reduction in 60S:40S subunit stoichiometry, the defect in subunit ratio was less dramatic than what has been previously reported in an *S. cerevisiae cgr1* mutant (39). This suggests that *A. fumigatus* may be able to compensate partially for the absence of CgrA function. To gain insight into potential compensatory pathways, the steady-state expression profile of the $\Delta cgrA$ strain was compared to that of the wt following a shift from 30°C to 37°C (4). All mRNAs that showed increased or decreased abundance in the $\Delta cgrA$ mutant relative to the level in the wt following the shift are summarized in Fig. 3. The largest category of down-regulated mRNAs in the $\Delta cgrA$ mutant was associated with metabolism, a finding consistent with the reduced growth rate of this strain at 37°C. In contrast, the largest number of up-regulated mRNAs in the $\Delta cgrA$ mutant belonged in the category of translational machinery, suggesting that these mRNAs constitute an adaptive response to loss of CgrA function. A heat map depicting the translational machinery-associated mRNAs that had increased abundance in the $\Delta cgrA$ mutant relative to that in the wt and r-wt strains is shown in Fig. 4. Two of these mRNAs were

randomly selected for validation by an independent method. As expected, RT-PCR analysis of the steady-state levels of *brx1* and *nop4* mRNAs showed increased abundance in the $\Delta cgrA$ mutant following a shift from 30°C to 37°C (Fig. 5).

Conidial germination and nuclear duplication. The transcriptional profile of the $\Delta cgrA$ strain at 37°C also revealed increases in the levels of some cell cycle-associated mRNAs relative to the levels in the wt (Fig. 3). Since mitosis is normally coordinated with the morphological changes that take place during germination of *Aspergillus* spp. (23, 37), the germination of wt and $\Delta cgrA$ conidia was examined in more detail. Conidia were inoculated into *Aspergillus* minimal medium and incubated until they had elaborated a germ tube that was approximately 30 μm in length. As expected for wt *A. fumigatus*, conidia that were germinated at 37°C underwent a period of isotropic growth before establishing a germ tube (Fig. 6, two ungerminated conidia are evident in the top right panel for comparison). In contrast, 90% of the $\Delta cgrA$ conidia incubated at 37°C swelled to over three times their normal diameter (Fig. 6), suggesting a defect in the establishment of polarized growth. This increased isotropic growth did not occur in $\Delta cgrA$ germlings that were cultured at 22°C (Fig. 6), indicating that the loss of CgrA induces a temperature-dependent polarity defect. However, once polarity was established, the maintenance of polarity in mature hyphae was apparently normal (data not shown). Excessive swelling was never observed in wt conidia at either temperature, although there was a slight increase in size at 37°C relative to 22°C (Fig. 6).

In *A. fumigatus*, wt conidia undergo the first mitotic division around the time at which polarized growth becomes established, giving rise to a pear-shaped cell with one or two nuclei (37). Nuclei continue to divide as the germ tube extends, and the first septum is usually formed around the fourth mitotic division (37). To determine whether the synchronization between morphogenesis and nuclear division was disrupted in the hyperswollen $\Delta cgrA$ germlings, the wt and $\Delta cgrA$ strains were transfected with a NopA-GFP expression construct. This GFP fusion encodes a nucleolar protein that fluorescently labels *A. fumigatus* nuclei (5). Conidia from both strains were germinated until the germ tube was approximately 30 μm in length (Fig. 7A). When cultured at either 22°C or 37°C, wt germlings of this size contained approximately eight nuclei distributed evenly along each hypha (Fig. 7A). At 22°C, the $\Delta cgrA$ mutant was indistinguishable from the wt (Fig. 7A). However, at 37°C, the enlarged conidia of the $\Delta cgrA$ mutant were filled with multiple nuclei (Fig. 7A). This hypernucleated phenotype was not apparent in mature hyphae (data not shown), suggesting that nuclear accumulation was specific to the early stages of germination. The same results were obtained when wt and $\Delta cgrA$ nuclei were stained with PI, indicating that this finding was not an artifact of NopA-GFP expression in the $\Delta cgrA$ background (data not shown).

To determine whether a similar defect in polarized growth could be induced pharmacologically by an agent that unfolds proteins and disrupts ribosomes (45), wt conidia were incubated in the presence of a sublethal concentration of DTT. The DTT delayed the onset of germination and was associated with increased swelling and nuclear accumulation (Fig. 7B). However, as seen in the $\Delta cgrA$ mutant, the hypernucleated phenotype was not evident in mature hyphae after they had grown

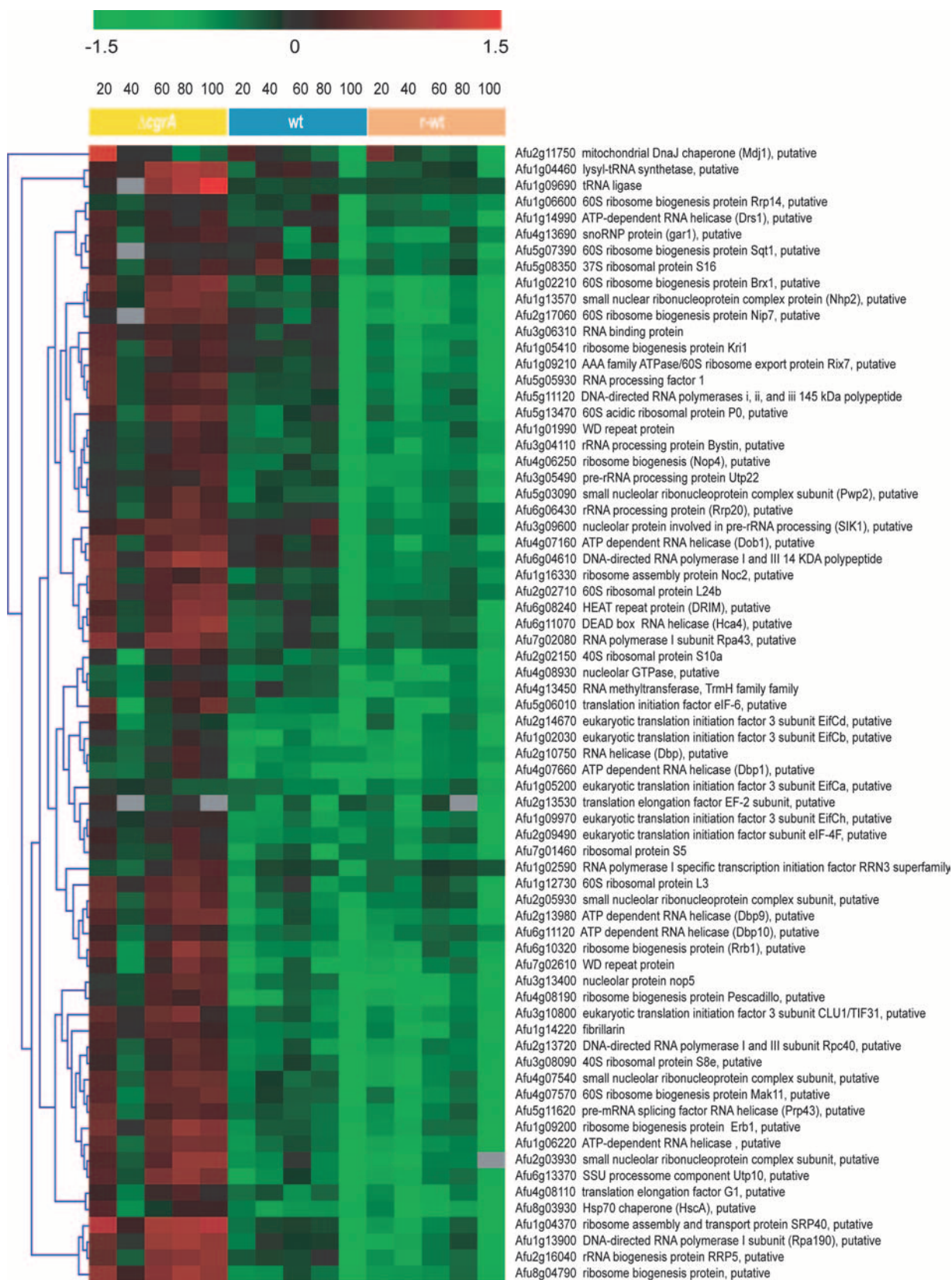


FIG. 4. Expression profile of mRNAs involved in the translational machinery. Clustered display of translational machinery mRNAs that were increased in the $\Delta cgrA$ mutant relative to the wt following a shift from 30°C to 37°C. Colors represent the observed expression ratios on a log₂ scale at 20, 40, 60, 80, and 100 min following the temperature shift. All measurements are relative to the measurement at time zero. A total of 70 translational machinery genes were clustered based on similar expression patterns using a hierarchical clustering algorithm.

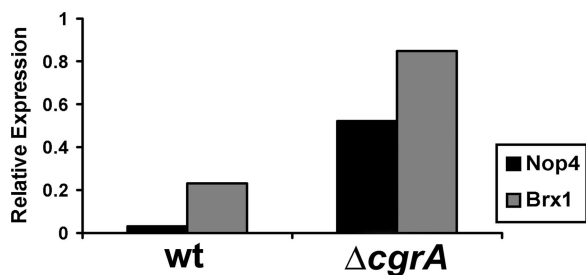


FIG. 5. Validation of microarray data by real-time PCR. Two mRNAs that showed increased abundance in the $\Delta cgrA$ mutant relative to the wt following the shift from 30°C to 37°C by microarray analysis were confirmed by qRT-PCR. C_t values were calculated for each gene 80 min following a shift from 30°C to 37°C. Relative expression values were normalized to the 0-min time point and to the reference gene *fks* as described in Materials and Methods.

beyond the germling stage (data not shown). This result suggests that the germination process is particularly vulnerable to a global disruption of protein function caused by either the loss of CgrA or DTT stress.

DISCUSSION

The success of *A. fumigatus* as an opportunistic fungal pathogen is due in large part to the ability of the fungus to grow rapidly in the host (2). Since infections with *A. fumigatus* continue to have a poor outcome (31, 44), there is a need for greater understanding of the pathways that sustain the growth of *A. fumigatus* in the host environment. One of these pathways includes ribosome biogenesis, a complex process that is tightly linked to growth rate (20, 33, 47, 59). We previously showed that the nucleolar protein CgrA is necessary for the growth and virulence of *A. fumigatus* at 37°C (4), but the contribution of the protein to ribosome biogenesis in this organism was not explored. Here, we reveal how a loss of CgrA function disrupts

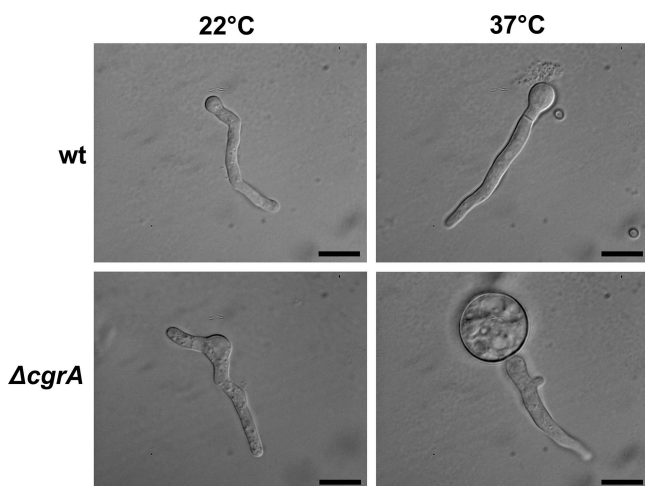


FIG. 6. Conidial morphogenesis. Conidia from the wt and $\Delta cgrA$ strains were inoculated into *Aspergillus* minimal medium and incubated at 22°C or 37°C until the conidia elaborated a germ tube that was approximately 30 μ m in length. (A) Differential interference contrast images of wt and $\Delta cgrA$ germlings grown at 22°C for 48 h or at 37°C for 12 h (wt) or 24 h ($\Delta cgrA$). Bars, 10 μ m.

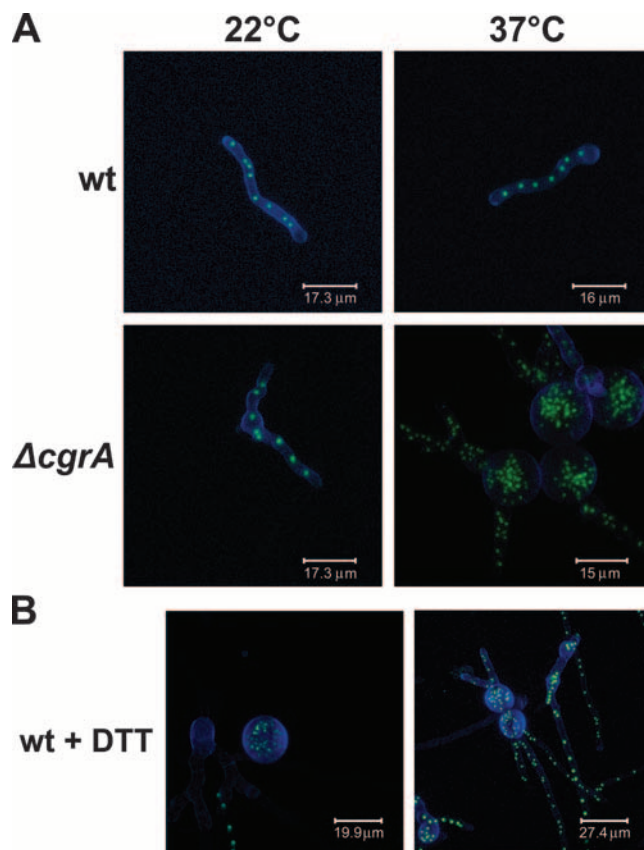


FIG. 7. Nuclear duplication. (A) Conidia from wt and $\Delta cgrA$ strains expressing the nucleolar marker NopA-GFP were inoculated into *Aspergillus* minimal medium and incubated at 22°C or 37°C until the conidia had elaborated a germ tube that was approximately 30 μ m in length. Nuclei were visualized by live-cell imaging with a 63 \times oil objective on a Leica TCS SP2 laser-scanning confocal microscope set for GFP detection. Sixteen optical sections, ranging in size from 7 to 23 μ m in thickness, were obtained through the z plane of each germling and assembled into the single compressed cell image shown. GFP is shown in green, and the calcofluor-stained cell wall is shown in blue. (B) wt and $\Delta cgrA$ conidia expressing NopA-GFP were inoculated into *Aspergillus* minimal medium containing 1 mM DTT and incubated for 24 h at 37°C.

ribosome biogenesis in *A. fumigatus* and highlight an unexpected response to such a defect.

The growth rate of wt *A. fumigatus* is approximately three times greater at 37°C than it is at 22°C (data not shown). In contrast, the $\Delta cgrA$ mutant grows normally at 22°C but is unable to increase its growth rate when cultured at 37°C (4). Therefore, we were surprised to find that half-mers were present in the $\Delta cgrA$ mutant at 22°C or 37°C. We interpret these data to indicate that the defect in ribosome biogenesis caused by the loss of CgrA is compatible with the limited demands for growth at 22°C but not with the heightened metabolic needs at 37°C. Half-mers, which are not detected under normal conditions, represent 40S preinitiation complexes stalled at the AUG and often arise in response to a defect in ribosome subunit stoichiometry (30). Analysis of total ribosome subunit levels confirmed that the loss of CgrA was associated with a disruption in subunit stoichiometry. This is consistent with the predominant 60S subunit synthesis defect

previously reported in an *S. cerevisiae cgr1* mutant (39), although the effect was less dramatic in *A. fumigatus*. The more subtle ribosome defect in *A. fumigatus* may indicate a level of redundancy in *A. fumigatus* that is not present in yeast. Alternatively, the presence of half-mers without a large change in stoichiometry could be indicative of a defect in subunit association (16). However, we were unable to identify a stable association of CgrA with fractionated ribosome subunits by Western blot analysis (data not shown), suggesting that if there is a role in subunit association, it is likely to be transient (16, 56).

A whole-genome microarray was used to determine how the loss of CgrA affects the expression profile of the organism under a condition of thermal stress that requires CgrA function to support optimum growth. The data revealed that a subset of translational machinery mRNAs were up-regulated in the $\Delta cgrA$ mutant. We hypothesize that these mRNAs are part of an adaptive response to CgrA deficiency, possibly involving the increased nuclear duplication phenotype noted in the $\Delta cgrA$ mutant. Interestingly, a number of these mRNAs showed decreased abundance in the wt following the shift to 37°C (Fig. 4), a finding that may reflect the temporary inhibition of the translational machinery previously reported following a heat shock (15, 55). A small number of translational machinery-associated mRNAs showed decreased abundance in the $\Delta cgrA$ strain relative to the wt following the temperature shift, including L22, L12, L1, L37, Rps29, S14, and P2 (data not shown). Most of these proteins have functions in ribosome structure or assembly (1, 8, 14, 46, 51). The decreased abundance of these mRNAs in the $\Delta cgrA$ strain suggests that their levels are dependent on intact CgrA function, although the mechanism for this is presently unclear.

An unexpected observation from this study was that $\Delta cgrA$ conidia swelled excessively and accumulated nuclei when germinated at 37°C, but not when germinated at 22°C. Thus, loss of CgrA function appears to disrupt the normal synchronization between size and nuclear duplication during the germination process, suggesting a defect in polarized growth that is temperature dependent. The multinucleated hyperswollen conidia of the $\Delta cgrA$ mutant are reminiscent of the phenotype displayed by polarity mutants of *Aspergillus nidulans* (22, 24, 26, 32, 48–50). A variety of genes are responsible for these defects in polarized growth, but *swc1* is of particular relevance to this study because it encodes an rRNA pseudouridine synthase (32), suggesting a link between ribosome biogenesis and the establishment of polarity. Further evidence to support this connection has recently been obtained in *S. cerevisiae*, where a dual function in ribosome biogenesis and polarized growth has been identified for the nucleolar protein Rrp14p (60). Although the mechanism by which ribosome biogenesis can influence polarity is not yet known, it is interesting to note that the *rrp14* mutant displays a ribosome defect similar to that of the *S. cerevisiae cgr1* mutant, including half-mers, decreased 60S subunits, and impaired processing of 27S pre-rRNA to 25S rRNA (39, 60). It is therefore intriguing to hypothesize that certain ribosome defects disproportionately interrupt the translation of mRNAs that encode gene products that influence the establishment of polarized growth. If nuclear duplication continues normally during this period of delayed polarized growth, nuclei would be expected to accumulate in

hyperswollen conidia, as was observed in the $\Delta cgrA$ mutant. The identities of gene products that could be selectively affected by ribosome disruption remain to be explored.

The presence of multinucleated hyperswollen conidia in the $\Delta cgrA$ mutant is also consistent with an alternative model in which nuclear abundance provides a mechanism to regulate ribosome output in proportion to the physiological demand for new proteins. In the vast majority of eukaryotes, the demand for ribosomes is met, in part, by maintaining multiple copies of the rDNA genes (21). However, variability in the number of rDNA genes has been reported within a species, suggesting that rDNA copy number can be modulated depending on the need for protein synthetic capacity. For example, only minor variations in the number of rDNA units are present among different subclones of *Candida albicans* and *S. cerevisiae* when grown slowly at 22°C, but increased rDNA content can be selected for by growing these yeasts more rapidly at their optimal temperatures of 37°C and 30°C, respectively (47). In filamentous fungi, the ribosome content of an ungerminated spore is insufficient to support polarized hyphal growth, so ribosome biogenesis is a major synthetic process during the early stages of germination (25, 36, 58). A defect in ribosome biogenesis is therefore likely to impair protein synthesis and disrupt this early stage of growth, particularly at high temperatures when metabolic needs are high. Since nuclei are the source of new ribosomes, the $\Delta cgrA$ mutant may accumulate nuclei until there are sufficient ribosomes to meet the demand for germ tube emergence and hyphal growth, a response that would require a parallel increase in size to accommodate the nuclei. Such a response would not be expected to completely rescue the growth defect, however, since each nucleus harbors the same mutation. This is consistent with the fact that the germination of the $\Delta cgrA$ mutant is impaired at 37°C (4). Regardless of the exact mechanism involved, the temperature sensitivity of the $\Delta cgrA$ phenotype clearly indicates that growth temperature can be an important variable to consider when studying polarity establishment during germination. Since temperature-sensitive alleles are often used for analysis of polarity in filamentous fungi, such experimental approaches should be interpreted with this caveat in mind.

The question remains as to why thermal stress induces $\Delta cgrA$ germlings to increase in size and accumulate nuclei but does not affect nuclear number or size in mature vegetative hyphae. A possible explanation for this difference is that the mechanism that coordinates nuclear duplication with size and septation is fundamentally different between these two stages of growth. For example, a germling that has not yet formed a septum, defined as “predivisional” (23), undergoes about four nuclear duplications before it lays down the first septum (37). This requires an uncoupling of cell division (septation) from mitosis in order to allow the uninucleated conidium to develop into a multinucleated hypha (23). In contrast, “postdivisional” hyphae are composed of multinucleated cellular compartments that are delimited by septa. These compartments exhibit autonomous nuclear duplication cycles that are tightly coordinated with size and septation (23). Thus, it is conceivable that the intracellular environment of a predivisional germling allows for nuclear accumulation, but the more rigid coupling between cell division and mitosis in postdivisional hyphae may be more restrictive. Interestingly, we found that the effects of

CgrA deficiency on conidial swelling and nuclear duplication could be replicated by germinating wt conidia in the presence of DTT, a reducing agent that disrupts many cell functions, including the translational machinery (45). As with the Δ cgrA mutant, continued growth allowed the nuclei to distribute normally in mature hyphal compartments, suggesting that it is the germination process that is particularly vulnerable to a widespread loss of protein function caused by either CgrA deficiency or DTT.

Taken together, the data outlined in this study establish a role for CgrA in ribosome biogenesis in *A. fumigatus* and provide new evidence that this is linked to the establishment of polarized growth during germination at 37°C. It will be of interest in future studies to determine the mechanism by which CgrA affects polarity and to elucidate the mechanism by which temperature influences this function.

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