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In the *Neurospora* circadian system, the White Collar complex (WCC) of WC-1 and WC-2 drives transcription of the circadian pacemaker gene *frequency* (*frq*), whose gene product, FRQ, as a part of the FRQ-FRH complex (FFC), inhibits its own expression. The WCC is also the principal *Neurospora* photoreceptor; WCC-mediated light induction of *frq* resets the clock, and all acute light induction is triggered by WCC binding to promoters of light-induced genes. However, not all acutely light-induced genes are also clock regulated, and conversely, not all clock-regulated direct targets of WCC are light induced; the structural determinants governing the shift from WCC's dark circadian role to its light activation role are poorly described. We report that the DBD region (named for being <u>d</u>efective in <u>b</u>inding <u>D</u>NA), a basic region in WC-1 proximal to the DNA-binding zinc finger (ZnF) whose function was previously ascribed to nuclear localization, instead plays multiple essential roles assisting in DNA binding and mediating interactions with the FFC. DNA binding for light induction by the WCC requires only WC-2, whereas DNA binding for circadian functions requires WC-2 as well as the ZnF and DBD motif of WC-1. The data suggest a means by which alterations in the tertiary and quaternary structures of the WCC can lead to its distinct functions in the dark and in the light.

Circadian clocks regulate a wide variety of physiological, behavioral, and molecular activities in eukaryotes. In fungi and animals, circadian systems at the molecular level consist of positive and negative factors and the negative proteins repress their own expression through inhibiting the positive proteins (1, 2). *Neurospora* has been an excellent model organism for studies of the circadian clock for several decades (3). In this organism, the White Collar complex (WCC) serves as a transcriptional activator complex for the pacemaker gene, *frequency* (*frq*), by binding to two DNA promoter elements: *Clock box* (*C box*) (4) in the dark and *Proximal Light-Response Element* (*pLRE*) in the light (5). The *frq* gene product, FRQ, binds with FRH (FRQ-interacting RNA helicase) to form the FRQ-FRH complex (FFC), which stably interacts with CKI (casein kinase I) and represses WCC activity to close the positive loop (6–9), presumably through promoting phosphorylation of the WCC (10, 11). Although three regions of FRQ have been identified as required for interaction with the WCC (12), the corresponding domain on the WCC that mediates binding to the FFC has not been discovered.

WCC consists of White Collar-1 (WC-1) and White Collar-2 (WC-2) (13), although we now know that the quaternary structure of this complex changes between the dark (D-WCC, the heterodimer) and the light (L-WCC, a heterotrimer as described below). WC-1 has a circadian transactivation domain (14), three Per-Arnt-Sim (PAS) domains (8), a domain described as a nuclear localization signal (NLS), and a zinc finger (ZnF) DNA binding motif (15). WC-2 contains a PAS domain that binds and stabilizes WC-1 and a ZnF DNA binding domain (16, 17). Within FRQ, a functional NLS (amino acids [aa] 194 to 199; PRRKKR) is required for nuclear localization of FRQ and for FRQ to repress the nuclear activity of the D-WCC (18). However, the identity of the actual NLS within the WCC is only based on conjecture.

WC-1 is also a flavin adenine dinucleotide (FAD)-binding photoreceptor (5, 19), and the dark and light activities of the WCC can be separated (14, 20). In the light, L-WCC is a heterotrimer believed to be composed of two WC-1 proteins and one WC-2 (5, 20) which senses light through the N-terminal most PAS domain of WC-1, a light-, oxygen-, and voltage-sensing (LOV) domain, and mediates light induction of about 5.6% of the *Neurospora* genome, including *frq*, *vvd*, *albino-3*, and *sub-1* (10, 21–25). Many questions remain about how the D-WCC turns into an L-WCC, how similar are the D-WCC and L-WCC structures and domain activities, and how the two ZnFs in the WCC act to bind the *frq* promoter and activate transcription of *frq* in the dark versus light.

A unique feature of the *Neurospora* system is that transcriptional activation driven by the photoresponse and that in the circadian rhythm are both carried out by the WCC, while in animals, different protein complexes execute the two different functions. For example, in *Drosophila*, cellular clocks, cryptochromes (dCRYs) sense light, while the Cycle-Clock complex activates transcription of the circadian pacemaker gene *period* (*per*) (26), and in mammals, circadian photoreception and pacemaker functions are anatomically separated (27). How the *Neurospora* WCC fulfills this dual role is elusive. Here we identify the FFC-interacting region on WCC and show how the *Neurospora* WCC utilizes different motifs to bind DNA and induce *frq* expression in the light versus dark. The many differences in domains required for DNA binding, in turn, shed light on the conformational differences between the L-WCC and D-WCC and suggest cross talk between the WC-1 LOV domain and ZnFs on WCC after the dimer formation or upon light exposure.

MATERIALS AND METHODS

Strains and growth conditions. 328-4 (*ras-1^bd^ A*) was used as a clock-wild-type (WT) strain in race tube analyses. The WT in the luciferase assays was 661-4a (*ras-1^bd^ A*), which contains the *frq C box* fused to codon-optimized firefly *luciferase* (transcriptional fusion) at the *his-3* locus. *wc-1* and *wc-2* mutants were generated as previously described (14). Recipient strains for generating *wc-1* and *wc-2* mutants were strains 141 (*ras-1^bd^*; Δ*wc-1::hph^+^*; Δ*mus-52::hph^+^ a*) and 173-1 (*ras-1^bd^*; Δ*wc-2::hph^+^*; Δ*mus-52::hph^+^ a*), respectively. *Neurospora* transformation was performed as previously reported (28). All *wc-1* and *wc-2* cassettes bearing mutations were targeted to their native loci. All *wc-1* and *wc-2* mutants were in the *ras-1^bd^* genetic background, and all *wc-1* mutants contain a V5 epitope tag at their C termini. For the experiment whose results are shown in Fig. 3D, WT@csr, ΔC box@csr, and ΔpLRE@csr in the genetic background of *wc-1^Δ918-925^* Δ*frq* were generated by transforming the *frq* open reading frame (ORF) fused with 3,794 bp of *frq* 5′ untranslated region (UTR) (WT), the *pLRE* alone (Δ*C box*, missing −2878 to −2491 of the *frq* 5′ UTR), or the *C box* alone (Δ*pLRE*, lacking −1846 to −1511), respectively, to the *csr* locus. The race tube medium was 1× Vogel's salts, 0.17% arginine, 1.5% Bacto agar, and 50 ng/ml of biotin with glucose at 0.1%, and liquid culture medium (LCM) contained 1× Vogel's salts, 0.5% arginine, 50 ng/ml of biotin, and 2% glucose. Unless otherwise specified, race tubes were cultured in constant light for 16 to 24 h at 25°C and then transferred to the dark at 25°C. All of the primers used for making *wc-1*, *wc-2*, and *frq* constructs are listed in Table 1.

Protein lysate and WB. Procedures for preparation of protein lysates and Western blots (WB) were followed as described previously (29, 30). For WB, equal amounts of whole-cell protein lysate (15 μg) were loaded per lane in an SDS gel. FRQ, WC-1, and WC-2 antibodies were from previous publications (5, 30, 31). V5 antibody (Thermo Pierce) was diluted 1:5,000 for use as the primary antibody.

IP. Immunoprecipitation (IP) was performed as previously described (29). Briefly, 2 mg of total protein was incubated with 20 μl of V5 agarose (Sigma-Aldrich) rotating at 4°C for 2 h. The agarose beads were washed with protein extraction buffer (50 mM HEPES [pH 7.4], 137 mM NaCl, 10% glycerol, 0.4% NP-40) twice and eluted with 50 μl of 5× SDS sample buffer at 99°C for 5 min.

***Neurospora* nuclear preparation.** Nuclear isolation was conducted as previously reported (32). Briefly, *Neurospora* mycelial pads were grown in LCM at 25°C in constant light or darkness as indicated below. The crude homogenate resulting from bead-beating about 5 g of the mycelial pads was filtered through cheesecloth. Nuclei pelleted by centrifugation were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) and loaded into a 4 to 12% bis-Tris SDS gel (Life Technologies) for Western blot analyses. Tubulin (the cytoplasmic marker) and histone H3 (the nuclear marker) antibodies were purchased from Sigma-Aldrich and Abcam, respectively.

ChIP. Chromatin immunoprecipitation (ChIP) experiments were done as previously described, with the exception of using fresh tissues (14, 33). The following primer sets were used for quantitative PCRs: *C box*, forward primer (−2790 to −2767 of the *frq* promoter), 5′-TCAAGTCAAGCTCGTACCCACATC-3′, and reverse primer (−2700 to −2678), 5′-CAATTTTGCAGCGTCATCGGTCT-3′; *pLRE*, forward primer (−1861 to −1836), 5′-GCTGGTCATCTCCTCAGCATTTTGTC-3′, and reverse primer (−1783 to −1760), 5′-ATACTTGTAGGCCCGCTCCCATC-3′.

Luciferase assays. Luciferase assays were conducted as previously described (34).

Peptide disruption assays. Lysate from the WC-1 V5 strain cultured in the light was prepared by grinding frozen *Neurospora* tissues and adding protein lysis buffer (50 mM HEPES [pH 7.4], 137 mM NaCl, 10% glycerol, 0.4% NP-40) and cleared by centrifugation at 11,750 × *g* for 10 min at 4°C. WCC from 2 mg of cleared lysate was isolated with 20 μl of V5 beads (Sigma-Aldrich) as described for the IP assay. The beads were washed four times with lysis buffer containing protease inhibitors [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, bestatin, E-64, leupeptin, and pepstatin A]. Synthesized DBD, 8before, and 8after peptides (GenScript) (see below) were applied to the washed beads at the concentrations indicated below. The mixture was rotated at 4°C for 1 h, and after a quick spin (11,750 × *g* for 10 s), the supernatant was removed. Fifty microliters of 5× SDS sample buffer was added to the beads and boiled to elute.
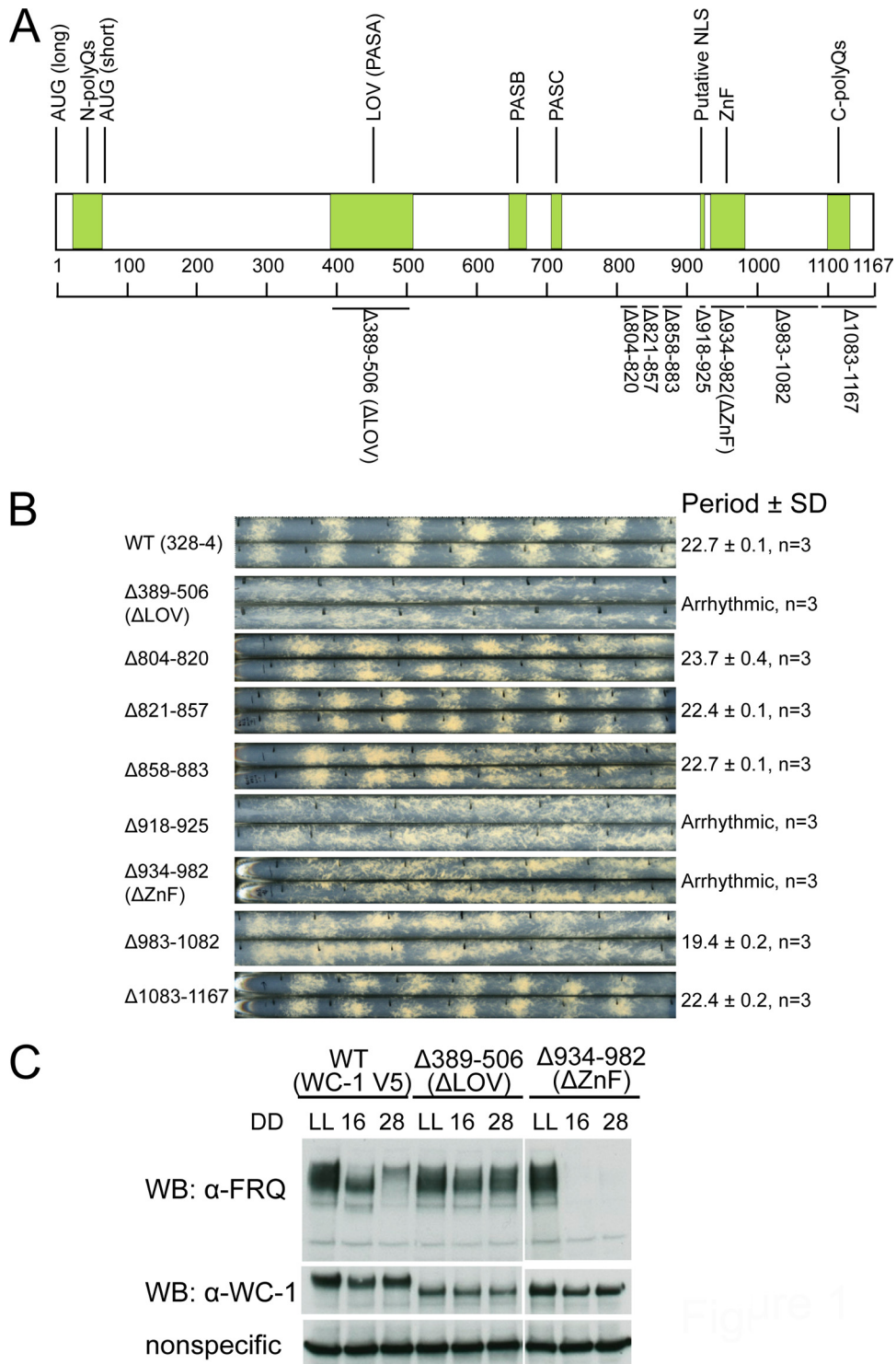
RESULTS

A WC-1 deletion series identifies regions required for the core *Neurospora* clock. In the search for motifs in WC-1 essential for the *Neurospora* circadian clock, strains bearing a deletion series within WC-1 were generated and analyzed by race tube assay and Western blotting (Fig. 1). One of these mutants, WC-1, missing the hypothetical NLS (aa 918 to 925) (15), showed an arrhythmic conidiation phenotype (Fig. 1B), suggesting that this motif on WC-1 was required for clock function. Although the LOV domain in WC-1 is required for light-dependent dimerization and activation (13, 35, 36) (data not shown), it is not essential for FRQ expression in the dark or light, whereas the ZnF is needed for FRQ expression in the dark (Fig. 1C).

The NLS in WC-1 does not function as a nuclear localization motif. One possibility for why WC-1 lacking the putative NLS failed to drive FRQ expression in the dark is that mutant WC-1^Δ918-925^ was unable to enter the nucleus. To test this possibility, WC-1 nuclear localization was monitored at DD16 (16 h in darkness, circadian time 5 [CT5] in the subjective morning), when the WCC dark activity peaks in the WT. Surprisingly, WC-1^Δ918-925^ was still highly enriched in the nucleus (Fig. 2A), consistent with unpublished data cited by Cheng et al. (20). It is also possible that the nuclear import of WC-1^Δ918-925^ was through "piggybacking" with its binding partner, WC-2. However, WC-1^Δ918-925^ was still seen in the nucleus even in the absence of WC-2 (Fig. 2B). Thus, the stretch of basic amino acids (KKKRKRRK) previously thought to be an NLS of WC-1 is not required for the nuclear localization of the protein. We sought the functional domain essential for WC-1 nuclear import by searching the WC-1 amino acid sequence, but no other basic stretch resembled a typical basic NLS, and indeed, there were no other basic clusters longer than 3 amino acids (data not shown). This suggested the existence of a noncanonical NLS. In an attempt to locate this, two additional WC-1 mutants were made: WC-1 1-579, missing from aa 580 to the C terminus (aa 1167), and WC-1 580-1167, which lacked the N-terminal region from aa 1 to 579. The level of WC-1 1-579 was comparable to that of WT and not dependent on WC-2 (Fig. 2C), while the level of WC-1 580-1167 was lower in the absence of WC-2, although it could be detected after enrichment by immunoprecipitation with V5 antibody (Fig. 2C); WC-2 may stabilize WC-1 aa 580 to 1167 through its interaction with the WC-1 PASC domain, which is located within aa 580 to 1167 (8). Nuclear preparation assays clearly showed that in the absence of WC-2, both the WC-1 1-579 and WC-1 580-1167 mutants were detected in the nucleus (Fig. 2D). Also, WC-1 580-1167 failed to associate with FRH in the Δ*frq* mutant or the FFC in the *frh*^R806H^ mutant (Fig. 2E), but it was still highly enriched in the nucleus (Fig. 2F), suggesting that nuclear import of WC-1 580-1167 was not through tailgating FRQ or FRH. It is apparent that intact WC-1, or the two halves of WC-1, aa 1 to 579 and aa 580 to 1167, are imported to the nucleus even without a typical NLS and in the absence of WC-2 or other known interactors. Although this conclusion was unantici-

TABLE 1 Primer sequences used for making constructs

Construct	Forward primer (5′–3′)	Reverse primer (5′–3′)
wc-1$^{\Delta389-506}$	CTTAAGCTCGGTGCCGTGGACGAATGCCCTGATGCCATAATC	GATTATGGCATCAGGGCATTCGTCCACGGCACCGAGCTTAAG
wc-1$^{\Delta804-820}$	CGTTTTTGTTGGCGCAAACAAAGAATATGTCTCCGGCGGTG	CACCGCCGGAGACATATTCTTTGTTGGCGCAACAAAACG
wc-1$^{\Delta821-857}$	CTCCCGCGACCGTAACGGTCACGGGGCCATGGTCTCAGC	GCTGAGACCATGGCCCCGTGACCGTTACGGTCGCGGGAG
wc-1$^{\Delta858-883}$	GAAGTCGGGAAGCAGCGATAGTGAACTCAAGACAACGCGTTGC	GCAACGCGTTGTCTTGAGTTCACTATCGCTGCTTCCGACTTC
wc-1$^{\Delta918-925}$	CTGGGCGCAGCTTTTGTCTAACGGAGGTAACATGGTGAG	CTCACCATGTTACCTCTCCGTTAGACAAAGCTGGGCCAG
wc-1$^{\Delta934-982}$	GGAGGTAACATGTGAGGGATTCGATGAGCAAGAAGAGCAATTC	GAATTGCTCTTCTTGCTCATCGAATCCTCAACCATGTTACCTCC
wc-1$^{\Delta983-1082}$	CCCGAGGTGGGAATGGTGATCTCCAGGGAACACACCTTAAC	GTTAAGGTGTGTTCCCTGGAGATCACCATTCCACCTCGGG
wc-1$^{\Delta1083-1167}$	CTCAACATCTTCCGCCTCATGGCCGGAGGGCGGGGAGGCGGAG	CTCCGCCTCCGCGCCTCCGCCATGAGGCGGAAGATGTTGAG
wc-1 1–579	GCACAAACAATCCTGGGATAAGGGAGGCGGAGGCGGTAAGCCTATC	GATAGGCTTACCGCCTCCGCCTCCCTATCCCAGGATTGTTTGTGC
wc-1 580–1167	CTTGGGCGACCGATGCAGCCACCATGCTTTTGGAGAACGCAGACG	CGTCTGCGTTCTCCAAAGCATGGTGGCTGCATCGGTCGCCCCAAG
wc-1$^{\Delta918-925-8A}$	CAGCGGCAGCAGCGGGGAGGAGGTAACATGGTGAG	CTGCCGCTGCTGCCGCGTTAGACAAAGCTGCGCCAG
	GTCTAACGCGGCAGCGGCAGCAGCGGGGAG	CTCCCCGCTGCTGCCGCTGCCGCGTGCCGCGTTAG
wc-1$^{KKK/AAA}$	GCAGCTTTTGTCTAACGCGGCAGCACGGAAACGAAGAAAG	CTTTCTTCGTTTCCGTGCCGCGCGTTAGACAAAAGCTGC
wc-1$^{RR/AA}$	GTCTAACAAGAAAAAACGGAAAAGCAGCAAAGGGAGGAGGTAACATG	CATGTTACCTCCTCCCTTTGCTGCTTTCCGTTTTTTCTTGTTAGAC
wc-1$^{926-929/AAAA}$	GAAAAAACGGAAACGAAGAAGGCAGCAGCTGCCATGGTGAGG	CAATTTGCACAATCCCTCACCATGGCAGCTGCTGCCTTTCTTCG
wc-1$^{930-933/AAAA}$	GAAGAAAGGGAGGAGGTAACGCGCGCGGCGGCTTGTGCAAAT	CTCGTATGGCAATTTGCACAGCCGCGCCGCGCGTTACCTCCT
wc-1 aa 918–925 KR invert	CTTTTTGTCTAACAGGAGAAAGAAAAGAGAAAAAAAGGGAGGAGGTAAC	GTTACCTCCTCCCTTTTTTTTCTCTTTCTCCTGTTAGACAAAAG
wc-1$^{2 \times DBD}$	AAAAAGAAGAAGAGAGGCGTAAAAAGAAAAAACGGAAAAGAAGAAAGGGGAG	TTTCTTTTTACGGCCTCTTTCTCTTCTTTTTGTTAGACAAAAGCTGGCCAG
wc-1$^{DBD\ 909}$	GAAAAGAAAAAAACGGAAACGAAGAAGGAGCTGGCGCAGCTTTTGTC	CTCCTTTCTCGTTTCCGTTTTTTCTTTTCGGCCAACATGCGATTCAC
wc-1$^{DBD\ 936}$	GATAAGAAAAAAACGGAAACGAAGAAAGTGTGCAAATTGCCATACGAGG	CACACTTTCTTCGTTTCCGTTTTTTTCTTATCCCTCACCATGTTACCTCCTC
wc-1 ZnF swap	GGAGGTAACATGTGAGGGATTGCACCGACTGCGGTACGCTC	GAGCGTACCGCAGTCGGTGCAATCCCTCACCATGTTACCTCC
	CAAGACACTATGCAATGCGCGGGGTTGAGAGTGGCAAAGCAG	CTGCTTTGCCACCTCAACCCGCAGGCATTGCATAGTGTCTTG
wc-2$^{\Delta456-461}$	GAGATCCCGGACGGGAGAGGTTGCAGAGGAATACGTGTGC	GCACACGTATTCCTCTGCAACCTCTCCGTCGGGGATCTC
wc-2$^{\Delta468-493}$	CAAGGTTGCAGAGGAATACGTGGGTCTTCGCTGGGCAAAGAAAG	CTTTCTTTGCCCAGCGAAGACCCACGTATTCCTCTGCAACCTTG
wc-2$^{\Delta499-504}$	CCTGCGGGTCTTCGCTGGGCAAAACGCCAACAACAACAATAACG	CGTTATTGTTGTTGTTGGCGGTTTGCCCAGCGAAGACGCGAGG
wc-2 ZnF swap	CAAGGTTGCAGAGGAATACGTGTGTGCAAATTGCCATACGAGG	CCTCGTATGGCAATTTGCACACACGTATTCCTGCAACCTTG
	CGAGATCTATGCAACAGTTGTGTGCTCTTCGCTGGGCAAAGAAAGAG	CTCTTTCTTTGCCCAGCGAAGACCACAACTGTTGCATAGATCTCG
frq@csr	GTAACGCCGAGGTTTTCCCAGTCACGACGGCCTAGGCTGGAGAAGCTCATTCCAC	GATGTGCCCTGTAACTTTCAGGTCACCTCAATGTCCATCTGATC
	GATCAGATGGACATTGAGGTGACCTGAAAGTTACAGGGACCATC	GTCAGAAGCCTGCGATGCTAC
	GTCTGCGGCTTCCATAGTCTC	CACATGACCTGCAAACTGTCCAAAAGCACACCGTTTGAAATC
	GATTTCAAAACGGTGTGGCTTTTGGGACAGTTTGCAGGTCATGTG	GCGGATAACAATTTCACACAGGAAACAGGCGTAGCCAACCGAGCACCAACTAAC
frqΔ*pLRE@csr*	GAGAGCTGCTGGTCATCTCCCCAGTCAGCTTTACACACTC	GAGTGTGTAAAGATTCAGACTGGGAGGAGATGACCAGCAGCTCTC
frqΔ*C box@csr*	GTTCGTAGGTCATGTACTTCGTGCCTGAGGCGACGCCTACGAAC	CAGGATCACAGGCTGCGCTCAGGCGAGTACAATGACCTACGAAC

FIG 1 Deletion scanning mutations within WC-1 detect domains of WC-1 required for circadian regulation in *Neurospora*. (A) Schematic depiction of the domain architecture of the WC-1 protein. Lines with corresponding numbers of amino acids show different deletions of WC-1: ZnF, zinc finger DNA binding domain; NLS, putative nuclear localization signal; LOV, light-, oxygen-, and voltage-sensing domain; PAS, Per-Arnt-Sim domain. (B) Race tube assays of WT (328-4) and WC-1 deletion strains. The strains were inoculated to 0.1% glucose race tube medium and synchronized in the light at 25°C overnight (12 to 16 h) prior to the transition to darkness. Images of replicate race tubes are displayed, and the vertical lines in race tubes mark the growth fronts of the strains every 24 h. Periods were calculated by the ChronOSX program, version 2.1, and are shown at the right in hours (SD, standard deviation; n, number of race tubes). (C) Western blot showing WC-1 and FRQ protein levels in WT and *wc-1* mutants. LL, constant light; DD, hours after the light to dark transition. Both WT WC-1 and mutants were C-terminally tagged with V5. DD16 = CT5 (circadian time 5); DD28 = CT17.
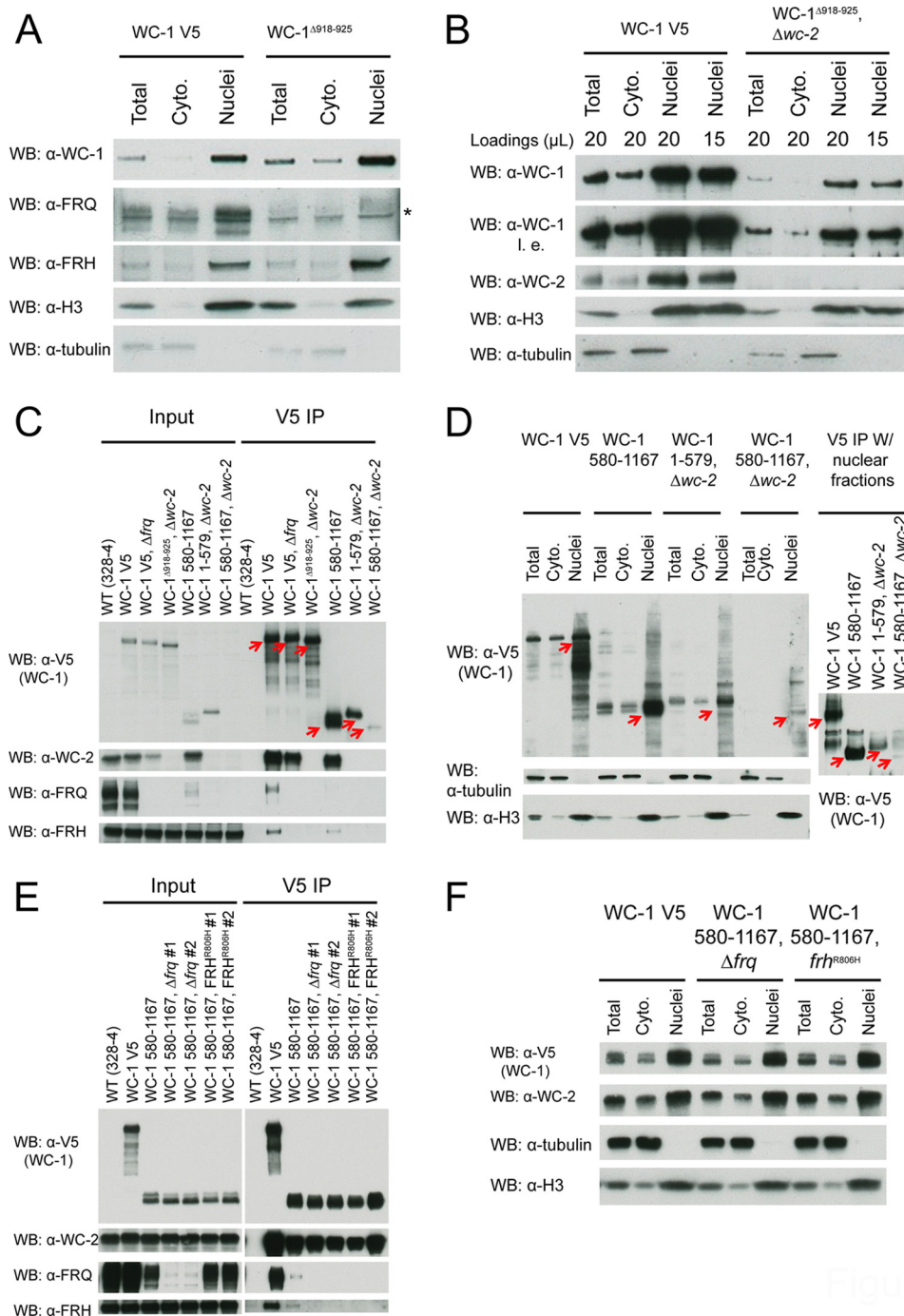
FIG 2 WC-1 does not use a tyical basic NLS for import into the nucleus. (A) Western blot analyses showing the enrichment of WC-1$^{\Delta918\text{-}925}$ in the nuclear fraction at DD16. Fresh *Neurospora* tissues were lysed by bead-beating, and nuclei were isolated from cytoplasm by centrifugation and lysed in RIPA buffer as described in Materials and Methods. Tubulin was used as a control to show that the nuclear fractions were not contaminated with cytosolic proteins, while H3 served as a positive control for enrichment of the nuclear fractions. The asterisk indicates a nonspecific band appearing in all fractions. (B) WC-1$^{\Delta918\text{-}925}$ was found in the nucleus in the absence of WC-2. Indicated volumes of lysate at the concentration of 1.5 μg/μl were loaded per lane to compare WC-1 levels in different fractions. l. e., longer exposure. (C) Immunoprecipitation assays using V5 antibody showing the levels of WC-1 1-579 and WC-1 580-1167. Arrows point to WC-1 bands. In the absence of WC-2, WC-1 580-1167 is undetected in the input lane (left) but appears as a weak band (right) after enrichment with V5 antibody. For the V5 IP samples, 5 μl out of the 50 μl of total eluate was loaded into each lane. WT (328-4) and WC-1 V5 served as negative and positive controls, respectively, for the IP assay. (D) Western blot analyses showing that in the Δ*wc*-2 background, both WC-1 1-579 and WC-1 580-1167 are located in the nuclear fraction (left). WC-1 580-1167 is also visualized after enrichment with V5 antibody from 2 mg of nuclear proteins (right). Arrows point to WC-1 bands. (E) Immunoprecipitation assays using V5 antibody showing the loss of interaction between WC-1 580-1167 and FRH in the Δ*frq* mutant or the FFC in the *frh*^{R806H} mutant. The *Neurospora* protein lysate was either analyzed directly by Western blotting analyses (input) or immunoprecipitated with V5 antibody (V5 IP). (F) Western blot analyses showing that WC-1 580-1167 in the Δ*frq* or *frh*^{R806H} background is located in the nuclear fraction.
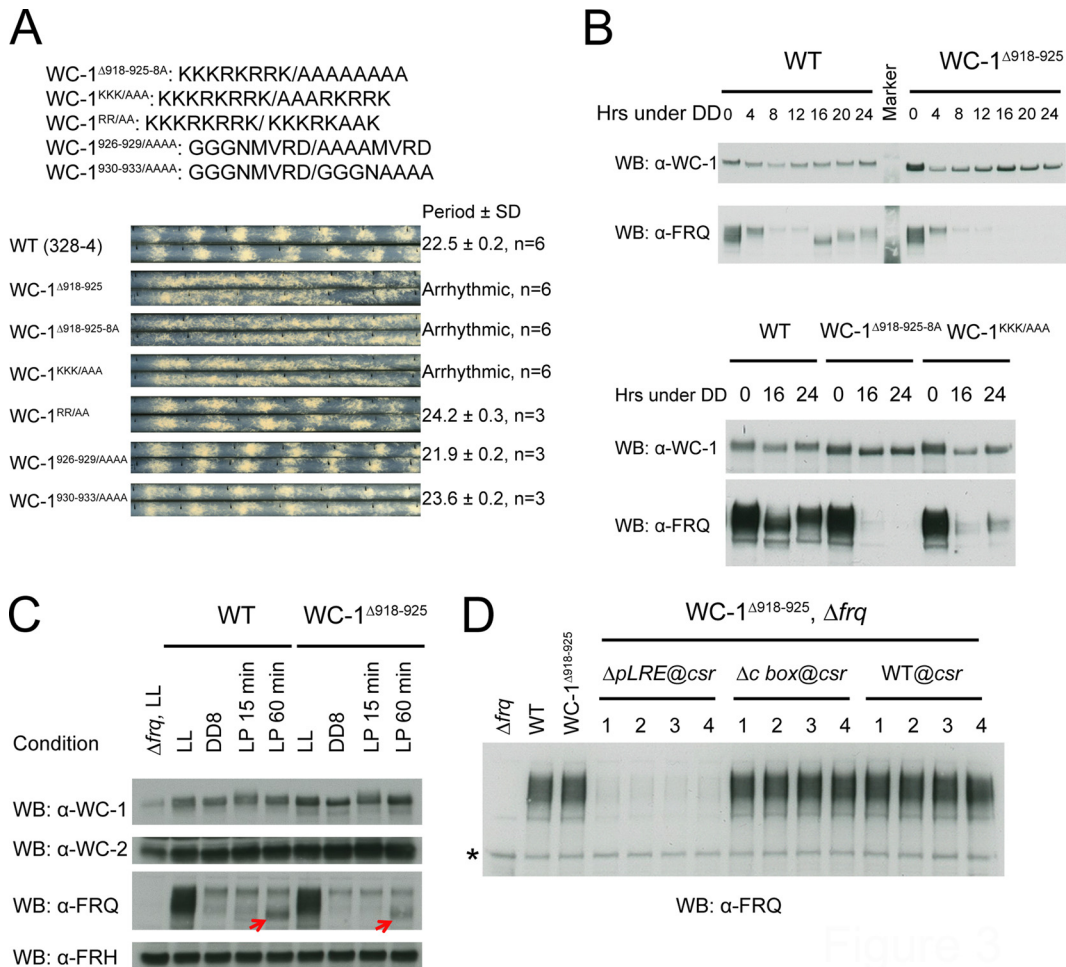
FIG 3 The WC-1 basic domain (aa 918 to 925) is essential for operation of the *Neurospora* clock. (A) Race tube analyses of WC-1$^{\Delta 918-925}$ mutants. The identities of the mutations in WC-1 in the region of aa 918 to 925 are listed at the top. The strains were cultured in 0.1% glucose race tube medium in the light at 25°C overnight and transferred to the dark. Vertical lines in race tubes indicate the growth fronts of the strains every 24 h. ChronOSX program version 2.1 was used to calculate circadian periods. WC-1$^{\Delta 918-925}$, WC-1$^{\Delta 918-925-8A}$, and WC-1$^{\Delta 918-925-KKK/AAA}$ are arrhythmic at 25°C. (B) Western blot analysis showing that FRQ expression in WC-1$^{\Delta 918-925}$ is impaired after transition to the dark (upper blots). In the lower blots, FRQ and WC-1 levels in WC-1$^{\Delta 918-925-8A}$ and WC-1$^{\Delta 918-925-KKK/AAA}$ were assayed at DD0 (constant light), DD16 (when new FRQ peaks in the WT), and DD24 (when old FRQ is extensively phosphorylated in the WT). (C) Western blot analysis showing light-induced FRQ expression in WC-1$^{\Delta 918-925}$ after a 60-min light exposure. The samples cultured at DD8 were treated with a 15- or 60-min light pulse and immediately harvested and frozen in liquid nitrogen. The arrows point to the FRQ bands. (D) Western blot analysis showing that FRQ expression in WC-1$^{\Delta 918-925}$ in response to light requires the *pLRE*. The *wc-1*$^{\Delta 918-925}$ Δfrq mutant as the recipient strain was transformed with the *frq* open reading frame (ORF) fused with the *C box* and *pLRE* (WT), the *pLRE* alone (ΔC *box*), or the *C box* alone ($\Delta pLRE$) targeting to the *csr* locus; see Materials and Methods. Expression of FRQ in the light was analyzed by Western blotting. The asterisk indicates a nonspecific band.

pated, it is consistent with observations that other proteins lacking a canonical NLS can still enter the nucleus (see Discussion).

Amino acids 918 to 925 of WC-1 are required for the circadian expression of FRQ. First of all, to address the concern that the WC-1$^{\Delta 918-925}$ phenotype derived from shortening the WC-1 polypeptide or disrupting the spacing between other domains, alanine scanning mutants within this region or between it and the ZnF were generated and analyzed. WC-1$^{\Delta 918-925-8A}$, in which the region was mutated to eight alanines, showed an arrhythmic phenotype similar to that of WC-1$^{\Delta 918-925}$ (Fig. 3A), and this arrhythmia was not rescued even with a 30°C synchronizing step prior to the dark transfer (data not shown). Sequence alignment of *Neurospora* WC-1 with orthologs from four other related fungi showed that the KKKRKRRK motif is highly conserved (data not shown), so other substitutions were crafted to explore the impor-

tance of the length, spacing, and identity of the 8 basic amino acids within this region (Fig. 3A). Rhythmicity was not apparent at 25°C in a strain with WC-1 bearing AAARKRRK (Fig. 3A) but was restored at 30°C, albeit with a period about 2 to 3 h longer than for the WT (data not shown). WC-1 bearing KKKRKAAK at 25°C displayed a period of about 24 h (Fig. 3A). We also probed the importance of the amino acids separating the basic motif from the ZnF: WC-1$^{926-929/AAAA}$ (aa 926 to 929 to AAAA) and WC-1$^{930-933/AAAA}$ (aa 930 to 933 to AAAA) supported a WT period (Fig. 3A). Together, the race tube data suggest that the phenotype of WC-1$^{\Delta 918-925}$ was caused by the motif itself rather than the neighboring sequences. We investigated the role of the motif on FRQ expression. In the WT, Western blot analyses using antibody against FRQ showed characteristically that FRQ phosphorylation isoforms accumulated in the light, that they
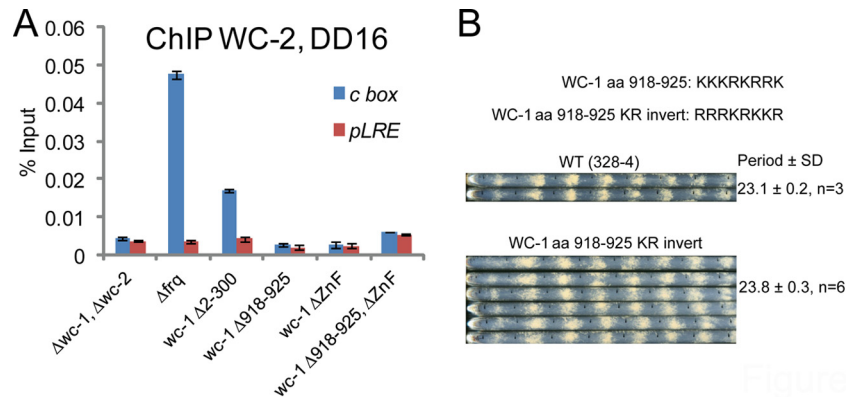
FIG 4 The WC-1 DBD motif is required for associating with the *C box* and for *frq* transcription in the dark. (A) ChIP at the *frq* promoter using WC-2 antibody on indicated *wc-1* mutants. The Δ*wc-1* Δ*wc-2* strain was the negative control, while the Δ*frq* mutant (in which the *frq* open reading frame was replaced by the *Escherichia coli* Hyg resistance-encoding gene [*hph*]) served as the positive control. Samples at DD16 were formaldehyde cross-linked and harvested by vacuum filtration prior to the addition of SDS lysis buffer and sonication. Quantitative PCR was performed using either the *C box*- or *pLRE*-specific oligonucleotides. The bars represent average values plotted as a percentage of the total, with error bars representing the standard errors of the means (SEMs) ($n = 3$). (B) Race tube assays of WT and WC-1 DBD KR invert strains. Race tubes were prepared as described in Materials and Methods.

were completely degraded by about 12 h after a light-to-dark transfer, and that subsequently, new FRQ peaked around DD16 (CT5) (Fig. 3B, upper blots, left). However, in the WC-$1^{\Delta918-925}$ mutant, although WC-1 and FRQ levels in the light were comparable to those in the WT, new FRQ was not detected at DD16 (CT5) or even later in the dark (Fig. 3B, upper blots, right), indicating that a loss of FRQ expression in the dark in the mutant caused the arrhythmic conidiation (see also reference 20). The FRQ level in WC-$1^{\Delta918-925-8A}$ in the dark was almost undetectable, but the FRQ level was higher in WC-1 bearing AAARKRRK (Fig. 3B, lower blots), suggesting that FRQ expression in the dark was positively correlated with the basic nature of this region in WC-1. In addition to driving FRQ expression in the dark, the WCC also drives transcription of light-responsive genes, including *frq*, *vvd*, *al-3*, and *sub-1* (see the introduction). In sharp contrast with the loss of dark activity in driving *frq* expression, upon light exposure, WC-$1^{\Delta918-925}$ was fully able to activate transcription of *frq*, *vvd*, *al-3*, and *sub-1*, as measured by reverse transcription-PCR (RT-PCR), with a level similar to that in the WT (data not shown), and reduced FRQ was confirmed by Western blotting expression after a 60-min light exposure (Fig. 3C). As expected, FRQ light induction mediated by WC-$1^{\Delta918-925}$ was through the *pLRE* element rather than the *C box* in the *frq* promoter (Fig. 3D). Overall, the basic region of amino acids 918 to 925 on WC-1 was not required for the light responses but was essential for driving *frq* expression and therefore for the *Neurospora* clock.

DNA binding at the *C box* is impaired in the WC-$1^{\Delta918-925}$ mutant. A normal nuclear localization but failure to promote transcription of *frq* by the WC-$1^{\Delta918-925}$ mutant led us to test whether it can bind the *frq* promoter in the dark. Because the observation of normal FRQ expression induced by light in *wc-$1^{\Delta918-925}$* indicated that the mutant protein was able to enter the nucleus and function normally in the light, ChIP experiments were conducted only using samples harvested at DD16 (CT5), when the WCC circadian activity in the dark peaks in WT. Using WC-2 antibody in the *wc-$1^{\Delta918-925}$* and *wc-$1^{\Delta ZnF}$* strains, the ChIP signal at the *C box* of the *frq* promoter was not above background (Fig. 4A), suggesting that the loss of FRQ expression in the mutant was mainly caused by the failure of DNA binding of WC-$1^{\Delta918-925}$.
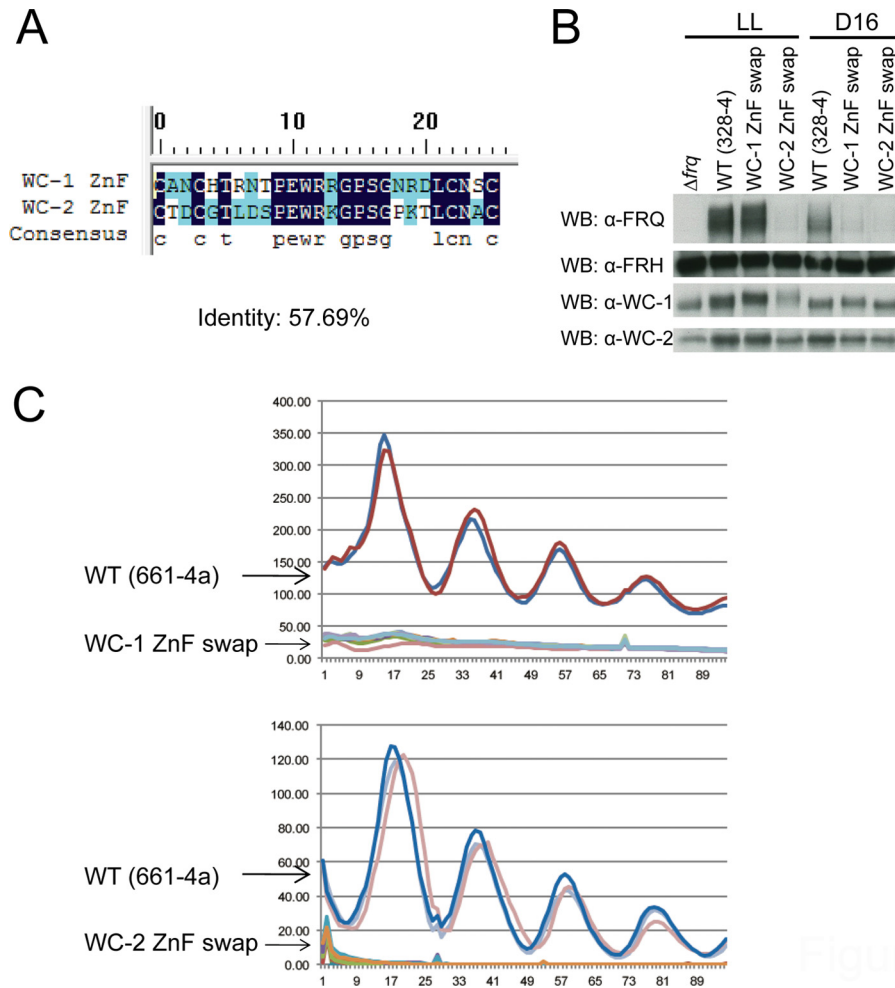
To determine whether charges or sequence specificity within the basic region mattered, we inverted the basic sequence (KKKR KRRK to RRRKRKKR) and found a WT circadian phenotype (Fig. 4B), indicating that the charges rather than the sequence of the basic region determined the phenotype. Based on the lack of DNA binding as indicated by the ChIP data, the basic region of amino acids 918 to 925 has been named DBD, for defective in binding DNA.

The ZnF and aa 499 to 504 of WC-2 are essential for WCC-mediated light induction of FRQ. Where is the functional DNA binding motif on the WCC in the light? The data presented above and in the work of Wang et al. (14) showed that the DBD and the ZnF on WC-1 were dispensable for the induction of the light-responsive genes tested. The WC-2 ZnF (aa 468 to 493), which is quite similar to that of WC-1 (58% identity as analyzed by DNA-MAN) (Fig. 5A and 6A), is a good candidate for mediating *frq* promoter association of the WCC in the light (16). In addition to the WC-2 ZnF, reminiscent of the role of the WC-1 DBD in DNA binding, we also searched the WC-2 amino acid sequence for potential NLSs and found two additional highly basic charged regions: aa 456 to 461(KKKKIK) and aa 499 to 504 (KKEKKK) (Fig. 5A). In the light, WC-$2^{\Delta499-504}$ and WC-$2^{\Delta ZnF}$ failed to bind to the *C box* of the *frq* promoter and FRQ expression was severely impaired (Fig. 5B); the DNA binding activity was significantly decreased, but FRQ expression was comparable to that of the WT in WC-$2^{\Delta456-461}$ (Fig. 5B), suggesting that the ZnF and aa 499 to 504 on WC-2 play a key role in transcription of *frq*, while aa 456 to 461 influence the WCC DNA binding capacity in the light. It was previously reported that the basic residues from aa 456 to 461 did not form a functional NLS (37), and the enrichment of the WCC in the nucleus in the *wc-$2^{\Delta499-504}$* strain disproved the possibility that aa 499 to 504 in WC-2 is a functional NLS despite its basic nature (Fig. 5C).

The ZnF, aa 456 to 461, and aa 499 to 504 of WC-2 are required for the circadian function of the WCC. Analysis of *C box*-driven luciferase in the *wc-$2^{\Delta ZnF}$*, *wc-$2^{\Delta456-461}$*, and *wc-$2^{\Delta499-504}$* mutants clearly showed all the three motifs of WC-2 were indispensable for the *Neurospora* clock (Fig. 5D). Consistent with the luciferase data, WC-$2^{\Delta ZnF}$, WC-$2^{\Delta456-461}$, and WC-$2^{\Delta499-504}$ failed

FIG 5 WC-2 ZnF (aa 468 to 493) and aa 499 to 504 are required for FRQ expression in the light. (A) Amino acid sequence of WC-2 highlighting aa 456 to 461, the ZnF, and aa 499 to 504, which are in bold and indicated by arrows (upper portion), and alignment of WC-2 ZnF domains from five related fungi (*Neurospora crassa*, *Gibberella zeae*, *Chaetomium globosum*, *Magnaporthe grisea*, and *Aspergillus nidulans*) (lower portion). aa 456 to 461, ZnF (aa 468 to 493), and aa 499 to 504 on WC-2s are boxed. WC-2 sequences were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/) and aligned using ClustalW2 (http://www.ebi .ac.uk/Tools/msa/clustalw2/). (B) FRQ, FRH, WC-1, and WC-2 levels and ChIP at the *frq* promoter using WC-2 antibody in the wc-$2^{\Delta456\text{-}461}$, wc-$2^{\Delta468\text{-}493}$, and wc-$2^{\Delta499\text{-}504}$ strains in the light. Asterisks indicate nonspecific protein bands. The bars represent average values taken as a percentage of the total, with error bars showing the SEMs ($n = 3$). The Δwc-2 strain was the negative control, while the WT (328-4) served as the positive control in the ChIP assay. (C) Western blot analyses showing that WC-$2^{\Delta456\text{-}461}$ is located in the nuclear fraction in the light. (D) *frq* transcription in WT (661-4a), wc-$2^{\Delta456\text{-}461}$, wc-$2^{\Delta468\text{-}493}$, and wc-$2^{\Delta499\text{-}504}$ strains was measured by the *frq C box* fused with codon-optimized firefly *luciferase* (transcriptional fusion). Strains cultured in race tube medium bearing luciferin were synchronized by growth in constant light for 2 days, followed by transfer to darkness. The luciferase signal, sampled every 30 min, was tracked for 6 days. Each strain was retested three or more times in the assay. (E) WC-2 DNA binding in the wc-$2^{\Delta456\text{-}461}$, wc-$2^{\Delta468\text{-}493}$, and wc-$2^{\Delta499\text{-}504}$ stains at DD16, when WCC binding to the *C box* peaks in WT was measured by ChIP assays using WC-2 antibody. The Δwc-1 Δwc-2 strain served as the negative control, while the Δfrq mutant, in which the WCC circadian activity is always on, was the positive control. Quantitative PCR was done using either the *C box*- or *pLRE*-specific primer sets (see Materials and Methods). The data are average values plotted as a percentage of the total, with error bars showing the SEMs ($n = 3$).

FIG 6 Identities of WC-1 and WC-2 ZnFs. (A) Sequence alignment of WC-1 and WC-2 ZnFs by DNAMAN. (B) Western blot analysis of FRQ, FRH, WC-1, and WC-2 levels in the light and at DD16 in the WT, *wc-1 ZnF swap*, and *wc-2 ZnF swap* strains. (C) *frq* transcription was measured using the *C box*-driven codon-optimized *luciferase* assays in the *wc-1 ZnF swap* (top) and *wc-2 ZnF swap* (bottom) strains. The luciferase signal was taken every 30 min for 4 days. Each strain was retested three times.

to bind to the *C box* of the *frq* promoter at DD16 (Fig. 5E). Together, these data suggested that the WC-2 ZnF along with aa 456 to 461 and aa 499 to 504 function as the DNA binding motif in promoting transcription of *frq*. To further probe these two mutants, the WC-1 ZnF swap (in which the ZnF of WC-2 replaced that of WC-1 such that WC-1 and WC-2 have the same WC-2 ZnF) and WC-2 ZnF swap (in which the ZnF of WC-1 replaced that of WC-2) mutants were examined (Fig. 6A). In the light, the WC-1 ZnF swap mutant had a WT level of FRQ expression, while FRQ expression in the WC-2 ZnF swap mutant disappeared (Fig. 6B), further indicating that the ZnF of WC-2 determines FRQ expression in the light. At DD16 (CT5), FRQ expression was impaired in both the WC-1 ZnF swap and WC-2 ZnF swap mutants (Fig. 6B), which suggests that both ZnFs play a role in driving *frq* expression in a circadian cycle. Consistent with the protein data, the *C box* promoter activity in the luciferase assay was extremely low and arrhythmic in both of the mutants compared with that of the WT (Fig. 6C).

The WC-1 DBD motif is additionally required for FFC interaction. Newly synthesized FRQ interacts with WCC and is

thought to recruit kinases to repress WCC transcriptional activity through phosphorylation of WC-1 (10, 11, 29). However, the FFC-interacting region within the WCC was previously unknown. To investigate this further, WC-1–FRQ interaction was investigated by V5 immunoprecipitation using the WC-1 deletion strains described in Fig. 1. Samples from cultures grown in constant light were used to study WCC-FFC interaction for three reasons. First, previous research studying WCC-FFC interaction similarly used samples from light-grown cultures. Second, in samples from constant light, no issues can arise from cultures being out of circadian phase or differentially modified. Finally, there was no detectable difference in our hands by Western blotting among samples cultured under different light and dark conditions (data not shown). Of the mutants tested, only WC-1$^{\Delta DBD}$ failed altogether to bind FRQ (Fig. 7A). Indeed, interactions between the WCC and either FRQ or FRH as the FFC, or CKI, were not detected in either WC-1$^{\Delta DBD}$ or WC-1$^{\Delta DBD-8A}$ and decreased in WC-1$^{\Delta ZnF}$, WC-1^{AAARKRRK}, and WC-1^{KKKRKAAK}, while interaction was similar to that of the WT in the WC-1 DBD KR invert mutant (Fig. 7B). FRQ-FRH interaction is not influenced by the
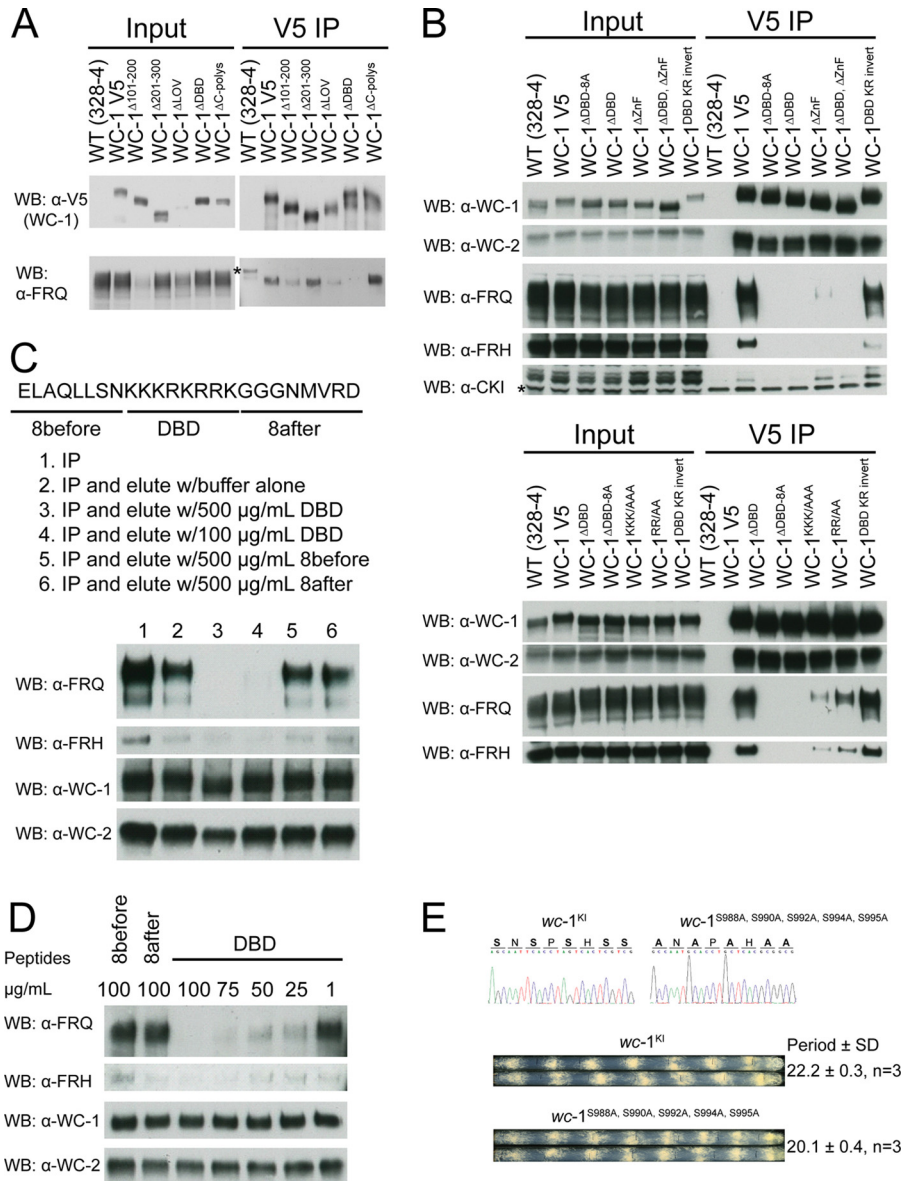
FIG 7 The DBD motif in WC-1 is essential for FFC interaction. (A) Immunoprecipitation assays using V5 antibody showing the interaction of WC-1 and FRQ in the WC-1 mutants. The *Neurospora* protein extracts were either assayed directly with Western blot analyses (left blot) or immunoprecipitated with V5 antibody (right blot). All *wc-1* constructs indicated were transformed into the *wc-1* native locus of the Δ*wc-1* strain. WT (328-4) and WC-1 V5 serve as the negative and positive controls, respectively. The immunoprecipitation assays were done as described in Materials and Methods. (B) Immunoprecipitation assays using V5 antibody showing that the DBD motif on WC-1 is required for the FFC-WCC interaction. (C) WCC-FFC interaction can be disrupted by the WC-1 DBD peptide *in vitro*. The WCC (lane 1) was isolated from WC-1 V5 lysate cultured in the light using V5 antibody as described in Materials and Methods. The washed beads were incubated with protein lysis buffer alone (lane 2), 500 μg/ml of DBD peptide (lane 3), 100 μg/ml of DBD peptide (lane 4), 500 μg/ml of 8before peptide (lane 5), or 500 μg/ml of 8after peptide (lane 6) at 4°C for 1 h, with rotating. After removal of the supernatant, SDS sample buffer was added to the beads prior to elution by boiling, and proteins were assayed by Western blotting analyses. (D) Similar to the case for panel C, WCC-FFC interaction was studied in a titration assay using the WC-1 DBD peptide at concentrations of 100, 75, 50, 25, and 1 μg/ml. (E) Race tube assays of *wc-1*[KI] (bearing a WT copy of WC-1) and *wc-1*[S988A, S990A, S992A, S994A, S995A] strains (period [in hours] ± 1 standard deviation; n, number of race tubes). Race tube assays were performed as described in Materials and Methods. The five point mutations in the *wc-1*[S988A, S990A, S992A, S994A, S995A] strain were confirmed by sequencing genomic DNA as shown in the upper portion.

presence or absence of the WCC (7), suggesting that the DBD motif is not involved in the interaction between FRQ and FRH. To test the specificity of the DBD motif in FFC interaction, WCC-FFC was isolated by immunoprecipitation first and then the interaction was disrupted by the addition of *in vitro*-synthesized DBD peptide; the interaction was not influenced by either of the control

peptides 8before (an *in vitro*-made peptide, ELAQLLSN, the 8 amino acids before the DBD motif) and 8after (the peptide GGG NMVRD, the 8 amino acids after the DBD motif) (Fig. 7C), suggesting that the WCC-FFC interaction was directly through the DBD motif on WC-1. A titration assay using the DBD peptide at different concentrations further showed that the peptide dis-

sociated FFC from the WCC but did not impact the interaction between WC-1 and WC-2 (Fig. 7D). In addition, WCC-FFC interaction was seen in the WC-2$^{\Delta456\text{-}461}$ strain (data not shown). It has been reported that the FFC represses the WCC circadian activity through mediating phosphorylation of WC-1 at five sites (S988, S990, S992, S994, and S995) located downstream of the ZnF and that mutating the five sites to alanines results in arrhythmic conidiation in the dark (10, 38). However, the *wc-1*$^{S988A, S990A, S992A, S994A, S995A}$ mutant showed a clear and robust rhythm, albeit with a 2-h-shorter period than for the WT in our hands (Fig. 7E), suggesting that phosphorylation of the five sites of WC-1 is not sufficient to repress the WCC.

DISCUSSION

WC-1 is a protein of central importance to *Neurospora*. As the principal photoreceptor, it initiates light responses for the organism by detecting the initial signal and then acting as a light-induced transcription factor to initiate the signaling cascade that brings about the organism's response to light. In the dark, the same WC-1 is a principal component of the complex that makes up the positive arm of the transcription-translation feedback loop comprising the clock. In this study, we used reverse genetics to dissect the structure and function of the WCC in general and of WC-1 in particular in both the light and clock systems. Regions of WC-1 required for light and dark DNA binding were identified, and surprising diversity in regions needed for DNA binding was revealed. Specifically, by complementing and expanding on previous published and unpublished work, we found that whereas light DNA binding by WCC requires only the ZnF and a proximal basic region within WC-2, dark circadian functions of the WCC require ZnFs and nearby basic regions of both WC-1 and WC-2. This implies that there are major quaternary structural changes needed to allow WCC to function alternatively as a circadian transcription factor or a light-sensing transcription factor.

A second unexpected finding to emerge from this work is the nonstraightforward nature of the process by which the WCC finds its way to the nucleus, wherein it must act to affect transcription. The data presented in this study clearly show that WC-1 does not need a typical NLS to be imported into the nucleus, nor does WC-2, based on previous analyses (20, 39). There are precedents for proteins being imported into the nucleus even without a typical NLS, although these generally require an interacting partner (40). For example, *Saccharomyces cerevisiae* Sof1p can enter the nucleus by piggybacking on Nop1p, which bridges Sof1p to the transporter, Kap121p (41); interleukin 5 (IL-5) can piggyback its alpha- and beta-receptor subunits to the nucleus *in vitro* (42); and hepatic glucokinase (GK) lacking an NLS enters the nucleus by interacting with the GK regulatory protein (GKRP) (43). In the case of WC-1, known interactors (WC-2, FRQ, or FRH) (Fig. 2) do not influence nuclear localization, and interaction with a single unknown protein cannot explain why proteins containing either aa 1 to 579 or 580 to 1167, each lacking a typical NLS, can be transported into the nucleus. Among clock proteins, FRQ has a well-characterized NLS (18), and in contrast to the WC-1 DBD motif, BMAL1, the mouse analog of WC-1, contains a functional bipartite NLS that is required for the nuclear entry that must precede transactivation of *per* genes (44). Also, mammalian mCRY2 has a

functional NLS for its nuclear localization through importin α/β (45).

Lastly, it is worth considering why evolution has yielded so much structural complexity involved with the seemingly straightforward processes of nuclear import and DNA binding by the WCC. The answer may lie in the need to keep distinct the different processes mediated by WC-1. In the light, WC-1 is the principal photoreceptor for *Neurospora*, and in the dark, WC-1 is the core transcriptional activator in the circadian feedback loop. Yet not all rapidly light-induced proteins are clock regulated, and not all clock-controlled genes are light induced. We previously showed that a part of this regulation lies in the use of different *cis*-regulatory elements in *frq*: the *pLRE*, which mediates light regulation, is right next to the transcription start site (TSS) and can act directly to aid in assembly of the transcriptional preinitiation complex, whereas the *Clock box*, which mediates transcriptional activation in a circadian context, is more than a kilobase distal from the TSS and requires the action of the SWI/SNF complex, presumably to bend DNA to bring the WCC bound to the *Clock box* in the dark into the vicinity of the TSS (14). A second part of this differential regulation is found in the quaternary structural changes observed in the WCC upon light induction and binding to the *pLRE* in which two WC-1 molecules interact with one WC-2 at the *pLRE* (5, 35); these are changes not seen at the *Clock box*. Based on the data presented here, we infer that a third part of this equation is the use of different DNA binding elements in the two complexes: light activation by the WCC requires only the WC-2 DNA binding ZnF and no part of the WC-1 DNA binding regions, whereas DNA binding to the *Clock box* required DNA binding regions from both WC-1 and WC-2. Through use of these mechanisms, the cell can use the same two proteins, WC-1 and WC-2, and a single photoreceptor, WC-1, to mediate responses in both the light and the dark.

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