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Physical association of the WC-1 photoreceptor and the histone acetyltransferase NGF-1 is required for blue light signal transduction in *Neurospora crassa*

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ABSTRACT In *Neurospora crassa* and other filamentous fungi, light-dependent-specific phenomena are regulated by transcription factors WC-1 and WC-2. In addition to its transcriptional activity, WC-1 is able to directly sense light stimuli through a LOV sensor domain. Its location in the nucleus and heterodimerization with WC-2, together with the presence of a zinc-finger DNA-binding domain and an environmental sensor domain, all resemble the functional evolutionary architecture adopted by vertebrate nuclear receptors (NRs). Here we describe a scenario in which WC-1 represents a functional orthologue of NRs and acts through association with the chromatin-modifying coactivator NGF-1, which encodes a homologue of the yeast Gcn5p acetyltransferase. To support this view, we show a direct association between WC-1 and NGF-1 that depends on a WC-1 region containing a conserved functional LXXLL motif, a signature previously described as being an exclusive feature of NR/coactivator interaction. Our data suggest that a WC-1/NGF-1 complex is preassembled in the dark on light-inducible promoters and that, after exposure to light stimulation, NGF-1-associated HAT activity leads to histone H3 acetylation and transcriptional activation. Finally, we provide evidence for a NGF-1-independent acetylated form of WC-1. Overall our data indicate that *Neurospora* and higher eukaryotes share a common mechanism for the signal transduction of environmental stimuli.

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INTRODUCTION

All organisms need to respond to rapid changes in their environment. During evolution various mechanisms have been selected to enable an organism to rapidly sense and respond to environmental light cues.

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Abbreviations used: NGF-1, *Neurospora* GCN Five-1; WCC, white collar complex.

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In *Neurospora*, one of the most important extracellular cues is light, and a proper response to this stimulus is crucial for its survival. Light-signal transduction in *Neurospora* is mediated by the White Collar complex (WCC), a heterodimer of WC-1 (Ballario et al., 1996, 1998) and WC-2 (Linden and Macino, 1997), proteins that form a photoreponsive transcription factor complex (Crosthwaite et al., 1997; Talora et al., 1999; Cheng et al., 2002). WC-1 is both a photoreceptor and a zinc-finger DNA-binding protein, whereas its partner, WC-2, contains transcriptional activation domains essential for the WCC to function properly (He et al., 2002; Cheng et al., 2003). When WCC is activated upon light stimulus, a transient expression of light-responsive target genes ensues (Baima et al., 1991; Ruger-Herreros et al., 2011).

A whole-genome microarray of *Neurospora* revealed that ~5.6% of transcript amounts rapidly increased after stimulation with a light

pulse (Chen *et al.*, 2009). Evidence suggests that such light-mediated transcription depends on White Collar proteins binding to responsive elements within the promoters of target genes (Carattoli *et al.*, 1994; Froehlich *et al.*, 2002; Liu *et al.*, 2003).

Neurospora light signaling also involves chromatin modifications. Histone H3-K14 at light-inducible promoters (i.e., *albino-3* and *vivid*) transiently increases its acetylation levels after photoinduction (Grimaldi *et al.*, 2006). This histone modification has been shown to be under the control of the *Neurospora* histone acetyltransferase (HAT) *Neurospora* Gcn Five-1 (NGF-1), homologous to *Saccharomyces cerevisiae* HAT Gcn5p. Several of these features described for *Neurospora* light signaling resemble the evolutionary architecture adopted by vertebrate nuclear receptors (NRs). In higher eukaryotes, for example, the signal transduction of several stimuli is orchestrated by the NR superfamily of transcription factors (Gronemeyer *et al.*, 2004). Canonical NRs seem to have appeared with the metazoans (Thornton *et al.*, 2003).

NRs change their transcriptional activity upon the binding of small molecules such as hormones (Bain *et al.*, 2007). Generally, NRs heterodimerize in the nucleus, and their association with coactivators or corepressors is essential for their functioning. NR and accessory proteins with chromatin-modifier activity are recruited to the promoter region of target genes (McDonnell *et al.*, 1991; Wiench *et al.*, 2011).

Among the coactivators, Gcn5-related *N*-acetyltransferase (GNAT) proteins (PCAF and GCN5) contribute to transcriptional activation in several physiological processes through their HAT enzymatic activity (Aoyagi and Archer, 2008).

A special signature represented by a consensus box (LXXLL motif, also called NR box, where L is a leucine and X is any amino acid) has been associated with coactivator–NR binding. The NR box can be present in single or multiple copies, and it can be found in the NR or its coregulator or both proteins (Savkur and Burris, 2004). Furthermore, in some cases the GNAT proteins also acetylate NRs, contributing to their response to signaling molecules (Wang *et al.*, 2011).

Here we investigate the possibility that WC-1 exemplifies a functional orthologue of vertebrate NRs.

RESULTS

WC-1 binds to NGF-1 in dark and light conditions

Our previous data support a model in which NGF1 could be part of the WCC. We therefore wanted to see whether NGF-1 and WC-1 proteins would be able to interact *in vivo* and whether this interaction could be modulated by light.

Protein extracts were prepared from a *Neurospora* strain expressing a Myc-tag version of WC-1 grown for 3 d in the dark, exposed to saturating white light for 5 min, and then returned to the dark for 25 min before harvesting (LP 30 min). WC-1 was then immunoprecipitated (IP) with an anti-Myc antibody, and the presence of NGF-1 in the IP samples was analyzed by Western blot with a commercial human GCN5 antibody. This analysis confirmed the *in vivo* association of NGF-1 with WC-1; of note, the two proteins coimmunoprecipitated in the dark (D) and after exposure to a light pulse (LP; Figure 1A, left). We also immunoprecipitated a wild-type (WT) strain (74A) but observed no signal with either the Myc or hGCN5 antibodies, confirming the specificity of the immunoprecipitation. Because we used a heterologous GCN5 (human) antibody, we tested this against a total *ngf-1* extract to confirm lack of cross-hybridization. As expected, the hGCN5 revealed a signal only in the WT strain or hGCN5 purified protein and not in the *ngf-1* mutant strain, confirming the specificity of the data (Figure 1B).

In addition, the level of WC-1/NGF-1 binding was apparently similar in dark and light-pulse conditions (Figure 1A, right). This suggests the existence in the dark of a preassembled WC-1/NGF-1 complex on the promoters of the WCC target genes. To explore this possibility, we evaluated the recruiting of NGF-1 to the light-responsive region (LRR) of the WC-1 target gene *albino-3* (*al-3*) in both D and LP conditions (Grimaldi *et al.*, 2006). As revealed by chromatin immunoprecipitation (ChIP) with anti-hGCN5 antibody and subsequent PCR amplification of the *al-3* LRR, a similar profile in the D and LP conditions was obtained (Figure 1C).

Remarkably, chromatin immunoprecipitation with anti-H3 and anti-AcH3 antibodies performed in the same samples showed a twofold increase in the acetylation of histone H3 at *al-3* LRR in the LP condition (Figure 1D). From this we inferred that it is not the WC-1/NGF-1 interaction per se present in D and L conditions that induces histone H3 acetylation, according to our previous report (Grimaldi *et al.*, 2006).

Overall these results support a scenario in which WCC-mediated gene activation is achieved by light-dependent modification of HAT activity of the NGF-1/WC-1 complex previously assembled in the dark on the target promoters.

This view is consistent with fast transcriptional response to light irradiation (He and Liu, 2005).

The C-terminal region of WC-1 dictates direct interaction with histone acetyltransferase

The *in vivo* interaction between WC-1 and NGF-1 (Figure 1A) indicates the presence in WC-1 of an as-yet-uncharacterized functional domain involved in NGF-1 binding. To identify this domain, we tested the ability of different recombinant WC-1 versions fused with glutathione *S*-transferase (GST) to interact with an S^{35} -radiolabeled yeast Gcn5p in pull-down assays. GNATs are highly conserved in evolution; Gcn5p shares strong homology with NGF-1, especially in the catalytic HAT domain (residues 107–219; 78% identity) and the bromodomain (residues 316–401; 63% identity; Grimaldi *et al.*, 2006). We used the yeast Gcn5p because the full-length NGF-1 product is not well characterized, thus raising the possibility of missing some regions important for its function.

One of the recombinant WC-1 products is a long version of WC-1 (WC-1 lv, amino acids [aa] 354–1000) that includes the LOV domain, the two PAS domains, and the zinc-finger (ZnF) domain (Figure 2A). The other two WC-1 versions lack the C-terminal region containing the ZnF domain (WC-1 sv, aa 354–838) and the N-terminal region containing the LOV and the PAS domains (WC-1 mini, aa 838–1000), respectively (Figure 2A).

When analyzed in the pull-down assays, the WC-1 lv was able to retain 30% of the input S^{35} -radiolabeled Gcn5p (Figure 2B). In marked contrast, the WC-1 version lacking the C-terminal region (WC-1 sv) retained only an amount similar to that obtained with the GST protein used as a negative control (Figure 2B). Of note, the WC-1 mini product expressing only the C-terminal domain preserved its ability to retain the labeled protein (Figure 2B).

These results identify the WC-1 portion between 838 and 1000 aa as the region directly involved in the association of WC-1 with HATs.

To further confirm that the C-terminal region of WC-1 dictates its interaction with Gcn5p also *in vivo*, we used a mutant of the *Neurospora* deletion mutant collection provided by Y. Liu (Cheng *et al.*, 2003). This collection contains a mutant strain that express a C-terminal-deleted, Myc-tagged WC-1 (WC-1 Myc Nru1 [WMN]) corresponding to the WC-1 short-version recombinant protein, which was unable to bind Gcn5p in our pull-down assays (Figure 2C, top).

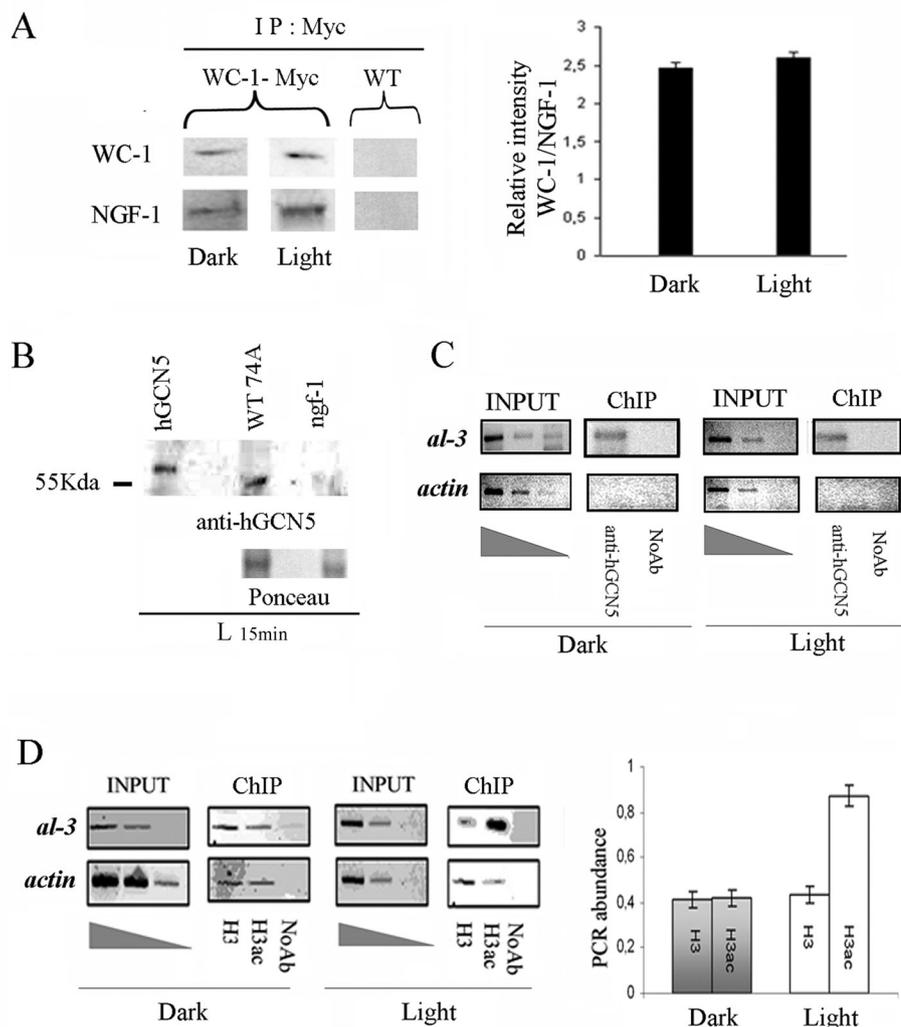


FIGURE 1: NGF-1 physically interacts with WC-1 in dark and light conditions. (A) Myc WC-1 was immunoprecipitated (IP) using a commercial anti-Myc antibody from 400 μ g of total protein extracts from *Neurospora* mycelia grown for 3 d in the dark before harvesting (Dark) or exposed to saturating white light for 5 min and then returned to the dark for 20 min before harvesting (Light 25 min). Precipitated WC-1 and coprecipitated NGF-1 were detected with anti-Myc and anti-hGCN5, respectively. As control of the specificity of the signal obtained, the same experiment was performed on the WT strain without the Myc epitope (left). Quantification of the ratio between the relative optical density of WC-1 and NFG-1 bands from three independent experiments under Dark and Light conditions (right). The data are the mean \pm SEM from three independent experiments. (B) Western blot with anti-hGCN5 against hGCN5 purified protein, *Neurospora* WT, and *ngf-1* strains confirms the absence of cross-hybridization of the heterologous antibody. Ponceau staining was used to check the presence of similar protein amounts on the membrane. (C) ChIP assay on *Neurospora* mycelia grown for 3 d in the dark before harvesting (Dark) or exposed to saturating white light for 5 min and then returned to the dark for 20 min before harvesting (Light 25 min). DNA immunoprecipitated with anti-hGCN5 antibody was amplified for the *al-3* light-responsive promoter region. A sequence for *actin* was used as negative control. INPUT, PCR on chromatin samples before immunoprecipitation. ChIP, PCR from anti-hGCN5 IP samples. Noab, PCR from samples without antibody. (D) ChIP analysis as described in C was performed with anti-histone H3 (H3) and anti-acetylated histone H3 (H3ac; left). Quantification of the PCR abundance for the *al-3* promoter was calculated as described in *Materials and Methods* (right). The data are the mean \pm SEM from three independent experiments.

Coimmunoprecipitation assays on protein samples from the WMN strain clearly showed that the WC-1 C-terminal region was required for association with NGF-1 *in vivo* (Figure 2C, bottom). In fact, the mutated WC-1 protein was unable to coimmunoprecipitate NGF-1

starting from two different input concentrations (800 ng; Figure 2C, lane A; and 400 ng; Figure 2C, lane B), although the amount of the WC-1 protein was high, as revealed by the anti-Myc antibody.

WC-1 C-terminal region is required for light-induced H3 acetylation

The C-terminal-deleted WC-1 protein failed to interact with NGF-1; accordingly, this HAT should not be recruited on light-induced promoters in the WMN mutant strain, and a light pulse should not increase H3 acetylation levels. To test this possibility, we performed ChIP assays with anti-H3 and anti-acH3 on the WMN strain kept in the dark or after exposure to a pulse of 5 min of light followed by 25 min of dark. The negligible differences in the H3 acetylation levels on the *al-3* LRR observed between dark and light-pulse conditions confirmed that the WC-1/NGF-1 association is required for light-induced HAT activity (Figure 3A).

To further assess whether the lack of WC-1/NGF-1 interaction and light-induced H3 acetylation impairs the activation of carotenoid biosynthesis and conidia formation, we evaluated the *al-3* transcription levels and the carotenoid production in the WMN and the isogenic wild-type strains.

Northern blot analysis of the two strains with a specific probe against *al-3* mRNA showed that light-induced activation of the *al-3* transcript is completely abolished in the WMN strain (Figure 3B). In addition, progressive dilutions of conidia from the WT and WMN strains spotted on a nitrocellulose filter showed a dramatic inhibition of conidiation in the strain harboring the WC-1-deleted mutant (Figure 3C, top). Cell count by hemocytometer revealed a 10-fold reduction of conidia in the WMN strain (Figure 3C, bottom). Overall these results indicate that the proper formation of a WC-1/NGF-1 complex on light-induced promoters is required for transcriptional responsiveness to light.

WC-1 is a fungal nuclear receptor-like transcription factor with a functional vertebrate NR box

Key to adaptation and survival has been the evolution of mechanisms that enable organisms to sense and rapidly respond to sudden environmental changes. In metazoans, the nuclear receptor superfamily of transcription factors comprises direct one-

step signaling sensors that typically bind and respond to small molecules to regulate gene expression programs governing numerous cellular processes (for review see van de Wijngaert et al., 2011).

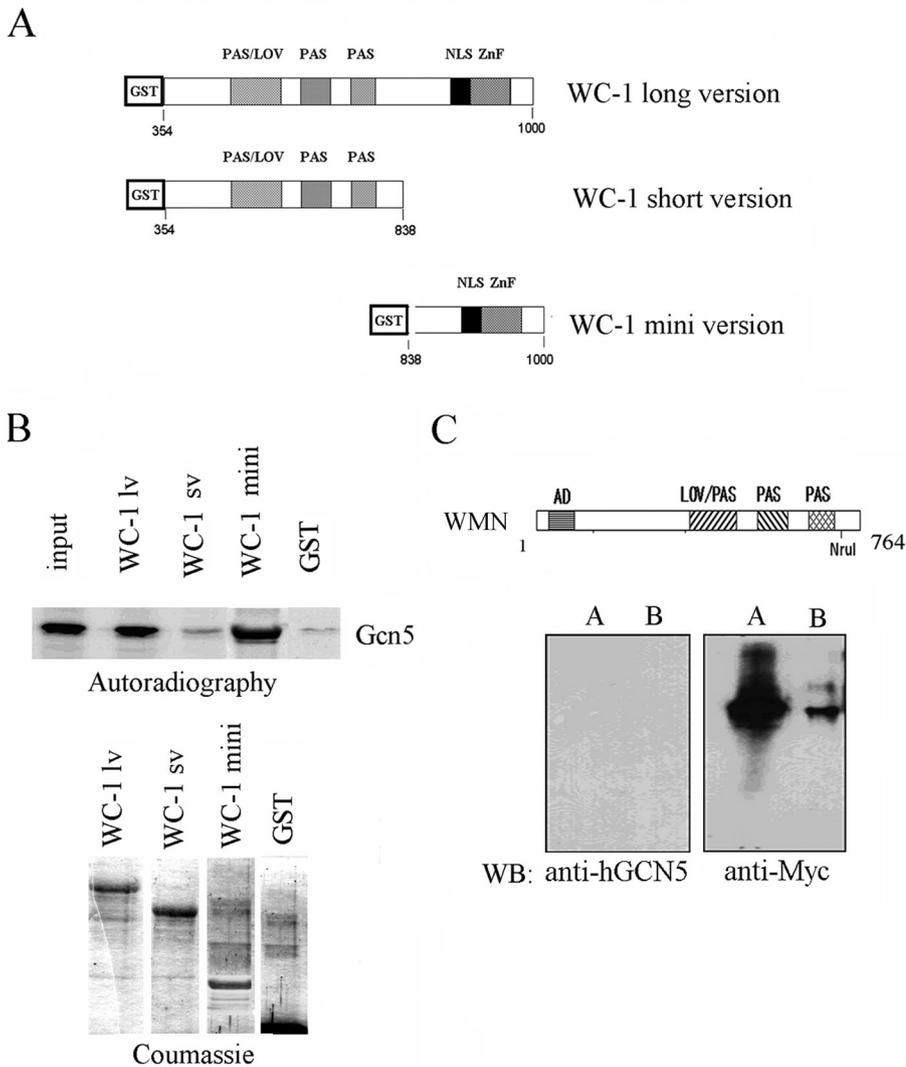


FIGURE 2: WC-1 needs the C-terminal region between 838 and 1000 aa to bind Gcn5p in vitro and in vivo. (A) Schematic representation of the functional domains of the recombinant proteins used in the pull-down experiments: GST, glutathione *S*-transferase; LOV, light sensor domain; NLS, nuclear localization signal; PAS, protein-protein interaction domain; ZnF, zinc-finger DNA-binding domain. The numbers refer to the amino acid residues of WC-1 in relation to the initial methionine in the full-length protein. (B) Pull-down experiments using the same recombinant proteins as shown in A and [³⁵S]Gcn5-radiolabeled protein. A representative autoradiography of the [³⁵S]Gcn5 protein retained by the GST fused proteins is shown (top). Input corresponds to 30% of total incorporation of [³⁵S]methionine in the GCN5. Coomassie staining of the SDS-PAGE illustrates the amount of the recombinant WC-1 proteins used for the pull-down experiment shown in the top (bottom). (C) Scheme showing the Nru1 deletion in the Myc-tagged WC-1 protein (WMN; Cheng *et al.*, 2003). Deleted WC-1 (aa 1–764) was immunoprecipitated with an anti-Myc antibody from 800 µg (lane A) and 400 µg (lane B) of total protein extract (bottom). IP samples were analyzed by Western blot with the indicated antibodies.

Although fungi and plants do not exhibit nuclear receptor orthologues, bioinformatics sequence analysis has shown that the transcription activity of members of the fungal zinc-finger transcription factor family is also modulated by small molecules, potentially representing environmental cues (for review see Näär and Thakur, 2009).

Although a photon cannot be considered a physical compound like nutrients or cellular metabolites, light constitutes an important environmental stimulus that can modulate the activity of the zinc-finger transcription factor WC-1 through a specific domain.

When the LOV domain is viewed as a ligand-binding domain (LBD) for light, extraordinary similarities emerge between the structural organization of metazoan nuclear receptors and *Neurospora* WC-1 (Figure 4A; Schaufele *et al.*, 2005). Indeed, both transcriptional factors present N- and C-terminal domains involved in promoting gene activation, a zinc-finger DNA-binding domain and an LBD domain accountable for response to stimuli (Figure 4A).

Of note, the molecular mechanism of action we describe here for WC-1 is also used by several NR members. It involves the direct interaction with chromatin-remodeling coregulators to execute their transcriptional activity (Green and Han, 2011). In vertebrates, several NRs, as well as several coactivators, harbor a leucine-X-X-leucine-leucine (LXXLL, where X is any amino acid) motif, also known as an NR box, which regulates the association of the transcription factors with the coregulators (Savkur and Burris, 2004; Jiang *et al.*, 2010).

Although NR-like analogues in nonvertebrate organisms have been shown to lack the LXXLL motif, our attention was attracted by the presence of two LXXLL sequences (LLDLL, aa 727–732, and LAQLL, aa 911–916) within the primary sequence of WC-1. Figure 4B shows the alignment of most WC-1- or WC-1-like identified proteins; ~50% of WC proteins examined contained the canonical LXXLL sequence and were members of Sordariomycetes, the largest class of Ascomycetes. Another 30% of WC-1 proteins were characterized by conservative substitution of polar neutral amino acids (leucine substituted by alanine or valine). The conserved LAQLL sequence is located in the C-terminal region of the WC-1 identified here to dictate its interaction with coregulator NGF-1. In contrast with the second conserved LXXLL, the first putative motif (LLDLL), located in the WC-1 truncated version lacking the NGF-1-interacting region (Figure 2A), appears not to be conserved (Figure 4B) and was not further investigated.

To assess whether the WC-1 LAQLL conserved region could work as an NR box mediating the association of WC-1 with its

coactivator, we constructed a mutated version of the WC-1 mini protein used in the in vitro pull-down assay shown in Figure 2, where the two leucines were substituted by two alanines (Figure 4C, bottom). GST pull-down assays demonstrated that mutation of the LXXLL motif resulted in a 45% decrease in the ability of WC-1 mini protein to retain labeled Gcn5 protein (Figure 4D), clearly indicating that the integrity of the LXXLL motif identified in *Neurospora* WC-1 is required for maximal association with the coactivator NGF-1 and confirming previous observations (Fan *et al.*, 2004).

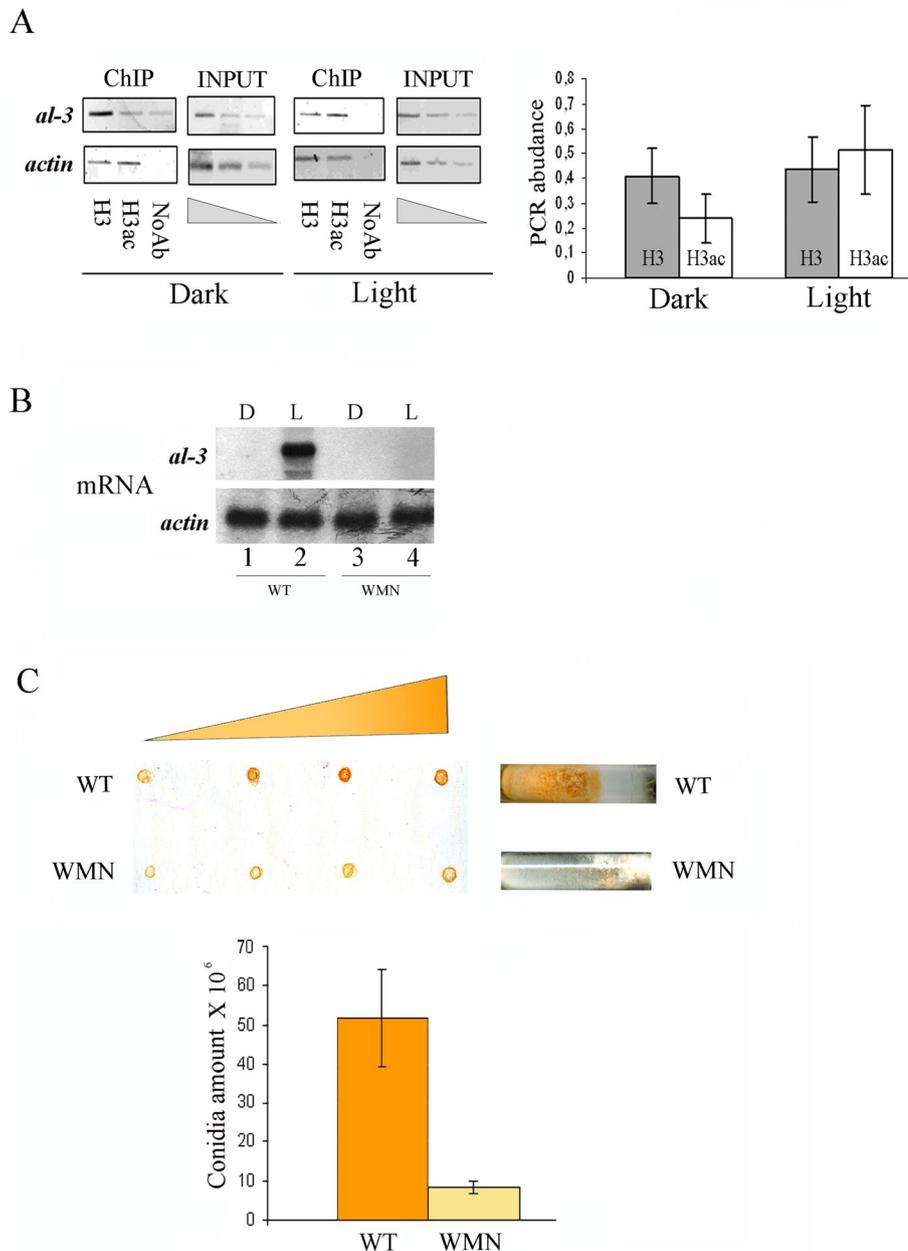


FIGURE 3: Impairment of light-mediated H3 acetylation in the WMN strain. (A) ChIP assay on *Neurospora* WMN mycelia grown for 3 d in the dark before harvesting (Dark) or exposed to saturating white light for 5 min and then returned to the dark for 20 min before harvesting (Light 25 min). DNA immunoprecipitated with the anti-histone H3 (H3) and anti-acetyl histone H3 (H3ac) antibodies was amplified for the *al-3* light-responsive promoter region. A sequence for *actin* was used as negative control. INPUT, PCR on chromatin samples before immunoprecipitation. ChIP, PCR from anti-hGCN5 IP samples. Noab, PCR from samples without antibody. Quantification of the PCR abundance for the *al-3* promoter was calculated as described in *Materials and Methods* (right). The data are the mean \pm SEM from three independent experiments. (B) Northern blot analysis of *al-3* mRNA in *Neurospora* WT and WMN strains kept in the dark (Dark) or after photoinduction (Light 25 min). The membrane was rehybridized using a probe against the *actin* mRNA as loading control (bottom). (C) WT and WMN strains were grown in slant for 4 d in the dark, illuminated for 5 h, and then kept in the dark for 8 h at 4°C to accumulate carotenoids. Increasing amounts of the conidia resuspended in water (5, 10, 20, and 25 μ l) were spotted on a nitrocellulose filter to observe the increment of the red color corresponding to carotenoid content (top). Conidia count in WT and WMN was measured using a Burkler cell counter (bottom). The data are the mean \pm SEM from three independent experiments.

To our knowledge, the box LAQLL is the first example of a nonvertebrate LXXLL motif mediating coactivator/NR-like interaction.

WC-1 is an acetylated protein

Because HATs acetylate many transcription factors and regulators, including NRs (Sternier and Berger, 2000; Roth et al., 2001; Wang et al., 2011), we decided to investigate whether WC-1 could be acetylated by the interacting acetyltransferase NGF-1.

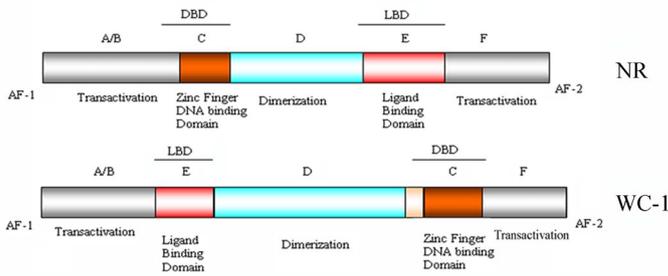
We first evaluated the ability of a recombinant WC-1 protein to be a substrate for an in vitro acetylation assay. Different amounts of the recombinant long-version GST-WC-1 were tested as a substrate for acetylation in the presence of human GCN5 acetyltransferase. As expected (see technical data sheet hGCN5 n.cat. 31204; www.activemotif.com/catalog/details/31204/gen5-active), hGCN5 was autoacetylated in each sample (Figure 5A, left). In addition, recombinant WC-1 was acetylated in this assay (Figure 5A). Addition of the HAT inhibitor anacardic acid (34 μ M) decreased the WC-1 acetylated band, confirming that the acetylated signal corresponding to WC-1 was produced in the in vitro reaction (Figure 5A, right).

To rule out the possibility that the observed WC-1 acetylation was an artifact of our in vitro acetylation assay conditions, we further used a different assay based on fluorescence emission (Trievel et al., 2000), which, in the presence of recombinant WC-1 as substrate, showed a significantly higher degree of acetylation with respect to background (Figure 5B). Overall these results indicate that WC-1 can be a substrate for HAT enzymatic activity.

To further evaluate whether WC-1 can be acetylated in vivo by NGF-1, we performed Western blot analysis on WT and *ngf-1RIP* mutant strains exposed to different time intervals of light stimulus (from 0 to 60 min; Figure 5C). As reported previously (Lee et al., 2000), the profile of WC-1 expression showed a transient kinetic activation in the WT strain. No signal was detected in the protein extract from a *wc-1* null strain (*wc-1^{KO}*), confirming the specificity of the WC-1 antibody (Figure 5C). When the same filter was tested with an antibody against anti-Pan acetylated-lysine, a band corresponding to WC-1 was detected for all time intervals in the WT but not in the *wc-1^{KO}* sample (Figure 5C).

In the *ngf-1RIP* mutant strain, the WC-1 protein signal was lower than that of the WT strain (Figure 5C), further confirming the role of NGF-1 in the activation of *wc-1* expression (Grimaldi et al., 2006). Nevertheless, the band revealed by the anti-acetylated lysine antibody was also very faintly detected in the *ngf-1RIP* mutant strain (Figure 5C), indicating that NGF-1 could not be entirely responsible for WC-1 acetylation in vivo and other

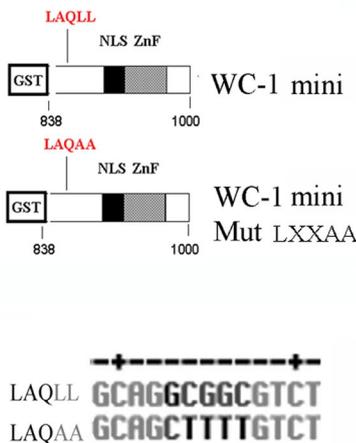
A



B

	881	900	1101	1120
<i>C. neoformans</i>	QAEELIGRNL	FSLPFGGQNT	TSLSQIGGI	GF.....
<i>C. gattii</i>	ITFGIDKVLG	YHAEELIGKS	NIAALKEKRA	GKAKRRK...
<i>N. crassa</i>	VSSNVRSLLD	LLPENLQGTS	ELAQLLSNKK	KRKRKRG...
<i>S. macrospora</i>	VSSNVRSLLD	LLPENLQGTS	ELAQLLSNKK	KRKRKRG...
<i>P. anserina</i>	VSSNVRSLLD	LERKDLGTS	ELAQLLSNRK	KRKRKRG...
<i>V. albo-atrum</i>	VSSNVRSLLD	LQPNDLIGTS	ELGGLSNKK	KRKRKRG...
<i>Colletotrichum</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>G. graminicola</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>N. haematococca</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>F. oxysporum</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>G. fujikuroi</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>G. zeae</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>H. jecorina</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>T. atroviride</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>M. anisopliae</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>M. acridum</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>M. oryzae</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>T. terrestris</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>C. globosum</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>M. thermophila</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>B. fuckeliana</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>S. sclerotiorum</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>C. zeae-maydis</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>M. graminicola</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>P. avenaria</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>C. miyabeanus</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>L. maculans</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>P. tritici-repentis</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>T. borchii</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>T. melanosporum</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>A. niger</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...
<i>A. nidulans</i>	VSSNVRSLLD	LQPDTLIGTS	ELGGLSNKK	KRKRKRG...

C



D

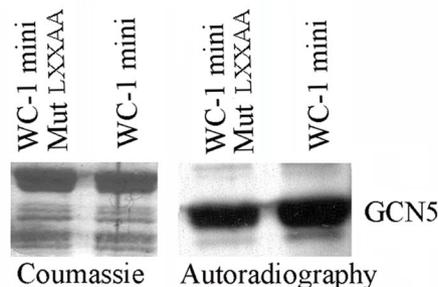


FIGURE 4: Identification of a WC-1 LXXLL motif involved in the association with HAT. (A) Schematic representation of a generic nuclear receptor (NR) and WC-1 structural organization, evidencing architectural and functional similarities. (B) Multialignment of the regions containing the LXXLL motifs within *Neurospora* WC-1 and several WC-1-like proteins present in the National Center for Biotechnology Information databank (LLDLL and LAQLL, indicated by a dashed line). Consensus analysis reveals conservation only for the LXXLL motif present at position 1008–1012 in *Neurospora* WC-1. Organisms and protein accession numbers: *Cryptococcus neoformans*, XP_567995.1; *Cryptococcus gattii*, XP_003193431.1;

HATs might compensate for this function in the *ngf-1RIP* mutant background.

DISCUSSION

In the eukaryotic model organism *Neurospora crassa*, blue light signal transduction is mediated by a photoreceptor formed by the two zinc-finger transcription factors WC-1 and WC-2 (WCC). The heterodimeric WCC controls a light-dependent gene expression program by binding specific GATA sequences within LRRs of target promoters (Talora *et al.*, 1999).

Chromatin modification and signal transduction have been extensively studied in several model organisms, including *S. cerevisiae* and filamentous fungi; however, the relevance of chromatin alterations in

Neurospora crassa, CAA63964; *Sordaria macrospora*, XP_003351224.1; *Podospora anserina*, CAD60767; *Verticillium albo-atrum*, XP_003001986.1; *Colletotrichum higginsianum*, CCF31796.1; *Glomerella graminicola*, EFQ30482.1; *Nectria haematococca*, XP_003040715.1; *Fusarium oxysporum*, ABY47609; *Gibberella fujikuroi*, CAO85915; *Gibberella zae*, XP_388117; *Hypocrea jecorina*, AAV80185; *Trichoderma atroviride*, AAU14171.1; *Metarhizium anisopliae*, EFY99524.1; *Metarhizium acridum*, EFY92414; *Magnaporthe oryzae*, XP_360995.2; *Thielavia terrestris*, XP_003654033; *Chaetomium globosum*, XP_001219613; *Myceliophthora thermophila*, XP_003665493.1; *Botryotinia fuckeliana*, XP_001547999.1; *Sclerotinia sclerotiorum*, XP_001586924.1; *Cercospora zeae-maydis*, AEH41590.1; *Mycosphaerella graminicola*, EGP83257.1; *Phaeosphaeria avenaria*, ACS74812.1; *Cochliobolus miyabeanus*, BAF35570.1; *Leptosphaeria maculans*, CBX98033.1; *Pyrenophora tritici-repentis*, XP_001933567.1; *Tuber borchii*, CAE01390.1; *M. Tuber melanosporum*, CAZ83744.1; *Aspergillus niger*, XP_001395039.1; *Aspergillus nidulans*, XP_661040.1.

(C) Schematic representation of the domains in the two WC-1 mini versions and WT and point mutation mutant (Mut LXXAA) used in the pull-down experiments. The LAQLL mutagenized version is changed in LAQAA. (D) Recombinant proteins (C) were used in pull-down assays to evaluate their interaction with a radiolabeled *Sc-Gcn5p*. A representative Coomassie-stained gel and its autoradiography are shown (top). Quantification of the relative amount of 35S-*ScGcn5p* retained by the two recombinant products. The signal corresponding to WC-1 mini/*ScGcn5p* interaction was set to 100% (bottom). The data are the mean \pm SEM from three independent experiments.

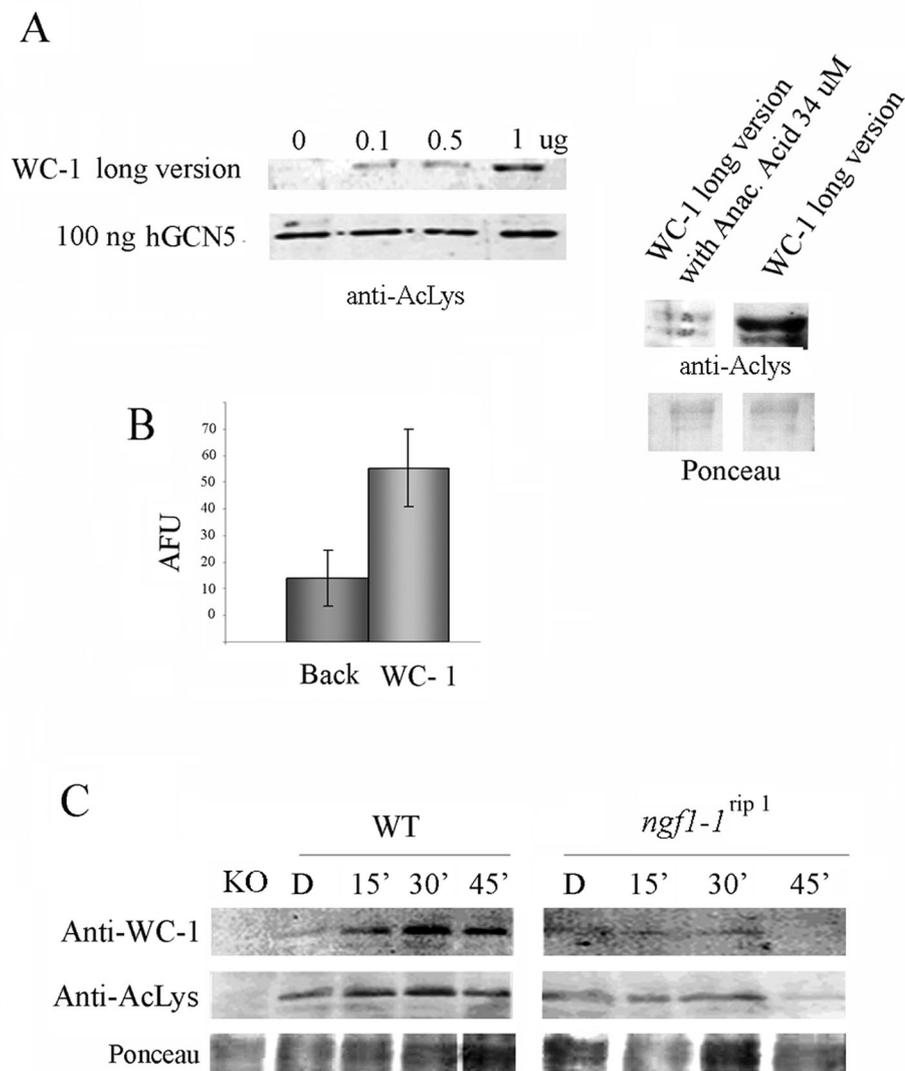


FIGURE 5: WC-1 is an acetylated protein. (A) Progressive amounts of GST–WC-1 long version (Figure 2A) were tested in an in vitro acetylation assay in the presence of human GCN5, as described in *Materials and Methods*, using an anti–Pan acetylated-lysine antibody. Anacardic acid, a common HAT inhibitor, was used to prove the dependence of the acetylated signal on the acetyltransferase reaction. Ponceau staining was used to check the presence of similar WC-1 amounts on the membrane (right). (B) WC-1 acetylation was confirmed by a fluorescence in vitro acetylation assay. The data are the mean \pm SEM from three independent experiments. AFU, arbitrary fluorescence units. (C) Western blot analysis with anti–WC-1 and anti–Pan acetylated-lysine antibodies of WT and *ngf-1* RIP strains grown for 3 d in the dark before harvesting (Dark) or exposed to saturating white light for 5 min and then returned to the dark for 10, 25, and 55 min before harvesting (Light 15, 30, 60 min). A *wc-1*–null strain (KO) was used as a control of the specificity of the anti–WC-1 antibody. Ponceau staining was used to check the presence of similar WC-1 amounts on the membrane.

response to external stimuli was analyzed only relatively recently (Belden *et al.*, 2007, 2011). In *Neurospora* the acetylation of histone H3-K14 at the light-inducible *albino-3* promoter is transiently induced by light (Grimaldi *et al.*, 2006). NGF-1, the *Neurospora* HAT homologue to *S. cerevisiae* Gcn5p, was shown to be responsible for this light-induced acetylation, and WC-1 was necessary for this process (Grimaldi *et al.*, 2006).

In addition, we reported that WC-1 directly forms a complex with NGF-1, switching HAT activity on the target promoters (Figures 1 and 3). Remarkably, we observed that the association of WC-1 with NGF-1 does not depend on the light stimulus (Figure 1A) and that

NGF-1 is present at the promoter of the light-inducible *al-3* gene already in the dark (Figure 1C). Therefore the light stimulus seems to mediate a switching of HAT activity of the preassembled NGF-1 associated with the WCC, as indicated by the chromatin immunoprecipitation results under dark and light conditions (Figure 1D).

One intriguing possibility is that light determines a conformational change that exposes NGF-1 to the histone regions to be acetylated (Figure 6). This idea takes into account the proposed model in which a light-dependent conformational change of the LOV sensor domain can regulate the activity of LOV-containing proteins such as WC-1 (Crosson and Moffat, 2002).

To confirm the importance of WC-1/NGF-1 interaction in light signaling and to localize the region involved, we performed GST pull-down experiments and identified a carboxy-terminal region in WC-1 responsible for its association with NGF-1 in vitro (Figure 2, A and B). This region also dictates WC-1/NGF-1 association in vivo, as indicated by the failure of the mutant WMN protein, lacking the C-terminus, to coimmunoprecipitate NGF-1 (Figure 2C).

Removal of the NGF-1–interacting region of WC-1 in the WMN strain completely impairs light-induced histone acetylation (Figure 3A) and abolishes light-mediated gene transcription (Figure 3B). As a result, the WMN strain presents a strong reduction of carotenoid biosynthesis (Figure 3C).

The association of WC-1 with the HAT NGF-1 reveals a functional architecture of the *Neurospora* photoreceptor with extraordinary similarities to the members of the nuclear receptor superfamily, as highlighted in Figure 4A.

As previously pointed out by Näär and Takum (2009), bioinformatics-based evolutionary studies were ineffective in recognizing NRs in lower eukaryotes. However, the study of functional domains of fungal transcription factors activated by environmental cues revealed the existence of a structure very similar to that of NRs. This finding can be interpreted as the product of the independent coevolution of vertebrate NRs and

fungal one-step signal-effector proteins, leading to the hypothesis that blue light behaves in WC-1–mediated photoreception signaling similarly to the small effector molecules in NR–mediated transduction.

Remarkably, WC-1 region responsible for NGF-1 association contains a conserved LXXLL motif (Figure 4B), also known as an NR box, reported to be involved in the interaction between NRs and coregulators (Savkur and Burris, 2004). Mutagenesis experiments confirmed the importance of WC-1 LXXLL-motif integrity in the association with the coactivator (in this case Gcn5p; Figure 4C). Our results show that upon the substitution of the terminal LL residues with the amino acid

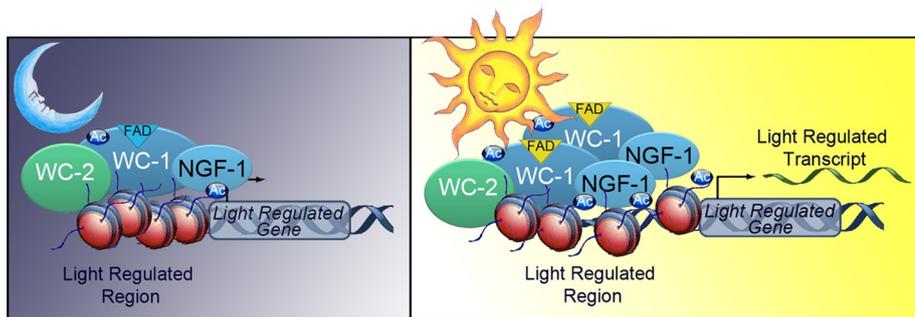


FIGURE 6: Diagram showing a possible working model. WC-1 and NGF-1 are preassembled in the dark on the promoter of light-inducible genes, but the histone tails are not accessible for NGF-1-mediated acetylation. (B) On photoinduction, the White Collar complex undergoes a conformational change that allows NGF-1 to acetylate histone H3 and promote transcriptional activation.

AA there is a 45% reduction of NGF-1/WC-1-binding activity, in accord with previous observations (Fan *et al.*, 2004) and partially in accord with others (Jiang *et al.*, 2010). VIVID is the other *Neurospora* protein containing a LOV domain (Heintzen *et al.*, 2001). However, it does not contain structural features reminiscent of nuclear receptor and the NR box. This suggests that the NR box marks only a subset of proteins involved in signal transduction. Other fungal and metazoan nonvertebrate zinc-finger factors have been reported to display functional similarity with vertebrate NRs. The lack of NR boxes in these proteins suggests that the LXXLL motif appeared with the evolution of higher eukaryotes (Näär and Takur, 2009).

For the first time, our findings revealed the involvement of the LXXLL motif in the association of transcription factors and coregulators in nonvertebrates. Overall our data indicate a conserved evolutionary mechanism in *Neurospora* and higher eukaryotes for one-step signal transduction responding to environmental stimuli, which involves the direct association of sensor proteins and chromatin-modifier coregulators.

Finally, we provide evidence, using two different HAT assays (Figure 5, A and B), that WC-1 can be acetylated *in vitro* and that this modification is blocked by the HAT inhibitor anacardic acid (Figure 5A).

Supporting the *in vivo* acetylation of WC-1, Western blot analysis with anti-WC-1 and anti-Pan acetylated lysine antibodies of WT protein extract showed an overlapping signal (Figure 5C). Although we cannot rule out the possibility that the acetylated band corresponds to a protein with the same molecular weight as WC-1, its absence in the *wc-1^{KO}* strain strongly suggests the existence *in vivo* of a WC-1-acetylated form. Of note, this acetylation does not change upon photoinduction and is unlikely to depend on *Neurospora* NGF-1 HAT, considering its presence in the *ngf-1RIP* strain (Figure 5). However, further evidence that the acetylated band corresponds to WC-1 is that its signal is reduced in the *ngf-1* mutant background, where WC-1 protein levels decrease due to defective photoinduction in the absence of NGF-1 (Figure 5). Taken together, the data suggest that WC-1 may be acetylated *in vivo*. Further studies are needed to elucidate the role of this modification in light signaling.

Figure 6 illustrates our working model for light-dependent transcriptional activation in *Neurospora*. It is a simply basal model for genes dependent on light activation.

The peculiarity of our model is the role of the histone acetyltransferase NGF-1 and of chromatin modification in blue light signal transduction.

MATERIALS AND METHODS

Neurospora strains and growth conditions

Neurospora wild-type strain 74OR23-1A (FGSC 987) was obtained from the Fungal Genetic Stock Center (Kansas City, KS). The *wc-1^{KO}* mutant (*matA; his-3; bd; wc-1 null*; FGSC 3081) was a gift from J. Dunlap (Dartmouth Medical School, Hanover, NH).

The WC-1 Myc strain was a *wc-1^{KO}* transformed with pDE3dBH derivative (Cheng *et al.*, 2001) containing the entire *wc-1* Myc tagged under the *qa2* promoter. The WMN strain expresses a WC-1 protein truncated at the carboxy-terminus (from aa 764 to the end of the protein; Cheng *et al.*, 2003). The strain *ngf-1RIP* has been described previously (Grimaldi *et al.*, 2006). For liquid

growth, 1×10^6 conidia were inoculated in Vogel's minimal medium supplemented with 1.5% sucrose, and cultures were incubated for 48–72 h in the dark at 28°C. For the preparation of dark-grown samples, mycelia were collected by filtration under a red safety lamp and frozen in liquid nitrogen. For the photoinduction experiments, mycelia were induced for 5 min with saturating light (10 W/cm²) and further grown in the dark for different time intervals before collection under a red safety lamp. The time intervals indicated in the figures refer to the time elapsed after exposure to the light-pulse stimulus.

Conidia count

Neurospora strains were grown in 250-ml flasks containing 100 ml of solid Vogel's minimal medium supplemented with 1.5% sucrose and then recovered in 25 ml of water. A dilution of conidia was observed with Burkler cell counter, and the count was performed according to the manufacturer's formula:

$$\left[\frac{\text{Number of conidia (Arith. Mean)}}{4} \right] \times 16 \times 10^4 \times \text{diluit. factor}$$

The chromatic effects (qualitative data) of the dilution were observed by spotting the conidia on a nitrocellulose filter.

Immunoprecipitation assay

Total protein samples from frozen mycelia were extracted with lysis buffer A (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 137 mM KCl, 10% glycerol containing 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM EDTA, and 1× protease inhibitor cocktail tablets [Roche, Indianapolis, IN]). For the immunoprecipitation assay, 5 µl of affinity-purified anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was previously incubated with 10 µl of protein A-Sepharose for 1 h at 4°C in 500 µl of lysis buffer A. After centrifugation, the antibody/protein A-Sepharose complex was washed twice with buffer A and then added to 400–600 µg protein extract precleaned with protein A-Sepharose for 1 h at 4°C. After incubation for 2 h at 4°C, the beads were washed five times with 500 µl of lysis buffer containing 0.05% Triton X-100. The beads were then resuspended in Laemmli's sample buffer, boiled for 10 min at 95°C, and loaded on 10% SDS-PAGE gel.

Purification of GST-WC-1 proteins

The WC-1 long version, which is a PCR fragment corresponding to aa residues 354–100 of WC-1, was cloned in pGex 2T in a *Bam*HI

cloning site. The primers were Bat1 (5'-GGGAATTCGATGAA-GGGGCGACGAGTG-3') forward and Bat3 (5'-GGG AATTCGACT-TCTCGATG-3') reverse, both with *Bam*HI restriction enzyme box as tails. The WC-1 short version and mini version, described in Ballario *et al.* (1998; WC-1 short version, GST-WC-1 LOV+PAS; WC-1 mini version, GST-WC-1 Zn), were transformed in *Escherichia coli* BL21(DE3 pLys). Isopropyl β -D-1-thiogalactopyranoside was added for 3 h, and the cells were collected by centrifugation. The cell pellets were resuspended in a GST protein buffer containing an anti-protease cocktail inhibitor (Roche) and 0.5 mM EDTA and then sonicated. Insoluble material was removed by centrifugation. The GST-fusion proteins were purified from the soluble extracts in a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

LXXLL mutagenesis

The protocol for mutagenesis followed the indications given in the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). For mutagenesis of LAQLL in LAQAA, we performed PCR using as template WC-1 mini DNA and the following mutagenesis primers:

Primer 1: 5'-GAAGAGCTGGCGCAGGCGGCGTCTAACAA-GAAAAACGG-3'

Primer 2: 5'-CCGTTTTTCTTGTAGACGCCGCTGCGCCA-GCTCTTC-3'

In vitro pull-down assay

GST-WC-1 long, short, mini, and mutated LXXAA constructs were expressed in the BL21 *E. coli* strain and purified to homogeneity with glutathione-agarose beads (Amersham) as described previously (Ballario *et al.*, 1996). Plasmid pGem 4-Z containing the full cDNA sequence of *S. cerevisiae* Gcn5 under the control of the T7 promoter was previously described (Ornaghi *et al.*, 1999). In vitro-radiolabeled Gcn5p was synthesized using reticulocyte reaction (Promega, Madison, WI) and incubated for 2 h with a similar amount of recombinant GST-WC-1 proteins resuspended in extraction buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 2 mM MgCl₂, 0.5 mM EDTA, pH 7.5, 0.5% NP40, 1 mM PMSF, and 1 \times protease inhibitor). The glutathione-Sepharose beads were sedimented by centrifugation and washed five times with washing buffer (50 mM Tris-HCl, pH 8.0, 300 mM KCl, 1 mM EDTA, 1 \times protease inhibitor, 1 mM PMSF, 5% glycerol). After the final wash, the beads were resuspended in Laemmli's sample buffer, and the proteins were run on 10% SDS-PAGE electrophoresis gel. After staining with Coomassie blue reagent, the gel was dried and subjected to autoradiography.

Chromatin immunoprecipitation assays

The ChIP experiments were performed as described in Grimaldi *et al.* (2006). The antibodies for immunoprecipitation were anti H3 (06-755; Upstate Biotechnology, Charlottesville, VA), anti-acetyl H3 (06-599; Upstate Biotechnology), and anti-hGCN5 (Santa Cruz Biotechnology). The obtained template was subjected to PCR using the following primers: 30 (5'-AGA TAG ATC TCT TGG CCT TG-3') and 31 (5'-CGA TTA TTG GAA ACC CGT CGG TA-3') for the promoter region of *al-3*, and ACT1 (5'-CCT CTC TCA GCC AAA GCA TC-3') and ACT2 (5'-GAA AGC TTA CCC CAT TGT CG-3') for the promoter region of the actin gene used for normalization. PCR products were amplified for 25 cycles and resolved on 2% agarose gel. To ensure that the amplified PCR products were in the linear range, the PCR conditions were calibrated with different amounts of immunoprecipitated samples and input DNA (derived from cross-

linked chromatin without immunoprecipitation). Band intensities were quantified by optical density analysis with OptiQuant Software (PerkinElmer Life and Analytical Sciences, Boston, MA). As negative controls, mock precipitations were performed in the absence of antibody. PCR products from these negative control samples were not detectable by ethidium bromide staining. The histograms in Figures 1C and 3A show the ratios between the values for the *al-3* and *actin* PCR products immunoprecipitated, divided by the same ratio obtained by PCR with input DNA [(*al-3/act*)IP/(*al-3/act*)input]. Three ChIP replicates were performed on different preparations for each experiment.

Protein extraction and Western blot analysis of proteins

Western blot analysis was performed as previously described (Crosthwaite *et al.*, 1997). Tissue was ground in liquid nitrogen with mortar and pestle and suspended in ice-cold lysis buffer A (50 mM HEPES, pH 7.4, 137 mM KCl, 10% glycerol containing 1 mM PMSF, 1 mM EDTA, 1 \times protease inhibitor) at a ratio of 0.5 ml of buffer to 0.1 g of tissue. Extracts were homogenized with three strokes of a Teflon/glass homogenizer, and cellular debris was removed by centrifugation at 10,000 relative centrifugal force. The protein concentration of the supernatant was determined with the Bio-Rad (Hercules, CA) reagent according to the manufacturer's instructions. Equal amounts of proteins (100 μ g) for different extracts were denatured in Laemmli's sample buffer and separated by 8% SDS-PAGE for WC-1 analysis. After transfer to nitrocellulose (Amersham Pharmacia Biotech), the blots were incubated for 2 h at room temperature (RT) with anti-Myc affinity-purified immunoglobulin G (IgG; diluted 1:300 in phosphate-buffered saline [PBS] 1 \times , Tween 0.5%, and milk 5%), anti-Gcn5 (Santa Cruz Biotechnology) purified IgG (1:500 in PBS 1 \times , Tween 0.5%, and milk 5%), or anti-Pan acetylated-lysine (Santa Cruz Biotechnology) purified IgG (1:300 in PBS 1 \times , Tween 0.5%, and milk 5%). Horseradish peroxidase-conjugated antibodies (1:10,000) were used as secondary antibody (Bio-Rad), and membranes were developed using chemiluminescence reaction (ECL; Amersham Pharmacia Biotech).

Northern blot analysis

Total RNA from the WT and WMN strains was isolated according to a previously published method (Baima *et al.*, 1991). For Northern analyses, a volume of 20 μ g of total RNA was fractionated on 1.2% denaturing agarose gels in 5% formaldehyde-3-(*N*-morpholino) propane-sulfonic acid buffer and transferred to positively charged nylon membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Hybridization was carried out according to the manufacturer's instructions. The *al-3* transcript was detected with a ³²P-labeled PCR fragment amplified with primers AL3F (5'-GTCCCTCCAAGGACTCTTC-3') and AL3R (5'-CCAGGAGGCTGTGTGTAGCA-3'). For normalization, the membranes were stripped in 0.1% SDS at 95°C and hybridized with an *actin-1*, ³²P-labeled probe obtained by PCR amplification with primers ACTF (5'-GCCTTCTACGTCTCCACCA-3') and ACTR (5'-GTCGGAGAGACCAGGGTACA-3').

In vitro protein acetylation assay

Different amounts of recombinant GST WC-1 were incubated at 30°C for 60 min in 30 μ l with 6 μ l of 5 \times HAT assay buffer (250 mM Tris, pH 8.0, 25% glycerol, 0.5 mM EDTA, 250 mM KCl, and 10 mM sodium butyrate), 0.5 mM acetyl-CoA, and 100 ng of human GCN5. After incubation, the reactions were stopped by adding 6 μ l of 5 \times Laemmli's buffer, and the proteins were separated on SDS-PAGE gel. After transfer to a nitrocellulose membrane, the filter was probed with an anti-Pan acetylated-lysine (Santa Cruz Biotechnology) antibody.

Acetyltransferase fluorescence assay

The HAT assay kit is a fluorescence method for assaying samples for histone or non-histone acetyltransferase (HAT) activity or to screen HAT inhibitors (Trievel et al., 2000). A volume of 30 μ l of reaction mix (1 \times buffer HAT, 0.5 mM acetyl-CoA, 125 ng of hGCN5) is needed per assay well. In brief, 10 μ l (1 μ g) of WC-1 long version is added to the assay well, without adding protein substrate to background wells so that autoacetylation activity of the acetyltransferase is included in the background measurement. The plate is covered and incubated for 30 min at room temperature. A 50- μ l amount of stop solution is added per well, followed by addition of 1 μ g of WC-1 to the background well. Then 100 μ l of developing solution is added to each well, followed by incubation for 15 min in the dark at room temperature. Fluorescence is read with excitation at 360–390 nm and emission at 450–470 nm.

Sequence analysis of WC-1 putative NR boxes

The sequences of WC-1 proteins from the different species (Figure 5) were aligned with GeneBee Multialignment software (www.genebee.msu.su). The local supermotif containing the putative NR boxes is shown in Figure 5.

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