Transcription Factors in Light and Circadian Clock Signaling Networks Revealed by Genomewide Mapping of Direct Targets for Neurospora White Collar Complex

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Light signaling pathways and circadian clocks are inextricably linked and have profound effects on behavior in most organisms. Here, we used chromatin immunoprecipitation (ChIP) sequencing to uncover direct targets of the Neurospora crassa circadian regulator White Collar Complex (WCC). The WCC is a blue-light receptor and the key transcription factor of the circadian oscillator. It controls a transcriptional network that regulates ~20% of all genes, generating daily rhythms and responses to light. We found that in response to light, WCC binds to hundreds of genomic regions, including the promoters of previously identified clock- and light-regulated genes. We show that WCC directly controls the expression of 24 transcription factor genes, including the clock-controlled adv-1 gene, which controls a circadian output pathway required for daily rhythms in development. Our findings provide links between the key circadian activator and effectors in downstream regulatory pathways.

light regulation of frq, and the distal LRE (dLRE) or “clock box” (C box), which is required for clock and light regulation (2, 18, 19, 24). This regulation results in rhythmic expression of frq mRNA and FRQ protein. FRQ protein dimerizes and binds to the FRQ-RNA helicase (FRH). The FRQ/FRH complex (FFC) functions as the negative element in the circadian negative-feedback loop (8, 23). Once FRQ protein is made, it becomes progressively phosphorylated, and when fully phosphorylated, it is degraded, allowing the cycle to restart the next morning (16, 32, 40).

The WCC is also required for all known blue-light responses, including resetting the circadian clock, carotenoid synthesis, asexual spore development, formation of female sexual structures, and ascospore release (47). In response to light, activated WCC functions as a TF and binds to LREs to regulate the expression of target genes. More than 100 light-responsive genes have been identified in neurospora, primarily through transcript microarray studies. Importantly, not all of these genes contain an obvious LRE (6, 28, 29, 31), which implies that a genetic regulatory cascade orchestrates expression of the light-responsive gene network. Furthermore, most light-regulated genes are also clock regulated. The identification of key molecules that function in light-regulated/circadian output pathways and control rhythms in target gene expression is one major goal of circadian biology. Despite significant efforts, these molecules have been difficult to identify. While the
predicted functions of the *Neurospora* light-inducible genes suggest which processes are regulated by light, microarray studies have failed to directly link the action of the WCC to these genes. Identifying the genes directly regulated by the WCC is an essential step toward generating hierarchical or network models to describe the molecular and physiological responses to light and the circadian clock.

To identify the direct targets of the light-activated WCC, we used chromatin immunoprecipitation (ChiP), followed by high-throughput sequencing of bound DNA on an Illumina genome analyzer (ChiP sequencing [ChiP-seq]) (25, 34). We found that the WCC binds to hundreds of sites, predominantly upstream of genes. WCC targets fell into various functional categories, but genes encoding TFs were overrepresented. We tested all TF genes and numerous other genes with WCC binding sites in their promoters for light induction and found that, as expected, most, but not all, responded to light. We show that one of the genes encoding a TF, *adv-1*, is a clock-controlled gene that is necessary for circadian rhythms in development. Our data suggest a “flat” hierarchical network in which ~20% of all annotated *Neurospora* TFs are regulated during the early light response by the WCC, the key TF factor of the circadian clock.

**MATERIALS AND METHODS**

**ChiP.** *Neurospora* cultures (FGSC2489) were grown at 25°C in minimal medium (1× Vogel’s salts, 2% glucose) in the light for 2 days, transferred to the dark for 12 h, light induced (160 μE) for 8 min, and cross-linked in constant light with 1% paraformaldehyde for 15 min. We isolated nuclei (33) and performed ChiP as described previously (26) on 6 mg of nuclear fraction with ~2 μg of WC-2 antibody (polyclonal antibody raised to a WC-2 protein fragment expressed in *Escherichia coli*) (44). Histone ChiPs were performed on germinated conidia with dimethylated H3 lysine 4 (H3K4me2) (Upstate; 07-030) antibodies as described previously (45). All ChiP experiments were validated by ChiP-PCR before (not shown) and after (see Fig. S1 in the supplemental material) ChiP-seq library construction to verify previously described results.

**ChiP-seq library construction and high-throughput sequencing.** DNA was end repaired and ligated to adapters (38). The 200- to 500-bp fragments were gel purified and amplified with 20 cycles of PCR using Phusion polymerase (Finzymes Oy). The PCR products were gel purified and sequenced on an Illumina 1G sequencer in the Oregon State University Center for Genome Research and Biocomputing (OSU CGRB) core laboratories.

**Sequence analysis to find WCC consensus binding sites.** Duplex PCR with [α-32P]dCTP was performed to determine enrichment in the ChiP samples relative to input DNA with region-specific oligonucleotide primers (see Table S1 in the supplemental material) (38, 45). An *hhf-1* segment was used as a control. Phosphorimager screens were exposed to dried gels and analyzed with a GE Storm 820 imager.

**RESULTS AND DISCUSSION**

**WCC ChiP.** To identify direct targets of the WCC, we performed ChiP-seq with anti-WC-2 antibody on cultures subjected to an 8-min light pulse. We verified that the ChiP-seq library was enriched for known targets of the WCC (see Fig. S1 in the supplemental material). Of 4,866,015 32-bp-long ELAND-processed (25) sequences, 92% were mapped to assembly 7 of the *Neurospora* genome by SOAP (30). Sequence reads are available through the NCBI sequence read archive (SRA010801.1).

The WCC binding sites were compared to ChiP-seq data for H3K4me2 (K. M. Smith, C. M. Sullivan, K. R. Pomraning, and M. Freitag, unpublished data) because the presence of this epigenetic modification is correlated with transcriptional activity. To establish a cutoff value for statistically significant WCC binding sites, we used the CASHX mapping algorithm (17) and sliding-window read counts to calculate the mean and standard deviation of reads per 500 bp for the entire genome. A count of 83 reads per 500-bp window was significantly above the mean level of background signal (*P* < 0.001 and *z* = 3.09).
In previous studies, only the *frq*, *vvd*, *al-3*, *fl*, and *sub-1* promoters had been identified as direct targets for the WCC (6, 18, 24, 36). By ChIP-seq, we identified >400 significant regions of WCC enrichment, with >200 falling in known or predicted promoters of at least one gene. Here, we focus on two groups: (i) the most significant peaks located in promoters and (ii) peaks in promoters of TF genes (Table 1). We also compiled a complete list of regions with significant reads, including genes involved in the circadian clock, chromatin function, kinases, phosphatases, cell cycle, DNA replication, DNA repair, and metabolism (see Table S1 in the supplemental material). Target genes were summarized according to their functional categories. While almost half of all target genes encode unclassified proteins, we noticed an enrichment for...
genes with functions in the cell cycle, transcription, protein binding, response to the environment, and cellular components when we compared the best 109 targets (z score = 5; P < 2.9e−7) to all 584 targets (z score = 3.09; P < 0.001) (Fig. 1A; see Fig. S3 in the supplemental material).

The Weeder (37) and SCOPE (4) algorithms were used to derive a consensus binding site for WCC based on 1-kb regions centered on the WCC ChIP-seq peak at 29 genes that were confirmed to be light induced (Fig. 1B). Both algorithms identified a common consensus binding site, GATCGA (with variability in the first and last bases), which extends the most recently published consensus (GATC) that was derived from studies with expression arrays (6). Every 1-kb fragment used to generate the consensus contained at least one, but often two or more, copies of this motif interrupted by a variable number of nucleotides.

For a known WCC target gene, frq, we observed peaks in two regions of the frq promoter that corresponded exactly to the previously identified pLRE and dLRE sites (18, 24) (Fig. 1C). Enrichment at the frq dLRE (or C box), which deviates from the consensus WCC binding motif, was less pronounced than at the pLRE. WCC binding at the pLRE correlated with increased frq transcription in response to light (Fig. 1D, black bars). This induction was absent in a Δwc-2 strain (Fig. 1D, white bars). We found an additional peak downstream of the frq coding sequence, which we named the “antisense LRE” (aLRE) because we predicted it would control light-induced expression of the antisense frq transcript (13).

The aLRE also falls in the promoter of NCU02264, encoding a predicted protein with a prefoldin chaperone domain. Binding of WCC to the aLRE is associated with induction of both the antisense frq transcript and the divergently transcribed NCU02264 (Fig. 1D). Approximately 15% of all WCC binding sites in promoters occur between two divergently transcribed genes, and most often, both genes are regulated by one shared binding site (Table 1). We measured the light induction of transcription by quantitative PCR (qPCR) and compared our results to previous results from microarray experiments (6) performed under similar conditions (Table 1). This list included known clock- and light-regulated genes and several novel genes. A replicate WC-2 ChIP was used to validate the ChIP-seq results by using duplex ChIP-PCRs for numerous regions. In all cases, enrichment shown by ChIP-PCR validated the WC-2 ChIP-seq results.

Of the other known WCC binding sites, both vvd and sub-1, listed in Table 1, have highly significant WCC binding sites in their promoters. Both were also light induced (Table 1; see Fig. S2A in the supplemental material for sub-1). The
vvd and al-3 promoter fragments were used in ChIP-PCRs to check the quality of the ChIP libraries prior to sequencing (see Fig. S1 in the supplemental material), and both were enriched in the library. Even though al-3 promoter sequence was present in the library, the number of reads sequenced from this region fell below our cutoff for significance. The fl promoter was recently shown to be a target of WCC (36), but we did not find enrichment at this promoter by ChIP-seq. The al-3 PCR product band was much fainter than the frq and vvd bands (see Fig. S1 in the supplemental material).

### TABLE 1. Regions of WCC enrichment after 8 min of light induction

| Contig<sup>a</sup> | Start nt | No. of reads | z score<sup>b</sup> | Gene(s)<sup>c</sup> | Array induction<sup>d</sup> | qPCR<sup>e</sup> |
|-------------------|---------|-------------|-----------------|----------------|----------------|----------|
| Largest peaks<sup>f</sup> |         |             |                 |                |                |          |
| 7.10              | 277601  | 738         | 45.66           | NCU02265 frq   | 2.35 (10)       | 13       |
| 7.12              | 422401  | 696         | 42.93           | NCU03867 vvd   | 4.4 (15)        | 130      |
| 7.2               | 298501  | 657         | 40.40           | NCU00582 cry   | 4.8 (15)        | 269      |
| 7.57              | 14601   | 415         | 24.68           | NCU08699 bli-4 | 4.9 (15)        | ND       |
| 7.9               | 872451  | 387         | 22.87           | NCU03071 os-4/NCU03072 | 1.6 (10)/2.3 (15) | 4/2.7  |
| 7.10              | 272251  | 378         | 22.28           | NCU02264/NCU02265 frqAS | 2.3 (15)/frqAS<sup>g</sup> | 2/6.13  |
| 7.48              | 193601  | 321         | 18.58           | NCU10063       | 6.5 (10)        | ND       |
| 7.12              | 623601  | 298         | 17.09           | NCU04021       | NF              | 2.7      |
| 7.22              | 197301  | 262         | 14.75           | NCU05594       | 8 (15)          | ND       |
| 7.7               | 782201  | 253         | 14.17           | NCU02800/NCU02801 | NF/3.8 (15) | 2/4/ND  |
| 7.45              | 138451  | 235         | 13.00           | NCU07541       | 2.8 (30)        | ND       |
| 7.2               | 196651  | 230         | 12.67           | NCU00552 al-1  | 14.3 (15)       | ND       |
| 7.12              | 426151  | 226         | 12.41           | NCU03968       | 1.8 (30)        | ND       |
| 7.21              | 6451    | 210         | 11.37           | NCU11300/al-6  | NF              | 4.7/1.7  |
| 7.2               | 305951  | 200         | 10.72           | NCU00584/NCU00585 al-2 | 3.7 (15)/5.1 (45) | ND     |

Peaks close to transcription factor genes<sup>h</sup>

| Start nt | No. of reads | z score<sup>b</sup> | Gene(s)<sup>c</sup> | Array induction<sup>d</sup> | qPCR<sup>e</sup> |
|----------|-------------|-----------------|--------------------|----------------|----------|
| 7.13     | 558401      | 19.81           | NCU04179 sah-1 TF  | 3.4 (30)       | 4.1      |
| 7.10     | 644051      | 13.65           | NCU02356 wc-1 TF   | 3.1 (10)       | 4.7      |
| 7.13     | 125751      | 13.06           | NCU04295 TF        | 1.4 (10)       | ND       |
| 7.81     | 66601       | 10.92           | NCU09615 TF        | NF              | 7.1      |
| 7.59     | 47101       | 9.56            | NCU09068 nit-2 TF  | NF              | NI       |
| 7.4      | 336501      | 8.84            | NCU05964 TF        | NF              | 2.2      |
| 7.88     | 31301       | 8.78            | NCU09829/NCU09830 TF | NF/11.6 (15) | 7.2      |
| 7.15     | 93601       | 8.27            | NCU01242/NCU01243 TF | NF/1.8 (10) | 2.4/1.3  |
| 7.66     | 66151       | 8.00            | NCU01871 TF/NCU01873 | NF              | NI/1.4<sup>i</sup> |
| 7.54     | 127201      | 7.48            | NCU08480 hsf-2 TF  | NF              | 1.8      |
| 7.6      | 191451      | 5.79            | NCU02713 csp-1 TF  | NF              | 14.6     |
| 7.60     | 44551       | 5.66            | NCU08807 cre-1 TF/NCU08806 rhp-55 | NF | 1.3/2.1  |
| 7.38     | 129051      | 5.20            | NCU00097 bec-ITF   | NF              | 3.8      |
| 7.1      | 389051      | 5.20            | NCU00097 bec-ITF   | NF              | 5.5      |
| 7.47     | 109401      | 4.69            | NCU07705 TF        | NF              | 1.2      |
| 7.15     | 391951      | 3.97            | NCU01154 sub-1 TF  | 1.8 (15)       | 8.1      |
| 7.28     | 101201      | 3.71            | NCU06334 TF/NCU06356 | NF              | NI/NI    |
| 7.16     | 202151      | 3.61            | NCU04731 sah-2 TF  | NF              | 1.2      |
| 7.47     | 25201       | 3.58            | NCU07728 sae TF    | NF              | 3.1      |
| 7.4      | 173001      | 3.52            | NCU07846 TF        | NF              | 2        |
| 7.1      | 1039001     | 3.39            | NCU00275 TF/NCU00276 mip-1 | NF/1.7 (30) | 2.5     |
| 7.3      | 949151      | 3.32            | NCU02094 vad-2 TF/NCU02095 | NF | 2.3/NI |
| 7.4      | 200401      | 3.32            | NCU05994 TF        | NF              | NI       |
| 7.9      | 175701      | 3.26            | NCU03273 TF/NCU03271 | NF              | NI/NI    |
| 7.9      | 498501      | 3.26            | NCU03184 TF        | NF              | 1.7      |
| 7.51     | 99701       | 3.19            | NCU08000 TF        | NF              | NI       |
| 7.52     | 161651      | 3.13            | NCU08159 TF        | NF              | 1.9      |
| 7.21     | 260701      | 3.13            | NCU06095 TF        | 1.8 (10)       | 6.2      |

**a** Assembly 7 of the neurospora genome (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html).

**b** z score calculated as described in Materials and Methods.

**c** If binding sites were located between two divergently transcribed genes, both locus names are listed.

**d** Level of induction (x-fold) calculated from raw data (6) at the time in minutes shown in parentheses. If two genes were near WC-2 binding sites, the fold induction levels are separated by a slash. NF, not found in array experiments.

**e** Expression level changes determined in this study by qPCR. If genes were known to be light induced from earlier studies and reference 6, qPCR was usually not done. NI, not induced; ND, not determined because there was previous evidence for light induction.

**f** Only regions with 200 or more reads/500-bp sliding window are listed (all peaks are shown in Table S1 in the supplemental material).

**g** The antisense frq transcript was not tested in the array experiments.

**h** Transcription factors are named or identified by their locus numbers and indicated by "TF." The WCC is enriched in promoter regions of 28 TFs, 24 of which are induced by light and/or their regulation is altered in a wc-2 deletion mutant (Fig. 2; see Fig. S2 in the supplemental material).

**i** Large standard deviation.
These disparities may be explained by weak and/or transient binding in these regions.

Identification of novel WCC binding sites in promoters of transcription factors. We chose to focus on WCC binding near TF genes as the first step in unraveling transcriptional networks that respond to light and are controlled by the circadian clock. We initially predicted that light signaling by the WCC would involve the activation of a few key downstream TFs that would, in turn, control a network of target genes. Instead, we identified 28 known or putative TF genes with significant (P < 0.001) WCC binding in their promoters (Table 1; see Fig. S2 in the supplemental material). To discover if these TF genes were true targets for the WCC, we analyzed transcript levels by quantitative RT-PCR in light regulated (6), and WC-2-dependent manner, suggesting that they are the actual targets of WCC. Transcription of cre-1, the neighboring genes (NCU01873, NCU03273, NCU05994, and cre-1) were excluded as WCC targets, at least under our conditions (see Fig. S2C in the supplemental material). No target has been identified for the peaks in the promoters of NCU05994 and NCU03273, but for NCU01871 and cre-1, the neighboring genes (NCU01873 and rhp55, respectively) were light inducible in a WC-2-dependent manner, suggesting that they are the actual targets for the WCC.

Of the 21 TF genes we identified here as WCC targets, only 4 (wc-1, sub-1, csp-1, and sath-1) had been previously identified as light regulated (6), and sub-1, csp-1, and sath-1 were not known to be involved in circadian output pathways. Conversely, two TF genes identified as light induced in the microarray study lacked high-confidence WCC binding sites in their promoters in our study (NCU06407 vad-3 and NCU03643, the gene encoding cutinase TF-1β). However, both genes had a single perfect match to the WCC consensus site (GATCGA) and several sites with a single mismatch (GATCCA) within 1 kb of their predicted initiation codons. Thus, while the two studies were largely congruent, ChIP-seq identified more direct first-tier targets and was more suitable for detecting regulation of TF genes, which are often expressed at low levels, or whose expression levels are changed within a narrow range.

Examples of genes regulated by single or multiple WCC binding sites are shown in Fig. 2, and data for the most significant peaks are summarized in Table 1. We found a single WCC binding site upstream of the gene for heat shock factor 2, hsf-2, and transcription of hsf-2 was light inducible and dependent on WC-2 (Fig. 2A). We found three WCC binding sites upstream of the wc-1 gene (Fig. 2B), which encodes the limiting subunit of the WCC. Transcription of wc-1 was induced by light and dependent on WC-2, in agreement with previous reports (1). Our data show that wc-1 is directly regulated by the WCC in a positive-feedback loop, as had been suggested by previous work (27). Transcriptional start sites of the wc-1 gene have been mapped at -924 and -1,222 bp (27). One WCC binding site is close to the transcriptional start site at -924. The other WCC binding sites at -3 and -4 kb are, however, located far upstream of the mapped transcriptional start sites.

WCC binds to sites far upstream of predicted transcriptional start sites in additional TF genes, e.g., sah-1, sub-1, nit-2, bek-1, and NCU05964 (see Fig. S2A in the supplemental material). In particular, a single strong WCC binding site is located about 10 kb upstream of the open reading frame (ORF) of sah-1 (Fig. 2C), which is light inducible in a WC-2-dependent manner. Thus, WCC binding appears to be capable of exerting long-range effects in the relatively compact Neurospora genome, where promoters are typically short and sometimes overlapping (20). The precise mechanism of the enhancer-like effects of WCC merits further investigation.

Developmental stages that have been associated with light regulation, i.e., asexual sporulation (conidiation) and sexual development, are represented by nine TFs whose mutation results in developmental phenotypes (hsf-2, adv-1, sub-1, sah-1, bek-1, vad-2, csp-1, and ghh) (11, 46). All nine genes are induced by light, and this response is abolished in a wc-2 deletion strain (Table 1; see Fig. S2A in the supplemental material).

WCC binding sites were associated with six TF genes involved in metabolism or stress response (nit-2, NCU05994, sre [regulator of iron uptake] [48], cre-1, NCU08000, and NCU05964 [homolog of Aspergillus vosA] [35]). Three (cre-1, vos4, and sre) were light induced, and this response was abolished in the Δwc-2 mutant, but the other three genes showed no light induction under our conditions (see Fig. S2C and D in the supplemental material). The expression levels of these genes in the Δwc-2 strain were decreased. These results suggest that WCC directly impinges on metabolic pathways.

The downstream targets of 11 TFs identified here remain completely unknown. These putative TF genes (NCU00275, NCU01243, NCU03184, NCU03273, NCU04295, NCU06534, NCU07705, NCU07846, NCU08159, NCU09615, and NCU09829) have no previously described function, but they encode motifs that match well-studied DNA binding domains (11). NCU00275, NCU01243, NCU03184, NCU04295, NCU07705, NCU07846, NCU08159, NCU09615, and NCU09829 were light induced, a response abolished in the Δwc-2 mutant (see Fig. S2A in the supplemental material).

The WCC activates an output pathway required for rhythmic spore development. To investigate the link between light and circadian clock pathways regulated by the WCC, we assayed available knockout mutants of the WCC target TFs for changes in circadian rhythms in development (data not shown). The most striking phenotype was observed in the Δadv-1 mutant, so we investigated this mutant further for clock defects. Loss of adv-1 had only minor effects on the development of spores, but development was no longer under the control of the clock (Fig. 3A).

To determine if arrhythmicity of the Δadv-1 mutant was due to an effect on the FRQ/WCC oscillator, we assayed rhythms in the accumulation of the FRQ protein in control and Δadv-1 strains over the course of 2 days in cultures grown in the dark (Fig. 3B and data not shown). No differences were observed in
we observed that the accu-

We have identified a large number of direct targets of the WCC, the key regulator of the circadian clock. These targets include 24 TFs that have the potential to control downstream target genes on a second hierarchical level. Genomewide identification of target genes for these second-tier TFs, together with genomewide expression studies by RNA-seq, will allow us to build a detailed network of the early and late light responses, as well as circadian clock output pathways. Substantial effort over the past several years has been spent on identifying key components of circadian output pathways in neurospora and other organisms, with only limited success (7, 43, 47). By applying WCC ChIP-seq to this effort, we have now uncovered excellent candidates for output pathway components, such as ADV-1, that lie directly downstream of the oscillator and that regulate distinct overt rhythms. In addition to gene products that may mediate posttranslational clock and light effects, we also found many WCC binding sites in promoters of metabolic genes that act in various anabolic and catabolic pathways.

Most, but not all, of the genes that are direct targets of the WCC were found in our experiments to be light induced. This may not be surprising for genes that are subject to complex regulation. For example, a gene may be repressed under most growth conditions and be activated by light and the bound WCC only when these conditions are not met and repression is removed. Similarly, while we expect most, if not all, genes directly regulated by the WCC to be rhythmically transcribed, other regulatory elements may take precedence under certain growth conditions.

While many of the downstream genes regulated by the WCC are not well studied or are uncharacterized, most of them have homologs in plants and mammals. Thus, our work provides key information to refine the growing network of light- and clock-regulated genes in a genetically and biochemically tractable model organism.

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