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The *Neurospora crassa* OS MAPK Pathway-Activated Transcription Factor ASL-1 Contributes to Circadian Rhythms In Pathway Responsive Clock-Controlled Genes

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Abstract

The OS-pathway mitogen-activated protein kinase (MAPK) cascade of *Neurospora crassa* is responsible for adaptation to osmotic stress. Activation of the MAPK, OS-2, leads to the transcriptional induction of many genes involved in the osmotic stress response. We previously demonstrated that there is a circadian rhythm in the phosphorylation of OS-2 under constant non-stress inducing conditions. Additionally, several osmotic stress-induced genes are known to be regulated by the circadian clock. Therefore, we investigated if rhythms in activation of OS-2 lead to circadian rhythms in other known stress responsive targets. Here we identify three more osmotic stress induced genes as rhythmic: *cat-1*, *gcy-1*, and *gcy-3*. These genes encode a catalase and two predicted glycerol dehydrogenases thought to be involved in the production of glycerol. Rhythms in these genes depend upon the oscillator component FRQ. To investigate how the circadian signal is propagated to these stress induced genes, we examined the role of the OS-responsive transcription factor, ASL-1, in mediating circadian gene expression. We find that while the *asl-1* transcript is induced by several stresses including an osmotic shock, *asl-1* mRNA accumulation is not rhythmic. However, we show that ASL-1 is required for generating normal circadian rhythms of some OS-pathway responsive transcripts (*bli-3*, *cgc-1*, *cat-1*, *gcy-1* and *gcy-3*) in the absence of an osmotic stress. These data are consistent with the possibility that post-transcriptional regulation of ASL-1 by the rhythmically activated OS-2 MAPK could play a role in generating rhythms in downstream targets.

Keywords

Neurospora crassa; Circadian output; MAPK pathway; ATF/CREB transcription factor; Osmotic stress; Oxidative stress

1. Introduction

The evolution of organisms in the cyclical environment of the earth has led to the development of internal biological clocks. Biological clocks provide a mechanism for

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organisms to anticipate cyclical daily stresses such as light, heat, and desiccation, and to mount appropriate physiological changes that will help the organism better adapt to their environment (Dodd et al., 2005; Ouyang et al., 1998; Woelfle et al., 2004; Yerushalmi and Green, 2009; Yerushalmi et al., 2011). We are beginning to understand that the circadian oscillator mechanism does this, at least in part, through the rhythmic activation of stress response pathways, even in the absence of obvious stress (de Paula et al., 2008). We previously showed that the *Neurospora crassa* clock transcription factor, the White Collar Complex (WCC), binds rhythmically to the promoter of the MAPK kinase kinase (Smith et al., 2010) of the OS-pathway (*os-4*), and drives daily rhythms in *os-4* mRNA and protein (Lamb et al., 2011). This then leads to a rhythm in the phosphorylation of the terminal MAPK, OS-2 (Vitalini et al., 2007). Daily rhythms in OS-2 phosphorylation occur in the absence of stress, and in constant conditions (constant temperature, constant darkness) under control of the endogenous circadian clock mechanism.

OS-2 is a homolog of both the Hog1 MAPK from yeast and the p38 MAPK from mammals (Zhang et al., 2002). This MAPK is critical for mounting responses that can help organisms adapt to increased extra-cellular osmotic pressure, oxidative stress, heat and many other stresses (Hohmann, 2002; Sheikh-Hamad and Gustin, 2004). In fungi, the OS-pathway provides the target by which many anti-fungal drugs work to promote toxicity (Ochiai et al., 2002; Vetcher et al., 2007; Zhang et al., 2002). In mammals, the p38 MAPK is a target of the pyridinyl imidazole class of compounds that have anti-inflammatory activity (Schindler et al., 2007). Furthermore, there are links between the p38 MAPKs and cell death, cell cycle progression, and cancer (Cuenda and Rousseau, 2007; Fu and Lee, 2003; Yaakov et al., 2009). Thus, understanding how the p38 MAPK pathway is connected to the circadian clock and downstream rhythmic gene expression may be important for many aspects of human health.

Several targets of the OS-2 MAPK have been discovered in *Neurospora*, and these are similar to the targets in yeast and mammals (Irmeler et al., 2006; Noguchi et al., 2007; Watanabe et al., 2007; Yamashita et al., 2007). In *S. cerevisiae*, Hog1 phosphorylates ion channels, kinases, phosphatases, and transcription factors that bring about the adaptive response and modulate pathway activity (Hohmann, 2002; Proft et al., 2006; Proft and Struhl, 2002, 2004; Rep et al., 2000). In mammals, a similar complement of p38 MAPK targets is also observed. One common target found in yeast and mammals is the bZIP transcription factor Sko1 or ATF/CREB. In yeast, Sko1 is phosphorylated by Hog1 (Proft et al., 2001; Proft and Struhl, 2002; Rep et al., 2001) and in mammals ATF/CREB is phosphorylated by p38 MAPK in response to various stresses (Hazzalin et al., 1996; Sen et al., 2005; Zayzafoon et al., 2002). Phosphorylation of Sko1 by the MAPK (Hog1) is known to convert it from a repressor to an activator, rapidly increasing the expression of many stress responsive genes (Proft and Struhl, 2002; Rep et al., 2001). *Neurospora* possesses a homolog of Sko1 designated ASL-1 for an ascospore lethal phenotype (Colot et al., 2006), and was subsequently referred to as ATF-1 for homology to the mammalian transcription factor (Yamashita et al., 2008). ASL-1 is required for the induction of some genes in response to the fungicide fludioxonil, and in response to osmotic stress (Yamashita et al., 2008).

Following the demonstration that the OS-2 MAPK is rhythmically phosphorylated during the course of a day in the absence of stress, it was of interest to determine if OS-2 phosphorylation rhythms would lead to rhythms in genes known to be stress-induced targets of the OS pathway. Indeed, some of the targets of the OS pathway, including *bli-3* (blue light induced-3, unknown function), *cgc-1* (clock controlled gene-1, unknown function), *cgc-9* (trehalose synthase), *cgc-13* (cell wall protein), and *cgc-14* (cell wall protein) are both induced by an activated OS-2 pathway and clock-controlled (Bell-Pedersen et al., 1996;

Loros et al., 1989; Shinohara et al., 2002B; Watanabe et al., 2007; Zhu et al., 2001). However, there are several other genes induced by the OS-pathway that have not been specifically studied for rhythmicity. Furthermore, the role of the ASL-1 transcription factor in generating rhythmicity of OS-pathway output genes has not been examined. In this study we investigate the connection between the OS-pathway and circadian gene expression, with a focus on the role of the ASL-1 transcription factor.

2. Materials and Methods

2.1. Strains and culture conditions

N. crassa strains utilized in this study were grown and maintained as previously described (Davis and de Serres, 1970). All strains are “clock strains” that contain the *ras-1^{bd}* allele, a mutation that reduces the growth rate, and clarifies the rhythmic asexual conidial banding pattern on racetubes (Belden et al., 2007; Sargent et al., 1966), but that does not alter the rhythm in phosphorylated OS-2 (Vitalini et al., 2007). Time course experiments were carried out as described (Lamb et al., 2011). The genotype of the circadian clock deficient Δ FRQ strain (*frq1⁰*) we analyzed was *ras-1^{bd}*, *Afrq::hph* (Aronson et al., 1994), which was obtained from the Fungal Genetic Stock Center (FGSC 7490). Stress treatments were performed on mycelial discs grown in 1x Vogels, 2% glucose, 0.5% arginine pH 6.0 liquid media in constant light at 25°C after 24 h of growth, except the 30 min light pulse was performed after growth in constant darkness for 24 h. The stress treatments were all for 30 min except the hydrogen peroxide treatment, which was for 15 min. The stress treatments consisted of 100% water, 4% NaCl, 1M sorbitol, 1µg/ml fludioxonil “Pestanal” (Sigma 46102), and 10 mM hydrogen peroxide. Tissue was harvested immediately following the stress treatments.

2.2. Deletion of the ASL-1 transcription factor

Deletion of the transcription factor ASL-1 causes an ascospore lethal phenotype (Colot et al., 2006), and thus a *ras-1^{bd}* *Asl-1* strain could not be obtained via crossing of the available *Asl-1* strain (FGSC 21336). Generation of the *Asl-1* strain in the *ras-1^{bd}* background required new strain construction. Primers used in the construction of the new knockout are identical to those used in the gene knockout project (see <http://www.dartmouth.edu/~neurosporagenome/primers.html>), however, rather than using yeast to recombine the 5' *asl-1* flank, the *hph* gene, and 3' *asl-1* flank, a hybrid PCR was performed to fuse them together. This hybrid PCR product was then used to transform a *ras-1^{bd}* strain (DBP1258). Heterokaryons were selected for on plates containing 200µg/ml hygromycin. Homokaryons of the *Asl-1::hph* genotype were identified by PCR screening of single colonies derived from the heterokaryons. Three homokaryons derived from three independent transformants called DBP889, DBP1314, and DBP1315 were obtained, and we have observed no differences between these strains. Consistent with previous data (Colot et al., 2006; Yamashita et al., 2008), we find that the *ras-1^{bd}* *Asl-1::hph* strain is ascospore lethal, has reduced conidial germination rates, and it is not sensitive to osmotic stress similar to the *Asl-1::hph* strain (data not shown).

2.3. Gene expression analysis

RNA was extracted and purified from ground tissue and 10 to 15 µg were run on denaturing formaldehyde gels (Bell-Pedersen et al., 1996). RNA from control and test strains were always run side by side on the same gel so that changes in gene expression relative to the

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control could be determined. Gels were blotted to NitroPure membranes (GE, WP4HY00010) and hybridized to gene specific probes: *cat-1*, *dak-1*, *fbp-1*, *gcy-1*, *gcy-3*, and *pck-1* were detected with [α - 32 P]-dATP-labeled DNA probes, while *asl-1*, *bli-3*, *ccg-1*, and *ccg-9* were detected with [α - 32 P]-UTP labeled anti-sense RNA probes. As is standard in the field, gene expression was normalized to the ribosomal RNA visualized on the membrane with ethidium bromide. For time course experiments, the average normalized expression level of a strain was set to one and then to permit sine wave curve fitting, one was subtracted from the expression level at each time point.

2.4 Statistical Analysis

Nonlinear regression to fit the rhythmic data to a sine wave (fitting period, phase, and amplitude) and a line (fitting slope and intercept), as well as Akaike's information criteria tests (Burnham and Anderson, 2002) 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, for instance, the sine wave fits the data better than a straight line.

3. Results

3.1. The OS-pathway stress induced genes, *cat-1*, *gcy-1* and *gcy-3* are circadianly regulated

Several *Neurospora* genes are induced after a salt shock or fungicide treatment, with induction being dependent on activation of the OS pathway. These include the catalase gene *cat-1*, the glycerol synthesis genes *gcy-1*, *gcy-3*, and *dak-1*, and the gluconeogenesis genes *fbp-1* and *pck-1*, amongst others (Noguchi et al., 2007; Watanabe et al., 2007; Yamashita et al., 2007). The OS-pathway is activated by the circadian clock in the absence of stress; therefore, we postulated that genes that are stress regulated by the OS pathway would also be circadianly regulated in the absence of stress. Thus, we examined if these mRNAs accumulated with a circadian rhythm in constant darkness and temperature, conditions in which the only time of day information comes from the organism's internal circadian clock. We observed rhythms in mRNA accumulation for *cat-1*, *gcy-1* and *gcy-3* with periods of 22.7 $\pm$ 1.1, 19.8 $\pm$ 0.8h, and 17.2 $\pm$ 0.5h, respectively (Figure 1). With measurements taken every 4 hours for roughly two days, these period values are imprecise ($\pm$ 8 h); however, all are within range of the endogenous 22-h period of the organism, while the *gcy-1* and *gcy-3* periods are somewhat shorter. No clear rhythms were observed for *dak-1*, *fbp-1* or *pck-1* mRNA accumulation (Supplementary Figure 1). Subjective morning peaks in expression were observed for *cat-1* and *gcy-3*, while *gcy-1* showed peak expression in the subjective night. Consistent with the idea that these genes depend on the *Neurospora* circadian oscillator, rhythms in abundance of *cat-1*, *gcy-1* and *gcy-3* were abolished in cells that lacked the core oscillator component, FREQUENCY (FRQ).

3.2. *asl-1* expression is regulated by stress, but not by the circadian clock

ASL-1 is involved in mediating part of the stress response downstream of the OS-pathway. Therefore, we examined if *asl-1* expression is regulated by stress. We found that *asl-1* mRNA levels increased after 4% NaCl, 1M sorbitol, 1 μ g/ml fludioxonil, 10 mM hydrogen peroxide treatments and after a light pulse, and decreased after water treatment (Figure 2A and B).

To define the role ASL-1 plays in mediating circadian output from the OS-pathway, we first tested if *asl-1* mRNA accumulates with a circadian rhythm. A time course experiment was conducted as described above, and *asl-1* mRNA was analyzed by northern blot (Figure 2B).

While there is some random variation in the levels of *asl-1* mRNA throughout the time course, no reproducible circadian rhythm was detected.

Next, we examined if ASL-1 is involved in generating the rhythmic pattern of asexual spore development (conidiation) observed in constant darkness on racetubes. To accomplish this goal, we first needed to generate a *ras-1^{bd} Δ;asl-1* strain. The *ras-1^{bd}* allele clarifies the developmental rhythm on racetubes, and therefore, serves as the standard laboratory clock strain (Belden et al., 2007; Sargent et al., 1966). To generate the *ras-1^{bd} Δasl-1* strain, the *asl-1* open reading frame was replaced with the hygromycin resistance (*hph*) gene (see Materials and Methods). Heterokaryotic *Δasl-1::hph* transformants were identified as hygromycin resistant colonies whose genomic DNA yielded the correct sized DNA fragments in PCRs with “insertion test” primer sets (Figure 3A and B). Homokaryons of the *Δasl-1::hph* genotype were isolated, and the absence of the WT *asl-1* gene in this strain was verified by PCR (Figure 3C). Furthermore, northern blots were used to confirm the absence of *asl-1* mRNA in the *ras-1^{bd} Δasl-1* homokaryon strain (Figure 3D). In racetube assays, we observed that the period of the conidiation rhythm in the *ras-1^{bd} Δasl-1* strain (22.3±0.3 h) was similar to the period in the control *ras-1^{bd}* strain (22.1 ± 0.2 h) (data not shown), indicating that the core FRQ/WCC oscillator functions normally in cells that lack ASL-1.

Together, these data suggest that circadian rhythms in ASL-1-target genes are not the result of clock-controlled *asl-1* promoter activation or mRNA stability, but are likely due to rhythms in the activation of ASL-1 through the rhythmically activated OS-2 MAPK. Furthermore, any effect that *Δasl-1* has on rhythmic gene expression will be due to its role in circadian output, rather than effects on the circadian oscillator.

3.3. The role of *asl-1* in stress induction of OS-pathway output genes

The effect of deleting *asl-1* on the stress induction of several OS-pathway output genes has been examined previously in wild type germinating conidia (Yamashita et al., 2008). However, because we are analyzing the effects of *Δasl-1* in the *ras-1^{bd}* background, as well as in mycelial mats grown in submerged culture, we needed to determine the role that ASL-1 plays in stress responses in this context (Figure 4). This was particularly important given the recent findings of Lara-Rojas et al (Lara-Rojas et al., 2011b), which showed cell-type-specific roles for the *Aspergillus* homolog of *asl-1*.

Various osmotic stressors (4% NaCl, 1M sorbitol, 1μg/ml fludioxonil) induced the expression of *bli-3*, *cat-1*, *ccg-1*, *ccg-9*, *gcy-1* and *gcy-3* in *ras-1^{bd}* mycelia. However, induction of these genes in the *ras-1^{bd} Δasl-1* strain was seriously impaired (Figure 4A and Figure S1). *cat-1* expression was nearly undetectable in all conditions in the *ras-1^{bd} Δasl-1* strain. *bli-3*, *ccg-1*, and *gcy-3* were not induced by salt (N) or fludioxonil (F) treatments, but were still weakly induced by sorbitol (S) treatment in the *ras-1^{bd} Δasl-1* strain. Induction of *gcy-1* by these treatments was impaired, but not abolished. *ccg-9* was still induced by salt and fludioxonil treatments, but not by sorbitol. Furthermore, *ccg-1*, *ccg-9*, and *gcy-3* expression levels were elevated in the *ras-1^{bd} Δasl-1* strain as compared to the *ras-1^{bd}* strain in the untreated samples (U). Shifting tissue from normal growth medium to 100% water (W) constitutes a hypoosmotic stress that may affect the expression of OS-pathway responsive genes. Expression of *bli-3*, *gcy-1* and *gcy-3* was strongly reduced after hypoosmotic stress treatment (Figure 4A and Figure S1). There was only partial reduction of *bli-3*, *gcy-1* and *gcy-3* expression in the *ras-1^{bd} Δasl-1* strain in response to a hypoosmotic stress, indicating an impaired response in the absence of ASL-1. We also examined the response of these genes to oxidative stress. Following a 15 min treatment with 10 mM H₂O₂ (X), *bli-3*, *cat-1*, *ccg-1*, *gcy-1* and *gcy-3* were induced, and this induction required *asl-1*. Finally, we examined the expression of *bli-3* and *cat-1* in constant darkness (D) and after a light pulse (LP) (Figure 4B). We found that both genes were induced by a light pulse

in the *ras-1^{bd}* strain, but only *bli-3* was induced in the *ras-1^{bd} Δ asl-1* strain. Thus, ASL-1 plays a crucial role in activating *cat-1* expression in all conditions, while for other genes, it is involved in activation after hyperosmotic (and drug induced) stress, repression after hypoosmotic stress, and repression in non-stressed conditions.

3.4. ASL-1 is required for proper circadian rhythms of *ccg-1*, *bli-3*, *gcy-1* and *gcy-3* and for *cat-1* expression

To determine the role ASL-1 plays in generating circadian expression of genes known to be regulated by the OS-pathway following stress, a time course experiment was performed with *ras-1^{bd}* and *ras-1^{bd} Δ asl-1* strains under constant conditions, and expression of OS-pathway output genes was examined (Figure 5). Similar to the stress response experiments, ASL-1 was found to be required for *cat-1* expression at all times of the day. Rhythms in *ccg-1* were still detected in the ASL-1 deletion; however, the amplitude of *ccg-1* expression rhythms was reduced 2-fold as compared to the control *ras-1^{bd}* strain. A similar expression profile was observed for *bli-3*, with a nearly 2-fold reduction in amplitude in the ASL-1 deletion strain. Alternatively, the rhythm in *gcy-1* expression depended on ASL-1. The rhythm in *gcy-3* was severely impaired in the *ras-1^{bd} Δ asl-1* strain, and the amplitude was reduced almost 2-fold. Lastly, ASL-1 was found to not be required for proper circadian regulation of *ccg-9*.

4.1 Discussion

In this paper we have explored the connection between the circadian clock and the osmotic stress pathway in *Neurospora*. We have identified three genes that were previously known to be induced by osmotic stress downstream of the OS-MAPK pathway, as also being under control of the circadian clock. We have defined a role of the ATF/CREB related transcription factor, ASL-1 in hyperosmotic, hypoosmotic, and oxidative stress responses. Finally, we have examined the role ASL-1 plays in driving circadian gene expression. Our results suggest that ASL-1 is receiving time of day information from the OS-pathway to contribute to circadian gene expression (Figure 6).

4.1 Identification of three new OS-pathway outputs as clock-controlled genes

The OS-pathway of *Neurospora* has been shown to receive time of day input from the circadian clock via the transcriptional control of the OS-4 MAPKKK (Lamb et al., 2011). This input then leads to the rhythmic phosphorylation of the OS-2 MAPK (Vitalini et al., 2007). One prediction from these findings is that genes that are regulated by the OS-pathway in response to osmotic stress would also be circadianly regulated. Several of the OS-pathway responsive genes were already known to be rhythmic: *ccg-1*, *ccg-9*, *ccg-13*, *ccg-14*, and *bli-3* (Bell-Pedersen et al., 1996; Loros et al., 1989; Shinohara et al., 2002a; Watanabe et al., 2007; Zhu et al., 2001). However, there are many other known OS-pathway outputs that were not known to be rhythmic. Here we tested several of these genes and found three more to be circadianly regulated in constant conditions (Figure 1). Furthermore, the rhythmicity of these genes was shown to depend on the canonical clock component, FRQ. Not all genes that are downstream of the OS-pathway have rhythms in their steady state transcript levels. No rhythms were detected in *dak-1*, *fbp-1* or *pck-1*. It is possible that the rhythms in OS-2 phosphorylation do not lead to rhythms in these genes because the amplitude of phosphorylated OS-2 does not reach a critical threshold for activating these genes in a time-of-day-dependent manner. Alternatively, the *semRNAs* may have stability characteristics that mask circadian regulation of transcription initiation or perhaps in the unstressed circadian conditions of our experiments, regulation of these genes by the OS-pathway is only a minor contributor compared to other factors.

Thus, while not an absolute predictor, other genes that are downstream of rhythmic stress signaling pathways (rhythmic phospho-OS-2), are likely to also be rhythmic. Rhythmicity of stress response genes is predicted to be important for the ability of the organism to rapidly respond to daily environmental stresses. Most of the genes examined in this study have peak expression in the late-night to early-morning, a time that anticipates the daytime associated stresses of light, UV light, heat, and desiccation. Elevated catalase (*cat-1*) is expected to detoxify the reactive oxygen species associated with UV light exposure and metabolism (Peraza and Hansberg, 2002). The glycerol dehydrogenases (*gcy-1* and *-3*) are expected to perform both forward and reverse reactions, so upregulation in the morning (*gcy-3*) could promote the production of the osmoprotectant, glycerol, from dihydroxyacetone (DHA) or glyceraldehyde (GA), while upregulation in the night (*gcy-1*) could be mediating recycling of glycerol back to DHA or GA when it is no longer needed (Viswanath-Reddy et al., 1978). The trehalose synthase gene (*ccg-9*) expression peaks in the mid-day and is expected to increase the production of trehalose, a storage form of glucose that is also an osmoprotectant (Iturriaga et al., 2009; Shinohara et al., 2002b; Soto et al., 1999). Moreover, rhythmicity of the OS pathway is functionally relevant as it promotes a more rapid induction of glycerol after salt stress treatment in the subjective morning compared to treatment in the subjective evening (Lamb et al., 2011). Over evolutionary time, or in competition with other organisms, it is tempting to speculate that the clock regulation of this pathway gives organisms an advantage.

4.2. The role of ASL-1 in stress responses

Although previous studies have examined the role of ASL-1 in output from the OS pathway, those studies were done on wild type strains in germinating conidia. As our studies focused on mycelia in the standard laboratory clock *ras-1^{bd}* strain, we reexamined the role ASL-1 plays in such conditions. Our results are largely in agreement with the published work (Yamashita et al., 2008), showing reduced induction of the OS output genes *bli-3*, *cat-1*, *ccg-1*, *gcy-1* and *gcy-3* in the ASL-1 deleted strain after NaCl, sorbitol, and fludioxonil treatments. One difference in our study is that we found ASL-1 to be unimportant for the NaCl induction of *ccg-9* (discussed below, see section 4.3). Furthermore, we extend the understanding of stress responses by examining expression of OS output genes in hypoosmotic (100% H₂O), oxidative stress (10 mM H₂O₂) and light pulse treatments. In the control strain, some of the output genes are repressed under hypoosmotic conditions, however in the *Δasl-1* strain, repression is impaired for *bli-3*, *gcy-1* and *gcy-3*. This suggests a function for ASL-1 in the hypoosmotic response, which could be related to the role the yeast homolog Sko1 plays in repressing osmotic shock genes in unstressed conditions (Proft et al., 2001; Rep et al., 2001). We also demonstrate that *bli-3*, *cat-1*, *ccg-1*, *gcy-1* and *gcy-3* are induced by oxidative stress and dependent on ASL-1 for induction. A role for ASL-1 in oxidative stress is thus conserved in fungi and mammals (Aggeli et al., 2006; Alonso-Monge et al., 2010; Kurata, 2000; Lara-Rojas et al., 2011a; Rep et al., 2001; Sakamoto et al., 2009). Finally we demonstrate that the light-induced expression of *bli-3* is not dependent on ASL-1, while *cat-1* expression remains completely dependent on ASL-1 even after a light pulse. Taken together these data suggest that ASL-1 plays several roles in stress responses. It is absolutely required for *cat-1* expression, it is important for repression of some genes in unstressed and in hypoosmotic conditions, it is required for hyperosmotic induction of several genes, and it is involved in the oxidative stress response of several genes.

4.3 The role of ASL-1 in circadian gene expression

Although *asl-1* steady state mRNA levels do not show a circadian rhythm (Figure 2), and *Δasl-1* strains have normal oscillator function, we propose that the ASL-1 transcription factor plays a role in circadian clock output by driving rhythmic expression of stress

response genes. Consistent with the demonstration that yeast HOG1 directly phosphorylates the ASL-1 homolog SKO-1 (Proft et al, 2001; Proft and Struhl, 2002), we hypothesize that rhythms in the levels of phosphorylated OS-2 MAPK (Vitalini et al., 2007) lead to rhythms in phosphorylation and activation of ASL-1, which drives rhythmic expression of some, but not all, OS-pathway output genes. While direct or circadian association of *Neurospora* ASL-1 with the OS-2 MAPK has yet to be demonstrated, our hypothesis is supported by the role ASL-1 plays in circadian gene expression and the known activities of ASL-1 homologs. ASL-1 is required for rhythmic *gcy-1* expression and is involved in the rhythms in *cgc-1*, *bli-3*, and *gcy-3* expression. Additionally, ASL-1 is necessary for *cat-1* expression under all conditions tested. Previous studies have shown the direct binding of purified ASL-1 protein to the promoters of the *cgc-1* and *cat-1* genes *in vitro* (Yamashita et al., 2008), suggesting direct regulation of these two genes. Because both *cgc-1* and *cat-1* genes peak in expression in the morning and are likely to be bound directly by ASL-1, the mechanism by which ASL-1 generates rhythms in the evening specific gene, *gcy-1*, is predicted to be indirect.

Now a nearly complete molecular pathway for circadian gene expression of *cgc-1* and *cat-1* can be constructed (Figure 6). The pathway begins with the circadian transcription factor (WCC) rhythmically binding to and driving expression of the MAPKK kinase (OS-4) (Lamb et al., 2011). Rhythms in the levels of the OS-4 protein lead to rhythms in the phosphorylation state of the MAPK (OS-2) in constant non-stress conditions (Lamb et al., 2011; Vitalini et al., 2007). Rhythms in OS-2 phosphorylation are expected to lead to rhythms in activity of the ASL-1 transcription factor, which directly binds the *cat-1* and *cgc-1* promoters (Yamashita et al., 2008) to drive expression and rhythms. During the daytime when the WCC activates *os-4* expression or after a hyperosmotic stress treatment, phospho-OS-2 MAPK levels increase. This activated MAPK promotes the activity of the ASL-1 transcription factor to drive *bli-3*, *cgc-1*, *cat-1* and *gcy-3* expression during the day or under osmotic stress conditions. It is not currently known whether *bli-3* or *gcy-3* are direct ASL-1 targets for activation. Osmotic stress also drives the expression of *cgc-9*, but ASL-1 is not required for that induction nor is it required for *cgc-9* rhythms. *gcy-1* is also induced by an osmotic stress, and that induction requires ASL-1, however expression in the evening of a circadian cycle is likely to be due to indirect circadian control by ASL-1. During the evening when the levels of active WCC are very low, or in unstressed conditions, there are very low levels of phosphorylated OS-2. This allows ASL-1 to function as a repressor on the *cgc-1* promoter directly and on the *cgc-9* promoter either directly or indirectly. While this model is consistent with current information, many aspects require clarification. Future studies will test if ASL-1 rhythmically associates with and is phosphorylated by OS-2. ChIP-seq analysis will define the direct targets of ASL-1 after osmotic and/or oxidative shock, which we predict will also identify additional ASL-1 circadian targets.

The only gene we tested that ASL-1 did not adversely affect following salt treatment or for circadian rhythmicity was *cgc-9*. ASL-1 did, however, play a clear role in repressing this gene. This suggests that multiple factors are controlling *cgc-9*. Rhythmicity of *cgc-9* is not likely to be the result of direct regulation by the WCC, as *cgc-9* was not identified as a direct target of the WCC in ChIP-seq studies (Smith et al, 2010). What drives the rhythm of *cgc-9* and the low amplitude rhythms observed in *cgc-1*, *bli-3*, and *gcy-3* in the absence of ASL-1? There are two additional transcription factors that are predicted to mediate output from the OS-pathway based on homology to yeasts, NCU02671 (*MSN2* homolog) and NCU02558 (*SMP1/RLM1* homolog) (Hohmann, 2002). These two transcription factors may also be involved in clock output from the OS pathway, and future studies will address this question.

4.4 Conservation of MAPK pathway rhythmicity and transcription factor targets

There is evidence that the OS-pathway is not the only rhythmic MAPK pathway in *Neurospora* (Bennett and Bell-Pedersen, in preparation). Furthermore, MAPK pathways in

other organisms may also be controlled by the clock (Coogan and Piggins, 2003; Dziema et al., 2003; Hasegawa and Cahill, 2004; Kako et al., 1996; Koyanagi et al., 2011; Obrietan et al., 1998). While a role for MAPK and ATF/CREB in mediating light input to the central oscillator has been extensively studied in mammals and birds (Butcher et al., 2002; Coogan and Piggins, 2004; Dziema et al., 2003; Ko et al., 2009; Koyanagi et al., 2011; Obrietan et al., 1998; Pizzio et al., 2003; Shimizu and Fukada, 2007) our findings with the fungal homolog, ASL-1, indicate a new role for this transcription factor in mediating circadian output. It is possible that a role for p38 MAPK and ATF/CREB in mammalian circadian output has been masked by its established role in mediating light input to the central pacemaker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Circadian clock control of the OS MAPK pathway leads to rhythms in expression of target genes.
- The ASL-1 transcription factor is necessary for normal stress responses of the target genes.
- ASL-1 is required for normal rhythmic expression of the target genes.
- Rhythmic posttranslational modification of ASL-1 is likely responsible for target gene rhythms.

Figure 1. *cat-1*, *gcy-1* and *gcy-3* are clock-dependent rhythmic targets of the OS-pathway
 The left panel shows representative northern blots of RNA harvested from a circadian time course. The time (h) after the shift to constant darkness (DD) is indicated at the top. The bars represent the phases that the circadian clock is experiencing with “subjective night” in black and “subjective day” in white. The *ras-1^{bd}*(WT) and clock mutant, *ras-1^{bd} & Delta;frq* (*Δfrq*), strains were examined for *cat-1*, *gcy-1* and *gcy-3* mRNA levels. The associated ethidium bromide stained rRNA loading control for each blot is shown underneath. The right panel indicates the normalized (gene signal/rRNA signal) average expression for the WT strain (black line) (N=4, ± SEM) and the *Δfrq* strain (gray line) (N=3, ± SEM). The best fit to a sine wave for the wild type strain is indicated by a red line for the morning phase genes (*cat-1* and *gcy-3*) and by a blue line for the late night phase gene (*gcy-1*) ($p < 0.0001$). The best fit for all of the genes examined in the *Δfrq* strain was a line(not shown).

Figure 2. Expression of *asl-1* is induced by multiple stress treatments, but is not clock-regulated at the level of transcript abundance

(A) Northern blots showing expression of *asl-1* in a *ras-1^{bd}* strain (WT) after treatment with the indicated stresses (N=2 with similar results in both experiments) and (B) in a circadian time course, with the hours in DD and the circadian phase indicated as in Figure 1. Symbols: U= untreated, N= 4% NaCl, W= 100% water, S= 1 M sorbitol, F= 1 μ g/ml fludioxonil, X= 10 mM H₂O₂, D= constant darkness, and LP = light pulse. In B, the ASL-1 deletion strain was used as a control (Δ) (N=3 with similar results obtained in all experiments). rRNA is shown as a loading control below each blot in both A and B.

Figure 3. Generation of a *ras-1^{bd}* Δ *asl-1* strain

(A) Schematic representation of the desired *asl-1* deletion locus is shown with the white bars representing the approximately 1 kb of homology flanking the *asl-1* gene used to recombine the knockout construct in which the *asl-1* ORF is replaced by the *hph* ORF conferring resistance to hygromycin. The black line represents the genomic locus outside the homology region. (B) PCR using the indicated primers in A to examine proper insertion of the knockout cassette. The expected 2376 bp fragment is observed using the 5'F and YG primers, and the expected 3378 bp fragment is observed using the HY and 3'R primers in a PCR using genomic DNA from strains containing the desired deletion of ASL-1 (Δ). No products are expected or observed from *ras-1^{bd}* (+) genomic DNA. Molecular weight standards are shown on the left (kb). (C) PCR using primer sets to detect the presence of the *asl-1* and the *os-4* gene open reading frames using genomic DNA from *ras-1^{bd}* (+) and a homokaryotic *ras-1^{bd}* Δ *asl-1* strain (Δ). The control *os-4* gene is present in both strains, but the *asl-1* gene is found only in the *ras-1^{bd}* strain, demonstrating that a homokaryon with all copies of *asl-1* deleted has been obtained. Molecular weight standards are shown on the left (kb). (D) Northern assay of *ras-1^{bd}* (+) and homokaryotic *ras-1^{bd}* Δ *asl-1* (Δ) RNA probed with a radio labeled *asl-1* riboprobe. rRNA is shown as a loading control.

Figure 4. ASL-1 is required for proper stress-induced gene expression of OS-pathway targets
 (A) The expression of *bli-3*, *cat-1*, *ccg-1*, *ccg-9*, *gcy-1* and *gcy-3* was examined by northern blot after various stress treatments in *ras-1^{bd}* (WT) and *ras-1^{bd} Δasl-1* ($\Delta asl-1$) strains. Quantitation of the expression levels from two biological replicates is shown in Supplemental Figure 1. Symbols: U= untreated, N= 4% NaCl, W= 100% water, S= 1M sorbitol, F= 1 μ g/ml fludioxonil, X= 10mM H₂O₂. (B) The expression of *bli-3* and *cat-1* in constant darkness (D) and after a light pulse (LP) was examined by northern blot in *ras-1^{bd}* (WT) and *ras-1^{bd} Δasl-1* ($\Delta asl-1$) strains. rRNA is shown in A and B as a loading control.

Figure 5. ASL-1 is involved in generating proper circadian rhythms of several downstream OS-pathway genes

Representative northern blots (left panels) showing accumulation of *bli-3*, *cat-1*, *ccg-1*, *ccg-9*, *gcy-1* and *gcy-3* mRNA in circadian time courses in the *ras-1^{bd} Δasl-1* strain, with the hours after dark shift (DD) and the circadian phase indicated as in Figure 1. rRNA is shown as a loading control. The right panel shows the average expression for each gene ($N \geq 3$, $\pm$ SEM). Results from the *ras-1^{bd}* strain (WT) are plotted in grey (the northern blots are not shown), *ras-1^{bd} Δasl-1* (*Δasl-1*) in black, and the best fit (line or sine) for the *ras-1^{bd} Δasl-1* strain is plotted in red. The predicted sine wave for each of the genes in *ras-1^{bd}* has a $p \leq 0.004$. In the *ras-1^{bd} Δasl-1* strain, the p value for the best fit to a sine wave for *ccg-1*, *bli-3* and *ccg-9* is $p \leq 0.001$, and for *gcy-3* is $p \leq 0.03$.

Figure 6. Model for the circadian and stress regulation of OS-pathway outputs

When the circadian transcription factor White Collar Complex (WCC) is in the subjective morning phase of activity, or when cells are exposed to elevated osmotic conditions, the OS-MAPK pathway is activated, leading to high levels of phospho-OS-2. Phospho-OS-2 activates several transcription factors, including ASL-1, which in turn stimulate the expression of numerous output genes either directly or indirectly. ASL-1 is required for both stress induction and normal circadian rhythms of *ccg-1*, *bli-3*, *cat-1*, *gcy-1* and *gcy-3*, although the levels of dependence on ASL-1 vary from gene to gene. Circadian control of the morning specific genes by ASL-1 may be direct, but control of the evening specific gene, *gcy-1*, is likely to be indirect (dashed line). The circadian expression of *ccg-9* is independent of ASL-1. When the WCC is in the subjective evening phase of inactivity or when cells are un-stressed, the OS-pathway MAPK cascade is inactive, leading to low phospho-OS-2 levels. Here, ASL-1 functions as a repressor, keeping the expression of *ccg-1* and *ccg-9* low. While repression of *ccg-1* is probably direct, it is unknown whether *ccg-9* is a direct target.