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Circadian Activation of the Mitogen-Activated Protein Kinase MAK-1 Facilitates Rhythms in Clock-Controlled Genes in *Neurospora crassa*

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The circadian clock regulates the expression of many genes involved in a wide range of biological functions through output pathways such as mitogen-activated protein kinase (MAPK) pathways. We demonstrate here that the clock regulates the phosphorylation, and thus activation, of the MAPKs MAK-1 and MAK-2 in the filamentous fungus *Neurospora crassa*. In this study, we identified genetic targets of the MAK-1 pathway, which is homologous to the cell wall integrity pathway in *Saccharomyces cerevisiae* and the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in mammals. When MAK-1 was deleted from *Neurospora* cells, vegetative growth was reduced and the transcript levels for over 500 genes were affected, with significant enrichment for genes involved in protein synthesis, biogenesis of cellular components, metabolism, energy production, and transcription. Additionally, of the ~500 genes affected by the disruption of MAK-1, more than 25% were previously identified as putative clock-controlled genes. We show that MAK-1 is necessary for robust rhythms of two morning-specific genes, i.e., *ccg-1* and the mitochondrial phosphate carrier protein gene NCU07465. Additionally, we show clock regulation of a predicted chitin synthase gene, NCU04352, whose rhythmic accumulation is also dependent upon MAK-1. Together, these data establish a role for the MAK-1 pathway as an output pathway of the circadian clock and suggest a link between rhythmic MAK-1 activity and circadian control of cellular growth.

As a result of the daily rotation of the Earth on its axis, organisms regularly face changes in their environment, including daily exposure to potentially harmful factors, such as UV light, high temperature, osmotic stress, and oxidative stress. Internal circadian clocks have evolved that allow organisms to coordinate internal biological processes with the cyclic environment, and thus provide the ability to anticipate and prepare for potential damage. External cues, which serve to reset and synchronize the clock with the environment, are perceived by a molecular circadian oscillator. Time-of-day information is transduced through output pathways to temporally regulate molecular rhythms, including transcription initiation, transcript stability, translation, and posttranslational processes, as well as physiology and behavior (1, 2). Significant progress has been made in understanding input pathways to the oscillator and the molecular mechanisms of the circadian oscillator; however, the mechanisms by which the oscillator regulates rhythmic output processes are just beginning to be unraveled (3–6).

In *Neurospora crassa*, the Frequency (FRQ)/White Collar Complex (WCC) oscillator (FWO) has been well characterized at the molecular level (7, 8). The FWO consists of a molecular feedback loop in which the WCC, composed of White Collar-1 (WC-1) and WC-2, acts as the positive element by directly activating transcription of *frq* mRNA (9). After translation of *frq* mRNA, the FRQ protein, in association with the FRQ-interacting RNA helicase (FRH), functions as a negative component by indirectly inhibiting the activity of the WCC (9–13). Over time, the FRQ protein is progressively phosphorylated and degraded, thereby relieving inhibition of WCC, allowing for the cycle to begin again (14). The WC-1 protein is also a blue-light photoreceptor and, with its partner WC-2, functions as a transcription factor to induce both light-responsive genes and clock-controlled genes (ccgs) (5, 15, 16). Studies in *Neurospora* have suggested that at least 20% of the genome is under the control of the circadian clock at the level of transcript abundance (17, 18). The WCC directly controls the expression of about 200 genes, many of which are ccgs that peak in expression in the subjective morning, coincident with the peak of WCC activity (5, 15). However, there are many ccgs that peak in expression at other times of the day, suggesting a complex output regulatory network. Consistent with this idea, several of the direct targets of the WCC are themselves transcription factors, including repressors that control evening-specific gene expression (5, 19). Additionally, clock regulation of major signaling pathways, such as mitogen-activated protein kinase (MAPK) pathways, provides mechanisms for circadian regulation of large sets of functionally related downstream target genes of the pathway. For example, we previously found that the *Neurospora* FWO controls rhythmic activity of the osmolarity-sensitive (OS) MAPK pathway and downstream ccg targets of the pathway (20–22).

The evolutionarily conserved MAPK signaling pathways are composed of 3 kinases, the MAP kinase kinase kinase (MAPKKK), the MAP kinase kinase (MAPKK), and finally the MAP kinase (MAPK). The pathway becomes sequentially phosphorylated in response to extracellular signals, and the phosphorylated MAPK then activates downstream effector molecules, including transcription factors that turn on target genes needed for the cell to respond, survive, and adapt to acute environmental signals (23). In *Neurospora*, the OS-2 MAPK is homologous to HOG1 in *Saccharomyces cerevisiae* and is responsible for survival during acute

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hyperosmotic conditions (24, 25). In studies to identify output pathways from the clock, we discovered that the WCC rhythmically binds to the promoter of *os-4*, encoding the OS-4 MAPKKK, and drives rhythms in the accumulation of *os-4* mRNA and OS-4 protein. Rhythmic accumulation of OS-4 protein is necessary for rhythmic phosphorylation and activation of the terminal MAPK, OS-2, and downstream ccgs (20, 21). Taken together, these data demonstrate that clock control of the OS pathway provides a mechanism for *Neurospora* to prepare for predictable daily hyperosmotic stresses.

In addition to OS-2, there are two other MAPKs present in *Neurospora*; MAK-1, a homologue of Slt2 in *S. cerevisiae* and extracellular signal-regulated kinase 1/2 (ERK1/2) in mammals, is predicted to be involved in cell wall integrity and maintenance, while MAK-2, a homologue of Fus3 in *S. cerevisiae*, and also ERK1/2 in mammals, has been implicated in the sexual cycle, conidiation, hyphal fusion, and vegetative growth (26–30). In this study, we show that the phosphorylation state of MAK-1 and MAK-2 is rhythmically controlled by the circadian clock; however, we focus on characterizing the circadian regulation of the MAK-1 pathway. The MAK-1 pathway is the least-characterized MAPK pathway in *Neurospora*. In *S. cerevisiae*, Slt2 modulates cell wall biogenesis, responses to cell wall perturbations and stress, and the cell cycle, while in mammals, ERK1/2 promotes cell growth and cell cycle regulation (31, 32). Although predictions can be made based on homology, there have been no extensive studies to identify the genetic targets of the MAK-1 pathway in *Neurospora*. Phenotypic analyses of deletion mutants of the MAK-1 pathway have shown various levels of perturbation in the cell wall, increased melanin biosynthesis, and decreased growth rate, polarity, hyphal fusion, conidiation, and sexual reproduction (28, 29).

Here we show that while MAK-1 protein levels are constitutive over the course of the day under constant conditions, the phosphorylation state, and thus activity, of MAK-1 is under the control of the clock. We also establish that MAK-1 is phosphorylated after an increase in temperature. Using microarray analysis, we identified potential target genes of the MAK-1 pathway and compared them to putative ccgs to determine the overlap in these processes. We verified expression levels of several of the MAK-1 downstream target genes and showed that MAK-1 is necessary for regulating mRNA levels in an unstressed environment. Furthermore, we demonstrated that 3 of the targets of the MAK-1 pathway are ccgs, with rhythms that are dependent on a functional MAK-1 pathway, establishing that the circadian clock signal is propagated through the MAK-1 pathway.

MATERIALS AND METHODS

Strains and culture conditions. *N. crassa* wild-type strain 74OR23-1 (FGSC 2489) and a Δ*mak-1* mutant strain (FGSC 11320) were obtained from the Fungal Genetics Stock Center. The Δ*mak-1* strain was created by replacing the *mak-1* coding region with a hygromycin resistance gene (33), and the knockout was verified using PCR (data not shown). The Δ*frq* and Δ*wc-1* strains were generated by replacing the coding region with the *bar* gene, conferring resistance to Basta (glufosinate-ammonium) in 74OR23-1 (34; T. M. Lamb and D. Bell-Pedersen, unpublished data). Correct integration of *bar* was verified using PCR (data not shown). The Δfrq^{BAR} and $\Delta wc\text{-}1^{BAR}$ strains (referred to here as the Δ*frq* and Δ*wc-1* strains, respectively) were backcrossed to 74OR23-1 to obtain homokaryons. A strain expressing a MAK-1::LUC translation fusion was created in a 3-way PCR using a fully codon-optimized luciferase gene (35; R. M. dePaula and D. Bell-Pedersen, unpublished data) and then transformed into 74OR23-1, where the *mak-1* gene was replaced with the MAK-1::LUC fusion by homologous recombination. Transformants displaying luciferase activity were chosen and crossed with the wild type to obtain homokaryons. Integration at the *mak-1* locus was verified by PCR (data not shown). Circadian time course experiments were performed in liquid culture as previously described (21), with the following modifications. Mycelial mats from the Δ*mak-1* strain required 4 additional days of growth in constant light (LL) compared to wild-type strains, because of poor growth. For RNA isolation, liquid cultures were grown in LL at 25°C for 24 h, transferred to constant darkness (DD) at 25°C for 24 h, and then harvested at the indicated times. For heat shock experiments, cells were shifted after growth in LL at 25°C for 24 h to DD at 42°C and harvested at the indicated times. To measure the linear growth rates of the wild-type and Δ*mak-1* strains, race tube analysis was done as previously described (36).

Protein extraction and Western blot assays. Total protein extracts, protein quantification, and Western blot analyses were done as previously described (21). To detect the phosphorylated state of MAK-1 (P-MAK-1), membranes were blocked with 5% nonfat milk in phosphate-buffered saline with 0.1% Tween 20 (PBST) overnight at 4°C and then washed 3 times for 5 min each in PBST before being incubated with rabbit anti-phospho-p44/42 (9101; Cell Signaling, Danvers, MA) at a 1:200 dilution in 5% bovine serum albumin (BSA)-PBST. After an overnight incubation at 4°C, membranes were washed 6 times for 10 min each in PBST and then incubated with a 1:10,000 dilution of secondary antibody [goat anti-rabbit IgG(H+L)–horseradish peroxidase (HRP) conjugate; Bio-Rad, Hercules, CA] in 5% nonfat milk-PBST at 4°C overnight. Membranes were washed 6 times for 10 min in PBST. Immunoreactivity was then visualized with a Super Signal West Pico chemiluminescence detection kit (Thermo Scientific, Waltham, MA). To detect the MAK-1::LUC protein, Western blots were performed as described above, except that membranes were probed with a primary goat anti-luciferase antibody (G7451; Promega, Madison, WI) at a 1:10,000 dilution and a secondary donkey anti-goat antibody (2020; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:5,000 dilution. To verify the phosphate-specific signal, protein extracts were treated with λ-protein phosphatase (New England BioLabs, Ipswich, MA) as indicated. After exposure to X-ray film, images were scanned at 600 dpi in TIFF format. Densitometry was performed using ImageJ software, and signal levels were normalized to the amido black-stained membrane.

Luciferase assays. Mycelial mats of the MAK-1::LUC strain were grown in 1× Vogel's salts, 2% glucose, 0.5% arginine-HCl, pH 6.0, in LL at 25°C for 3 days. Mycelial mats were cut with a cork borer, generating disks with a 0.5-cm diameter. The disks were washed in sterile water for 30 min before transfer to 96-well plates. Each well was inoculated with 170 μl medium containing 1× Vogel's salts with 25 μM luciferin (LUCNA-300; Gold Biotechnology, St. Louis, MO) and no carbon source. The plates were incubated in LL at 30°C for 4 h and then transferred to a Packard Topcount microplate scintillation and luminescence counter (Perkin-Elmer Life Sciences, Boston, MA). The plates were kept in DD at 25°C, and light production was assayed every 90 min. Data were collected and analyzed using the Import and Analysis (I&A) program designed by the Kay laboratory (37).

RNA extraction and Northern blot assay. Total RNA was extracted from tissue harvested and ground in liquid N_2 as described previously (38). All transcripts were detected by Northern blotting using [α-^{32}P]UTP-labeled RNA probes as described previously (5). Primer sequences used to make gene-specific templates are listed in Table S1 in the supplemental material. Images were processed as described for Western blots and normalized to rRNA levels. To determine significant differences in expression of selected genes between the wild-type and Δ*mak-1* strains, Student's *t* test was performed, and *P* values were required to be ≤0.05.

Microarray analysis. Wild-type and Δ*mak-1* strains were grown in LL for 24 h and then transferred to DD for 24 h before being harvested and having the total RNA extracted. RNA samples were treated with DNA-free (Ambion, Grand Island, NY) to remove residual genomic DNA and then

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amplified using SenseAmp (Genisphere, Hatfield, PA). The high quality of the original and amplified RNAs was confirmed with an Agilent Bioanalyzer (Santa Clara, CA) using RNA 6000 nanochips. The amplified RNA was used to prepare Cy-dye-labeled aminoallyl cDNA targets, using a Pronto indirect labeling system (Promega, Madison, WI). Targets obtained from mutant and wild-type cells at designated time points were labeled with either Cy5 or Cy3. The 70-mer oligonucleotide probes were synthesized by Illumina (San Diego, CA) and represented 10,036 open reading frames (ORFs) identified by the Broad Institute (http://www.broadinstitute.org/annotation/genome/neurospora). The printed arrays were cross-linked to the gamma amino propyl silane coating of Ultra-GAPS slides (Corning Life Sciences, Tewksbury, MA) with 600 mJ of UV energy in a Stratagene Stratalinker (Agilent Technologies, Santa Clara, CA). The arrays were presoaked and prehybridized to lower background fluorescence and then hybridized with the labeled cDNAs by using the reagents supplied in a Corning Pronto universal hybridization kit according to the manufacturer's specifications. As an internal control, Cy5- and Cy3-labeled wild-type-derived cDNAs were hybridized together in duplicate. Wild-type-to-Δ*mak-1* sample hybridization was performed in triplicate. Two of the replicates were carried out with Cy5-labeled mutant-derived cDNAs and Cy3-labeled wild-type-derived cDNAs. In the third replicate, the dyes used to label the cDNAs were swapped. Following hybridization, the arrays were washed with the buffers provided in a Pronto universal hybridization kit (Promega, Madison, WI) as recommended by the manufacturer. The washed arrays were quickly dried under a stream of nitrogen and then scanned using an Affymetrix (Santa Clara, CA) 428 array scanner. Image and data analyses were carried out with GenePix 6.1 and GeneSpring 7.3 software as previously described (39, 40). Data were filtered using the following criteria: the ratio of the wild-type value to the wild-type average value was required to be between 0.5 and 2.0. Genes that met these conditions were then subjected to the following restrictions: in comparing RNAs from the Δ*mak-1* and wild-type strains in triplicate, values for each of the three experiments were required to be <0.67 or >1.5 relative to the wild-type value, or the average values for the 3 replicates were required to be statistically different from the wild-type value, using Student's *t* test and a *P* value cutoff of 0.05.

Statistical data. Nonlinear regression was used to fit the rhythmic data to a sine wave (fitting period, phase, and amplitude) or a line (fitting slope and intercept). Akaike's information criterion tests (41) to compare the fit of each data set to the 2 equations were carried out using the Prism software package (GraphPad Software, San Diego, CA). The *P* values reflect the probability that the sine wave fits the data better than a straight line.

Microarray data accession numbers. The raw microarray data are available at the Filamentous Fungal Gene Expression Database (http://bioinfo.townsend.yale.edu/), under experiment ID number 225, and the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), under series GSE41778.

RESULTS

The levels of phosphorylated MAK-1 and MAK-2 are regulated by the circadian clock. In previous studies of *Neurospora*, the phosphorylation state of MAK-1 and MAK-2 has been detected using an available phospho-specific antibody against the mammalian homologs p44 and p42 (29, 42). As expected, using this antibody in Western blots, bands were observed corresponding to the predicted sizes for MAK-1 (46.8 kDa) and MAK-2 (40.8 kDa) that were absent in the Δ*mak-1* and Δ*mak-2* strains, respectively (Fig. 1A). Under these growth conditions, the levels of phosphorylated MAK-2 (P-MAK-2) were lower than those of phosphorylated MAK-1 (P-MAK-1) in the wild-type strain, and the levels of P-MAK-2 were increased in the Δ*mak-1* strain compared to the wild-type strain.

To determine if the phosphorylation state of the MAPKs is under clock control, the levels of P-MAK-1 and P-MAK-2 were

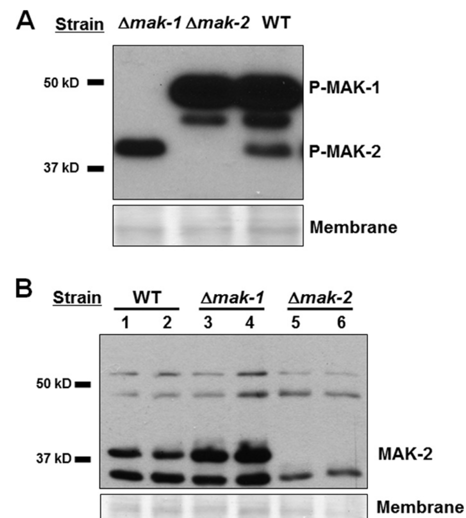

FIG 1 Detection of MAK-1 and MAK-2. (A) Western blot showing that P-MAK-1 and P-MAK-2 are detectable using an anti-phospho-p44/42 antibody. Proteins were isolated from the indicated strains, and the stained membrane is shown as a loading control. (B) Western blot showing that total MAK-2 protein, but not total MAK-1 protein, is detectable using the anti-p44/42 antibody. Proteins were isolated from the indicated strains, and the stained membrane is shown as a loading control. No specific bands were observed for the wild-type (WT) or Δ*mak-2* strain that were absent in the Δ*mak-1* strain, indicating that the antibody does not recognize MAK-1. Lanes 2, 4, and 6 were treated with λ-phosphatase in an attempt to increase the resolution of the bands, while lanes 1, 3, and 5 were left untreated.

examined in cultures grown in a circadian time course, in which the cells were maintained in DD and constant temperature and harvested every 4 h over 2 days. Under these conditions, the only time cue is from the endogenous circadian clock. A daily rhythm in both P-MAK-1 and P-MAK-2 levels was observed in wild-type strains, peaking in the subjective afternoon (about DD h 16 [DD16] and DD40/DD44) (Fig. 2A). Consistent with control of MAK-1 and MAK-2 phosphorylation by the circadian clock, the rhythms were disrupted in cells that lacked the core oscillator component FRQ (Fig. 2A and B). Additionally, unlike with P-OS-2, where the levels of P-OS-2 were significantly reduced in Δ*wc-1* cells and increased in Δ*frq* cells (22), we did not observe a significant change in overall levels of P-MAK-1 or P-MAK-2 in Δ*frq* and Δ*wc-1* strains compared to the wild type (Fig. 2C), suggesting a different mechanism of clock regulation.

The MAK-1 pathway is the least-characterized MAP kinase pathway in *Neurospora*. Therefore, we focused further efforts on defining the MAK-1 pathway. MAK-1 was previously shown to be phosphorylated after treatment with a β-1,3 glucan inhibitor, aculeacin A (29), suggesting that the MAK-1 pathway functions in the cell wall stress response pathway in *Neurospora*, similar to Slt2 in *S. cerevisiae* (43). Slt2 is also activated in response to increased temperatures, which can destabilize the integrity of the cell wall (44). Under constant conditions, the peak in phosphorylation of MAK-1 occurs in the subjective afternoon, a time of day when environmental temperatures are likely to be higher; therefore, MAK-1 may be activated under similar conditions. Indeed, Vogt and Seiler observed an increase in P-MAK-1 after a shift in temperature from 25°C to 37°C (45), and we determined that after a temperature shift from 25°C to 42°C, wild-type cells consistently

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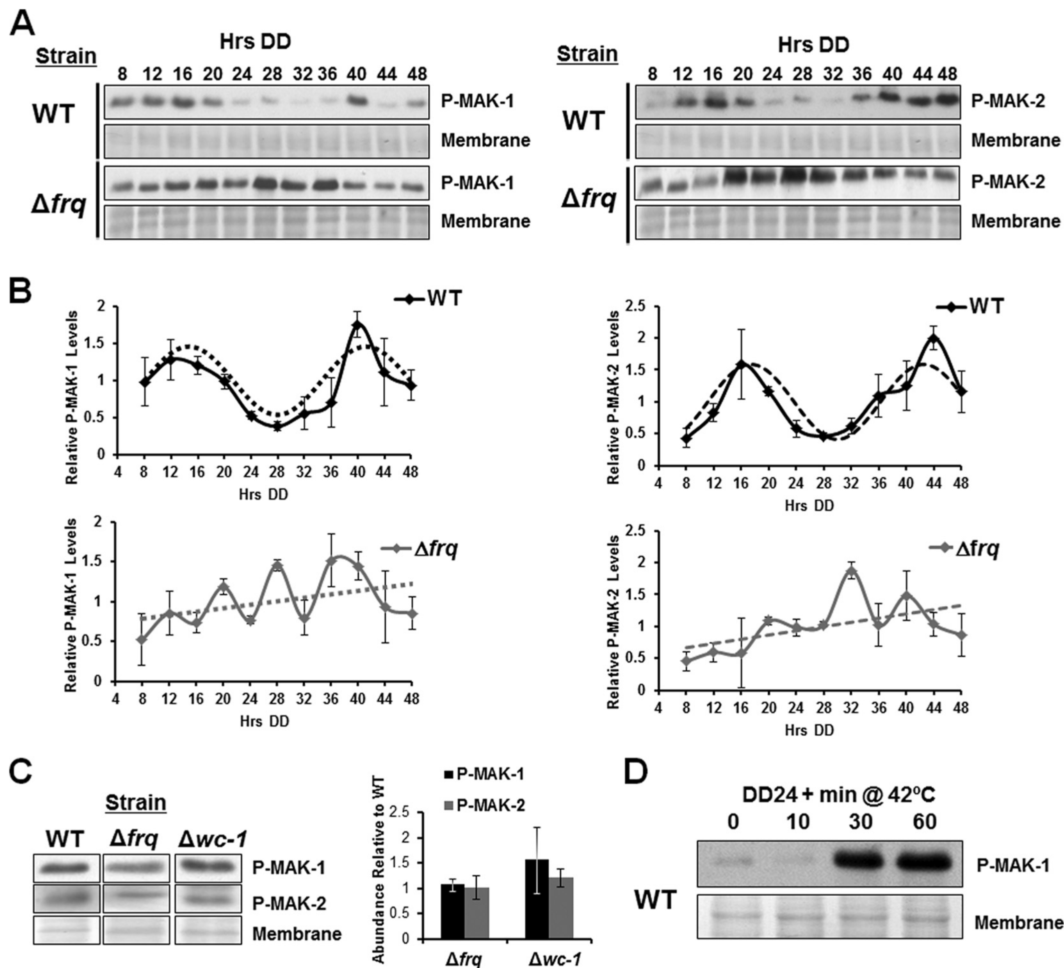

FIG 2 Circadian regulation of MAK-1 and MAK-2 phosphorylation. (A) The accumulation of P-MAK-1 (left) and P-MAK-2 (right) is shown in representative Western blots of total protein from the indicated strains grown in a standard circadian time course in constant dark (DD) and harvested every 4 h. The blots were probed with an anti-phospho-p44/42 antibody. The stained membranes were used as loading controls. (B) Plots of normalized P-MAK-1 and P-MAK-2 data from panel A. The WT data (solid black line) were better fit to a sine wave (dotted black line) (for P-MAK-1, $P < 0.001$; for P-MAK-2, $P < 0.0001$), whereas the Δ*frq* data (solid gray line) were better fit to a line (dotted gray line) (values are means ± standard errors of the means [SEM]; $n = 3$). (C) The levels of P-MAK-1 and P-MAK-2 are not altered in clock mutant strains. Western blotting was performed on total proteins harvested from the specified strains grown in DD for 16 h, and the membrane was probed with an anti-phospho-p44/42 antibody. The stained membrane is shown as a loading control. The data are plotted on the right. The levels of P-MAK-1 and P-MAK-2 in WT cells were set to 1 (values are means ± SEM; $n = 3$). (D) Representative Western blot showing the levels of P-MAK-1 at 25°C and after 10, 30, or 60 min at 42°C. Stained membranes are shown as a loading control. This experiment was performed 3 times with similar results.

showed a large increase in the levels of P-MAK-1 after 30 and 60 min at the elevated temperature (Fig. 2D).

To resolve whether the rhythm in P-MAK-1 is due to rhythms in the accumulation of total MAK-1 protein, we first examined if the available antibody against total mammalian p44/42 would recognize MAK-1 (42); however, this antibody detected MAK-2, not MAK-1 (Fig. 1B). Therefore, to detect MAK-1, a translational fusion between MAK-1 and luciferase was generated. No circadian rhythms in MAK-1::LUC were observed by Western blotting using anti-Luc antibodies or assays to measure luciferase activity (Fig. 3). While it is possible that the tag interfered with the stability of the protein, the levels of P-MAK-1 cycled normally, and no obvious phenotype was associated with this strain (Fig. 3 and data not shown). Furthermore, using a different tag (V5), no circadian rhythm in MAK-1::V5 was observed (data not shown).

A Δ*mak-1* strain, generated by replacing the *mak-1* locus with a gene conferring resistance to hygromycin (33), has a severe defect in growth, hyphal fusion, protoperithecium formation, and asexual conidiation, indicating that MAK-1 is important for the overall growth and health of the cell (29). Using the race tube assay, the circadian phenotype and linear growth rate of the Δ*mak-1* strain grown in DD at 25°C were assayed. The Δ*mak-1* strain grew at 0.27 ± 0.01 cm/day, which was significantly reduced relative to the wild-type growth rate of 6.6 ± 0.57 cm/day. As expected, the Δ*mak-1* strain did not conidiate, and therefore a circadian rhythm in development could not be observed (data not shown). To ensure that the FWO was functional in the Δ*mak-1* strain, FRQ protein levels were assayed in the wild-type and Δ*mak-1* strains over a circadian time course, and no major difference was observed between the two strains (Fig. 4A). We also tested the rhythmicity of the anchored cell wall protein 1 (*acw-1*) gene, also known as *ccg-15*, a ccg regulated by the FWO (46) and

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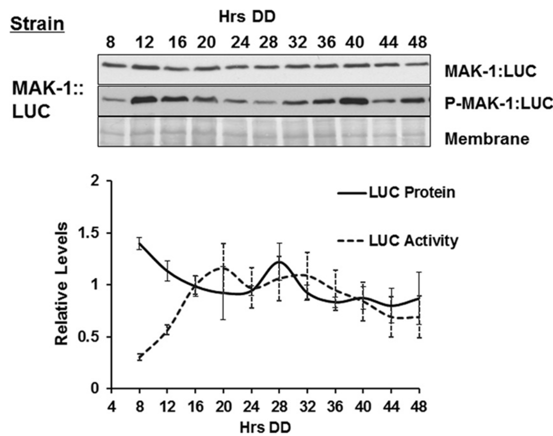

FIG 3 The total level of MAK-1 is not clock controlled. Western blotting was performed on total protein from MAK-1::LUC cells grown in a circadian time course (see the legend to Fig. 2). The blots were probed with anti-LUC or anti-phospho-p44/42. The stained membrane was used as a loading control. Plots of MAK-1::LUC protein levels and luciferase activity are shown below the blots (values are means ± SEM; $n = 3$).

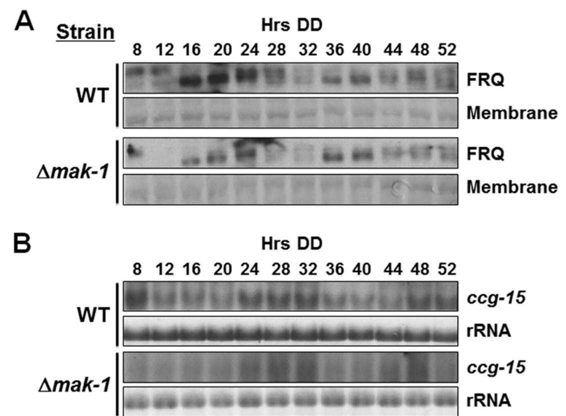

FIG 4 The FWO remains functional in the absence of MAK-1. (A) Western blots probed with anti-FRQ, showing both the levels and phosphorylation state of FRQ protein in WT and Δ*mak-1* cells grown in a circadian time course (see the legend to Fig. 2). The stained membrane was used as a loading control. (B) Northern blots probed for *ccg-15* expression from WT and Δ*mak-1* strains grown in DD and harvested every 4 h. rRNA was used as a loading control. Consistent results were obtained from two independent experiments.

encoding a protein homologous to the extracellular mutant (Ecm33) regulated by the Slt2 pathway in *S. cerevisiae* (47). No major differences in expression levels or rhythms were observed between wild-type and Δ*mak-1* cells (Fig. 4B). Based on these results, we concluded that although the Δ*mak-1* strain grows poorly, the FWO remains functional, and any effects observed on ccg rhythms in the mutant strain can be attributed to a defect in output from the clock.

Identification of downstream targets of P-MAK-1. Phosphorylation of MAPKs is associated with activation of target transcription factors, kinases, and other regulatory networks (48). The demonstration that P-MAK-1 is clock regulated suggested that at least some of the downstream targets of P-MAK-1 are likely clock controlled. To test this idea, we first needed to identify downstream genes of the MAK-1 signaling pathway. To accomplish this, microarray analyses were carried out to identify genes with altered expression in the Δ*mak-1* strain compared to the wild-type strain grown in DD and harvested at 24 h. At this time of day, the levels of P-MAK-1 are decreasing. Thus, any observed changes in mRNA levels may reflect clock-dependent (such as a change in period or phase of the rhythms) or clock-independent events. The microarray analyses identified 517 genes that were misregulated in the Δ*mak-1* strain (see Table S2 in the supplemental material), including 111 that were downregulated and 406 that were upregulated (Table 1), suggesting a role for MAK-1 in both activation and repression of gene expression. The genes were sorted into functional categories by using the Munich Information Center for Protein Sequences Functional Catalogue (MIPS FunCat), and they included genes in 15 of the 20 major functional classifications represented in the *Neurospora* genome (Table 1) (49). Genes with significant enrichment under the control of MAK-1 included genes involved in metabolism, energy production, biogenesis of cellular components, transcription, and protein synthesis, suggesting a role for MAK-1 in these processes. Interestingly, of the 111 genes with decreased levels of mRNA at DD24, 38 (34%) were classified as being involved in protein synthesis, indicating a positive role for MAK-1 in protein synthesis; specifically, in the absence of MAK-1, genes encoding many ribosomal proteins were downregulated. Furthermore, mutations in the MAK-1 homolog Slt2 in *S. cerevisiae* result in defects in the cell wall and growth (50), and a loss of MAK-1 MAPK pathway components also reveals weak cell walls and a significantly reduced growth rate (29). Although few of the MAK-1 candidate downstream target genes overlapped those known to be regulated by Slt2 in *S. cerevisiae*, the positive regulation of genes necessary for protein synthesis by the MAK-1 pathway correlates with the decreased growth rate observed when the pathway is disrupted.

Using Northern blot analysis, mRNA levels for 14 candidate genes identified as misregulated in Δ*mak-1* cells, and representing 8 of the 15 functional categories, were analyzed (Fig. 5A). We focused primarily on those genes that had decreased expression in the Δ*mak-1* strain at DD24 and are involved in cellular processes predicted to be regulated by MAK-1, including biosynthesis of cellular components, metabolism, cellular communication/signal transduction, and stress response/interaction with the environment, as well as the most highly enriched category, protein synthesis (Table 1). mRNA levels of the candidate genes were examined in wild-type and Δ*mak-1* cells from cultures harvested under the same growth conditions used for the microarray analyses. In accordance with the microarray data, NCU06871, NCU04352, *acw-5*, NCU02044, NCU08923, *hsp88*, *hsp70* (NCU02075), NCU03980, *crp-4*, and *ccg-1* mRNA levels were reduced, while the levels of NCU07465 and *acw-2* were elevated, in the Δ*mak-1* strain compared to the wild type. The expression of 2 predicted MAK-1 downstream target genes, NCU05429 and NCU07472, was inconsistent with the microarray results (Fig. 5A and B). To differentiate between genes that are misregulated in the Δ*mak-1* strain at all times of the day and those that show misregulation due to variations in the period or phase (ccgs) in Δ*mak-1* cells, mRNA levels of 2 of the downstream target genes, *acw-2* and NCU06871, were assayed at 3 different times of the day, including subjective afternoon (DD16), subjective late afternoon (DD20), and subjective evening (DD24). Consistent with the microarray results, *acw-2* and NCU06871, neither of which is predicted to be a ccg, were misregulated in Δ*mak-1* cells at all 3 time points (Fig. 5C). Three

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TABLE 1 MAK-1 regulation of gene expression

Classification	Observed regulation[a]			Expected regulation[b]	Predicted ccg(s) verified by Northern blotting	No. of regulated ccgs[c]		
	Down	Up	Total			Down	Up	Total
Metabolism	27	81	108	68	NCU06871	10	27	37
Energy	6	14	20	7		3	5	8
Cell cycle and DNA processing	3	10	13	21		0	1	1
Transcription	3	28	31	24		1	6	7
Protein synthesis	38	5	43	14	*crp-4*	20	2	22
Protein fate	1	11	12	26		0	1	1
Protein with binding function or cofactor requirement	1	7	8	7		0	0	0
Regulation of metabolism and protein function	1	2	3	2		0	1	1
Cellular transport, facilitation, and routes	2	28	30	24		1	4	5
Cellular communication/signal transduction mechanism	5	11	16	2	NCU02044, NCU08923, *acw-5*	0	3	3
Cell rescue, defense, and virulence	2	18	20	7	*hsp88*, NCU03980	0	3	3
Interaction with the environment	1	5	6	6	*hsp70*, NCU07465	0	0	0
Transposable elements, viral and plasmid proteins	0	1	1	0		0	0	0
Biogenesis of cellular components	5	9	14	3	NCU04352, *acw-2*	2	1	3
Unclassified proteins	16	176	192	307	*ccg-1*	6	48	54
Total	111	406	517	517	10	43	102	145

[a] Number of genes observed to be up- or downregulated in the Δ*mak-1* strain compared to the WT.
[b] Total number of genes expected to be represented in each classification compared to the abundance within the genome.
[c] Number of genes observed to be up- or downregulated in the Δ*mak-1* strain compared to the WT and predicted to be rhythmic.

additional genes were assayed every 4 h during a circadian time course. *ccg-1* and NCU04352 showed decreased expression in Δ*mak-1* cells over the course of 2 days in DD, while NCU07465 mRNA levels were increased in Δ*mak-1* cells at all times of the day (Fig. 6). These data indicated that the MAK-1 pathway is necessary for normal mRNA accumulation of at least a subset of its target genes, including known and predicted ccgs.

To identify rhythmic targets of the P-MAK-1 pathway, we

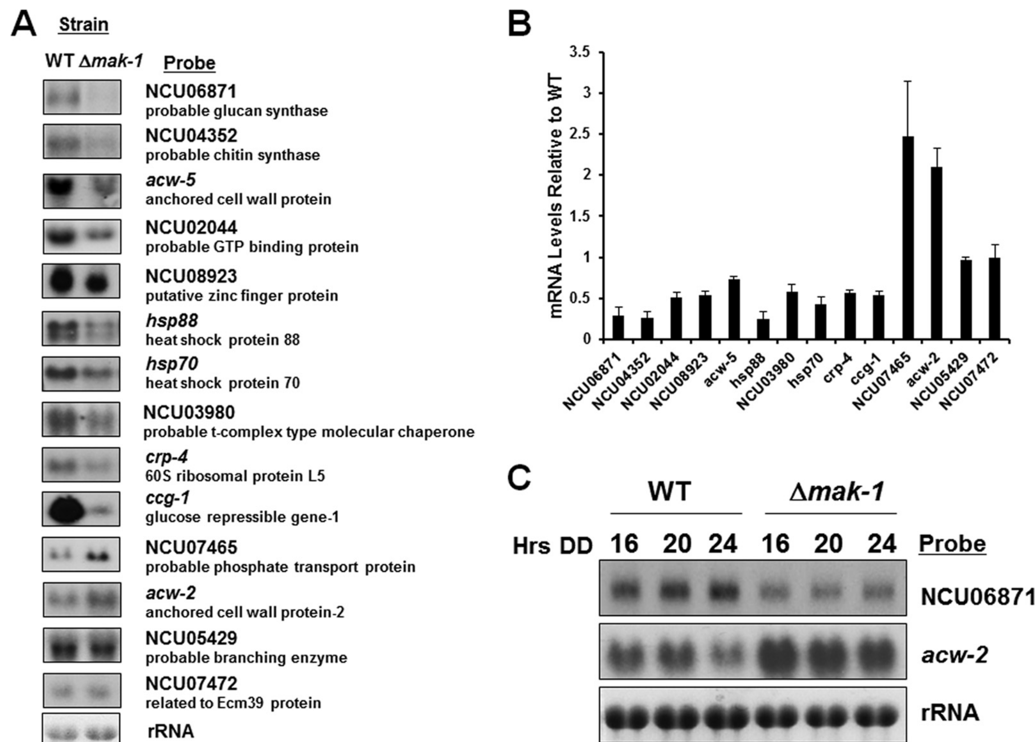

FIG 5 MAK-1 is required for regulation of target genes. (A) Representative Northern blots of total RNAs from the indicated strains, validating the MAK-1-dependent regulation of 12 of 14 genes identified by microarray analysis. The membranes were probed with the indicated genes. The known or predicted functions or domains of the genes are shown below the gene names. rRNA was used as a loading control. (B) Plots of mRNA levels of the indicated genes in Δ*mak-1* cells, normalized to rRNA (see panel A) (values are means ± SEM; $n = 3$), relative to the levels in WT cells. The level of mRNA in WT cells in each blot was set to 1. Twelve of 14 genes tested showed significantly different expression levels between the WT and Δ*mak-1* strains (for NCU06871, $P \leq 0.02$; for NCU04352, $P \leq 0.01$; for NCU02044, $P \leq 0.05$; for NCU08923, $P \leq 0.04$; for *acw-5*, $P \leq 0.02$; for *hsp88*, $P \leq 0.02$; for NCU03980, $P \leq 0.05$; for NCU02075, $P \leq 0.03$; for *crp-4*, $P \leq 0.004$; for *ccg-1*, $P \leq 0.01$; for NCU07465, $P \leq 0.02$; for *acw-2*, $P \leq 0.04$; for NCU05429, $P \leq 0.39$; and for NCU07242, $P \leq 0.21$). (C) Northern blots probed for NCU06871 and *acw-2* expression from WT and Δ*mak-1* strains grown in DD for the indicated times. rRNA is shown as a loading control.

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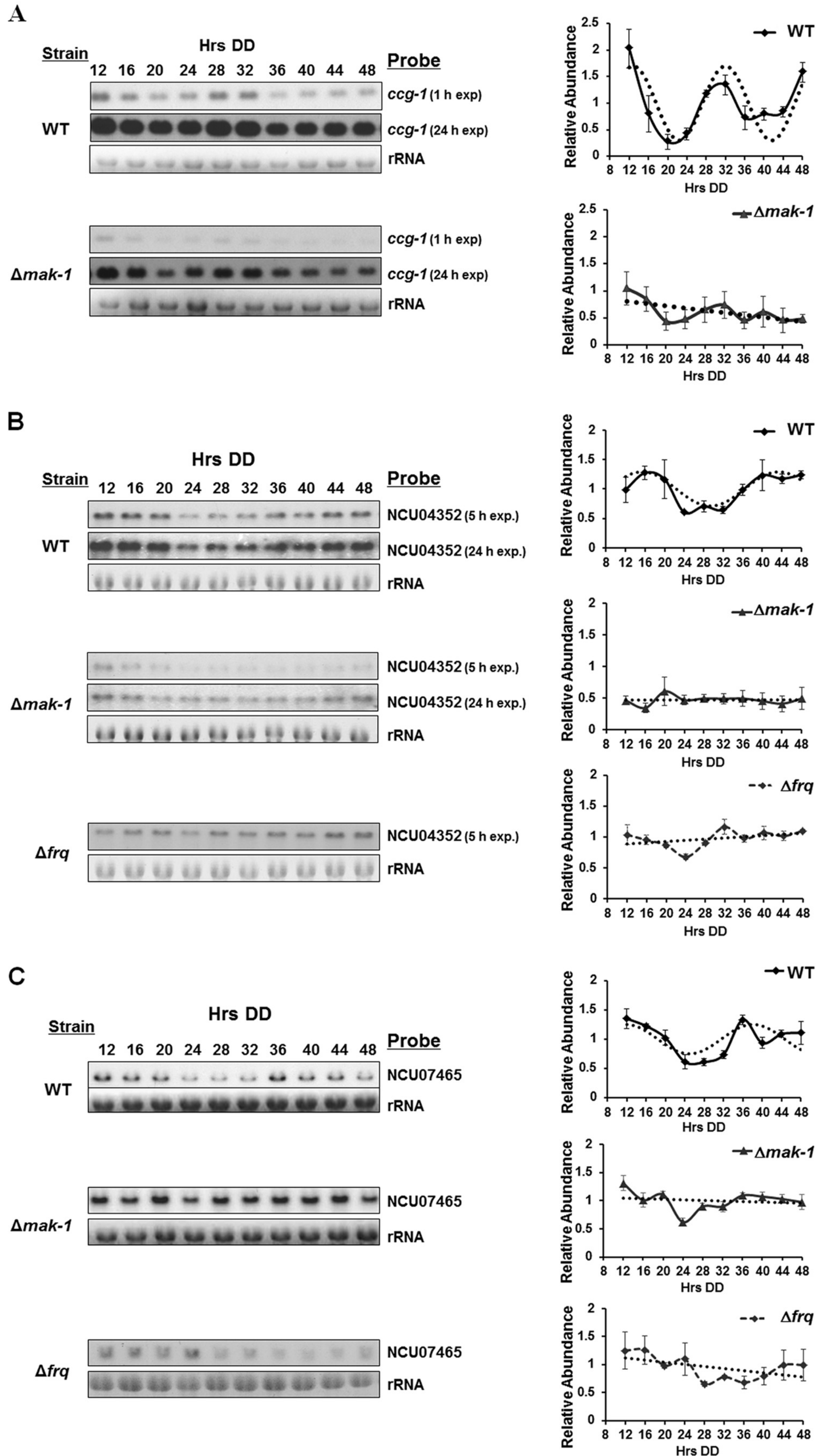

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compared the Δ*mak-1* microarray data to 3,818 unique genes that were previously classified as putative ccgs by use of microarrays (17, 18, 51, 52; R. M. dePaula, R. Gomer, P. Beremand, T. L. Thomas, and D. Bell-Pedersen, unpublished data) and found that, of the 517 genes predicted to be expressed differentially in the Δ*mak-1* strain at DD24, at least 145 genes were also predicted to be ccgs (Table 1; see Table S2 in the supplemental material). Similar to the total number of MAK-1-responsive genes, an enrichment of ccgs involved in metabolism, energy, and protein synthesis was observed. Taken together, these data support the idea that the clock regulates rhythms in the activity of MAK-1, which, in turn, controls rhythmic expression of many target genes of the pathway.

MAK-1 is required for rhythmic expression of some pathway target genes. To determine if ccgs that are targets of the MAK-1 pathway require MAK-1 for rhythmic mRNA accumulation, a subset of MAK-1 target ccgs was examined for circadian rhythms in mRNA accumulation in the Δ*mak-1* strain. As shown in Fig. 3, levels of the *ccg-1* transcript were reduced in the Δ*mak-1* strain under non-stress-inducing conditions, suggesting that MAK-1 is important for *ccg-1* mRNA accumulation. This led us to investigate if MAK-1 is necessary for rhythmic *ccg-1* mRNA accumulation. The amplitude of the observed rhythms in *ccg-1* accumulation was reduced in Δ*mak-1* versus wild-type cells (Fig. 6A), and a rhythm was not reliably reproducible; low-amplitude rhythms were detected in half of the experiments. We previously showed that the OS-2 MAPK pathway regulates rhythms in *ccg-1* expression; thus, it was not unexpected that when MAK-1 was deleted in cells, some elements of rhythmicity were maintained under the control of the OS pathway, which peaks in phase with *ccg-1*. Regulation of *ccg-1* by at least 2 MAPK pathways may also underlie the observation that the peak in *ccg-1* expression precedes the peak in MAK-1 phosphorylation rhythms (Fig. 2 and 6A).

We next focused on genes known or predicted to be a part of the biogenesis of cellular components. The structural elements of the cell wall in *Neurospora* consist primarily of polymers made of glucan and chitin (53). There are at least 2 proteins required for glucan synthesis in *Neurospora*; the enzyme glucan synthase 1, encoded by the *gs-1* gene (NCU06871), and the regulatory subunit RHO-1, encoded by the NCU01484 gene (54, 55). Transcripts for both genes were misregulated in Δ*mak-1* cells; transcript levels of *gs-1* were reduced, while *rho-1* levels were elevated in Δ*mak-1* cells compared to wild-type cells at DD24 (Fig. 5 and data not shown). Although there are 10 genes predicted to be involved in chitin synthesis in *Neurospora*, only the NCU04352 gene, encoding a predicted chitin synthase, was found to be misregulated in the Δ*mak-1* strain in the microarray experiments. As with *gs-1*, NCU04352 mRNA levels were reduced by about half in the Δ*mak-1* strain under non-stress-inducing conditions. While neither of these cell wall biogenesis genes had previously been reported to be clock controlled, based on their regulation by the rhythmic MAK-1 pathway, we assayed mRNA levels of *gs-1* and NCU04352 in wild-type and Δ*mak-1* strains over a circadian time course. No rhythm was observed for *gs-1* mRNA levels in wild-type cells (data not shown); however, a rhythm in mRNA accumulation was observed for NCU04352 in wild-type cells, with peaks occurring around DD16 and DD40 (Fig. 6B), a phase similar to that of P-MAK-1 (Fig. 2). Consistent with clock regulation of the MAK-1 pathway, the rhythm in NCU04352 was abolished in Δ*frq* and Δ*mak-1* strains (Fig. 6B). The levels of NCU04352 mRNA were low in the Δ*mak-1* strain compared to wild-type cells at all times of day, supporting the idea that P-MAK-1 positively regulates NCU04352 expression. Although the levels were decreased, transcript levels of NCU04352 were detectable in Δ*mak-1* cells after a 24-h exposure to film, but with no obvious circadian rhythm in mRNA levels (Fig. 6B).

Finally, we tested the necessity for MAK-1 in the rhythmic accumulation of an additional putative ccg, NCU07465, encoding a predicted mitochondrial phosphate carrier protein. As shown in Fig. 3, the levels of NCU07465 mRNA were increased when *mak-1* was deleted, which indicated that MAK-1 contributes to the repression of NCU07465. In wild-type cells, accumulation of NCU07465 mRNA was rhythmic; however, the rhythm was abolished in the Δ*frq* and Δ*mak-1* strains (Fig. 6C). Similar to that of *ccg-1*, the peak phase of NCU07465 mRNA was in the subjective early morning (DD12 and DD36), occurring earlier than the peak of P-MAK-1 levels. Taken together, these data support the hypothesis that the clock signals through the MAK-1 MAPK pathway to regulate rhythmic gene expression via positive and negative regulation, with peak phases at different circadian times.

DISCUSSION

Endogenous circadian clocks provide a mechanism for organisms to anticipate environmental stress and prepare cellular response pathways needed for survival. Here we show that in *Neurospora*, the circadian clock regulates the activity of the MAK-1 and MAK-2 MAPK pathways. Under constant conditions and in the absence of stress, the levels of active P-MAK-1 and P-MAK-2 peak during the subjective afternoon. At this time of day, in the naturally changing environment, the ambient temperatures would generally be higher; thus, activation of P-MAK-1 at this time may provide a mechanism for the cell to anticipate stress on the cell wall induced by increased temperatures. Consistent with this idea, increased levels of P-MAK-1 were observed when wild-type cells were subjected to an increase of temperature. A role for P-MAK-2 in the regulation of vegetative growth, conidiation, and mating was previously established (27, 42), and these processes are under the control of the circadian clock in *Neurospora* (36, 56, 57). Thus, the clock may signal through the MAK-2 pathway to control rhythms in genes involved in these biological processes. However, the biological functions of the MAK-1 pathway were not known.

FIG 6 MAK-1 is necessary for robust rhythmic accumulation of downstream target ccgs. (A) Representative Northern blots of *ccg-1* mRNA from WT and Δ*mak-1* strains grown in a circadian time course (see the legend to Fig. 2). rRNA is shown as a loading control. A 24-h exposure was used to detect *ccg-1* mRNA in Δ*mak-1* cells. The data are plotted on the right (values are means ± SEM; $n = 3$). The WT data are represented by a solid black line, and the Δ*mak-1* data by a solid gray line. *ccg-1* data from WT cells were fit to a sine wave (dotted black line) ($P < 0.02$), whereas *ccg-1* data from the Δ*mak-1* strain were better fit to a line (dotted black line). (B) Representative Northern blots of NCU04352 mRNA from the indicated strains, labeled and plotted as described for panel A. The data for the Δ*frq* strain are represented with a dotted gray line. NCU04352 data from WT cells were fit to a sine wave (dotted black line) ($P < 0.0001$), whereas NCU04352 data from Δ*mak-1* and Δ*frq* cells were better fit to a line (dotted black line). (C) Representative Northern blots of NCU07465 mRNA from the indicated strains, labeled and plotted as described for panel A. NCU07465 data from WT cells were fit to a sine wave (dotted black line) ($P < 0.0001$), whereas for Δ*mak-1* and Δ*frq* cells, NCU07465 data were better fit to a line (dotted black line).

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In *S. cerevisiae*, not only is Slt2 activated in response to stress that affects the cell wall but it is also activated during cell cycle transitions, leading to budding and polarized pseudohyphal growth (58). The ERK1/2 pathways in mammals are activated in response to stress as well as extracellular signals leading to the regulation of cellular growth and polarity (59). To better understand the role of the MAK-1 pathway in *Neurospora*, we performed microarray analysis to identify genes that are misregulated in the Δ*mak-1* strain under normal growth conditions. About 500 genes were misregulated in Δ*mak-1* cells, with independent validation of the downstream targets suggesting an accuracy of 85% (12/14 targets tested showed significant results). We did not observe considerable overlap of genes regulated by MAK-1 in our arrays and those regulated by Slt2 in *S. cerevisiae* (47, 60, 61). These results may be explained by differences in morphogenesis of these organisms: *Neurospora* hyphae grow as syncytia (62), while budding is observed in *Saccharomyces* (63). However, the slow growth of Δ*mak-1* cells, as well as the decreased expression of a significant number of genes encoding ribosomal proteins, suggests that similar to ERK1/2 in mammals, MAK-1 may be needed for cells to proceed through the G_1 phase of the cell cycle, a process requiring an increase in protein synthesis and the biogenesis of structural components of the cell membrane or wall (31). Although the microarray results yielded fewer MAK-1 pathway-regulated genes with characterized roles in cell wall biogenesis than might be expected, some genes with predicted roles in cell wall biogenesis are classified by FunCat as metabolic or energy-related genes. Furthermore, some of the MAK-1 target genes that are uncharacterized may turn out to be important in cell wall biogenesis and maintenance. The MAK-1 downstream target gene and ccg NCU07465 encodes a mitochondrial phosphate carrier protein homologous to Pic2 in *S. cerevisiae*, which is induced in response to stress (64). This gene is not known to be regulated by the Slt2 pathway; however, this family of carrier proteins is necessary for transport of inorganic phosphate into the mitochondria in order to generate ATP, supporting a role for the MAK-1 pathway in rhythmic energy production (65) and cellular growth control.

Of the genes affected in the Δ*mak-1* strain, 28% were previously characterized in microarray experiments as putative ccgs, supporting the idea that by controlling rhythmic activation of MAPK pathways, the clock can coordinately control a subset of functionally related genes. However, similar to the case in the OS pathway (20), not all downstream targets of MAK-1 are ccgs. For example, no circadian rhythm in mRNA accumulation was observed for the two heat shock genes *hsp88* and *hsp70* (data not shown), despite these two genes showing different levels of mRNA in Δ*mak-1* and wild-type cells. In agreement with this idea, all 3 MAPK pathways in *Neurospora* are clock controlled, albeit with various phases. These pathways are likely responsible for controlling both unique and overlapping cellular processes and stress responses that occur at various times in the day, consistent with our finding that not all ccg targets of the MAK-1 pathway behave arrhythmically in Δ*mak-1* cells: circadian rhythms in mRNA accumulation of 2 downstream targets of MAK-1, *ccg-7* and NCU08949, were maintained in the Δ*mak-1* strain (data not shown). P-OS-2 peaks in the morning, at a time when the cell is beginning to encounter potential osmotic changes in the environment (22), while P-MAK-1 and P-MAK-2 peak later in the afternoon, at a time when the growth rate of the cells is increasing and conidiation is repressed (57). Interestingly, when MAK-1 was deleted, the levels of *ccg-1* mRNA were greatly reduced. In spite of this, low-amplitude *ccg-1* mRNA rhythms were observed in the deletion strain, albeit with less reproducibility than in wild-type cells. We previously reported that *ccg-1* rhythmicity depends upon a functional OS-2 pathway (22), and *ccg-1* is induced in response to a variety of stresses (66–69). Thus, it appears that both MAPK pathways contribute to robust rhythmicity in the expression of some target genes, including *ccg-1*. This may not be surprising, as previous studies have demonstrated cross talk between MAPK pathways (70, 71) and, more specifically in *S. cerevisiae*, have indicated that dual activation of the Hog1 and Slt2 pathways is required for cell survival during cell wall stress (72). In contrast to MAK-1 pathway regulation of *ccg-1*, the levels of target gene NCU07465 mRNA were increased in the Δ*mak-1* cells at all times of the day, and circadian rhythms in mRNA accumulation were abolished. Taken together, these data illustrate the complexity of clock output mechanisms, showing that MAK-1 is necessary to both promote and repress gene expression and, through these activities, control rhythmic gene expression. Additionally, both *ccg-1* and NCU07465 mRNA levels peak in the morning, prior to the peak of P-MAK-1, supporting the existence of dual MAPK module inputs to some of the MAK-1 downstream targets and/or a complex, hierarchical regulation of downstream effector molecules that transduce the rhythmic signal from MAK-1 to the downstream target genes. Alternatively, the correspondence of peak levels of NCU04352 mRNA and P-MAK-1 suggests a short regulatory pathway from P-MAK-1 to NCU04352.

In summary, we have shown the activity of the ERK1/2-like MAP kinases MAK-1 and MAK-2 to be under the control of the circadian clock in *Neurospora*. The conservation of the clock and these signaling cascades underscores their relevance in the health of eukaryotic organisms. Both the clock and MAP kinase pathways have been linked to diseases, including cancer; defects in either can lead to uncontrolled growth and tumor formation (73, 74). The circadian activation of MAK-1 suggests that clock regulation of rhythmic growth and development in *Neurospora* may act in part through the MAK-1 MAPK pathway. Therefore, in both *Neurospora* and mammalian cells, these ERK-like kinases may provide a mechanism to temporally limit cellular growth, such that progression through the cell cycle occurs at times of the day when cells are the least susceptible to genotoxic stress.

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