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Genome-wide analysis of light-inducible responses reveals hierarchical light signalling in *Neurospora*

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White collar-1 (WC-1) and white collar-2 (WC-2) are essential for light-mediated responses in *Neurospora crassa*, but the molecular mechanisms underlying gene induction and the roles of other real and putative photoreceptors remain poorly characterized. Unsupervised hierarchical clustering of genome-wide microarrays reveals 5.6% of detectable transcripts, including several novel mediators, that are either early or late light responsive. Evidence is shown for photoreception in the absence of the dominant, and here confirmed, white collar complex (WCC) that regulates both types of light responses. VVD primarily modulates late responses, whereas light-responsive submerged protoperithecia-1 (SUB-1), a GATA family transcription factor, is essential for most late light gene expression. After a 15-min light stimulus, the WCC directly binds the *sub*-1 promoter. Bioinformatics analysis detects many early light response elements (ELREs), as well as identifying a late light response element (LLRE) required for wild-type activity of late light response promoters. The data provide a global picture of transcriptional response to light, as well as illuminating the *cis*- and *trans*-acting elements comprising the regulatory signalling cascade that governs the photobiological response.

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Introduction

Neurospora crassa has served as a model organism to study light responses in eukaryotic cells for several decades (Dunlap and Loros, 2004; Purschwitz *et al*, 2006; Bahn *et al*, 2007; Corrochano, 2007; Heintzen and Liu, 2007; Herrera-Estrella and Horwitz, 2007). Two GATA family zinc finger transcription factors (TFs), white collar-1 (WC-1) and white collar-2 (WC-2), have been shown to play an essential role in diverse UV/blue light regulated physiological processes, including maintenance and resetting of the circadian clock, carotenoid biosynthesis, asexual conidiation and, in the sexual cycle, the formation of protoperithecia and the direction of ascospores release. Molecularly, WC-1 is both a flavin-adenine dinucleotide-binding photoreceptor and a TF. WC-1 interacts with WC-2 through its Per-Arnt-Sim domain and, after sensing light, forms a large white collar complex (L-WCC) that activates downstream target genes, presumably through the recognition of a consensus sequence, the light responsive element (LRE) (Froehlich *et al*, 2002; He *et al*, 2002; Cheng *et al*, 2003). Notably, despite extensive efforts to identify blind strains in several labs, only *wc-1* and *wc-2* mutants have been isolated (Linden *et al*, 1997), strongly suggesting their unique and dominant role in mediating light signals. In addition to WC-1, VVD is the other blue light photoreceptor that has been implicated in light sensing. In *vvd* loss-of-function strains, the induction of light responses is largely normal, but there subsequently appears a defect in photoadaptation, the ability of the organism to sense incremental changes in light intensity (Heintzen *et al*, 2001; Schwerdtfeger and Linden, 2001, 2003; Shrode *et al*, 2001).

In the *Neurospora* genome sequence, several putative photoreceptors have been identified based on sequence similarities, including a cryptochrome and two phytochrome homologues (PHY-1 and PHY-2) (Galagan *et al*, 2003). The presence of phytochromes was a surprise, as all known light responses in *Neurospora* are restricted to blue light (Dunlap and Loros, 2006). Studies further show that PHY-2 not only can covalently bind either biliverdin or phycocyanobilin but also is capable of undergoing a photocycle *in vitro*. However, due to the absence of a detectable phenotype in the knockout strains (Froehlich *et al*, 2005), the biological function of PHY-1 and PHY-2 remains to be discovered. Another potential photoreceptor, NOP-1, is structurally related to archaeal rhodopsins and is also capable of absorbing light and undergoing a photocycle. However, like other putative photoreceptor mutants in *Neurospora*, the knockout stain has only a weak phenotype at best (Bieszke *et al*, 1999a, b; Furutani *et al*, 2004). Undoubtedly, a more comprehensive and systematic approach is needed to elucidate functions of these light-sensing proteins.

Here, using microarrays with full-genome coverage (Dunlap *et al*, 2007; Tian *et al*, 2007) and large-scale quantitative analysis, we provide a catalogue of the 314 genes showing strong early or late light responses and describe functional and sequence analysis, suggesting a clear correspondence between the timing of induction and gene function. Data confirm the dominant role of WC-1 and WC-2 in

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initiating the light responses and of VVD in modulating the responses but show that residual light responsivity remains in a *wc-1* loss-of-function mutant. Identification of SUB-1 as a novel light-responsive TF and of novel *cis*-acting early light response elements (ELREs) and late light response elements (LLREs) allows a more global understanding of the regulatory cascade governing the response of the organism to light.

Results

Unsupervised hierarchical clustering of light-inducible responses identifies ELRGs and LLRGs

We took a systematic approach using long-oligomer constructed microarrays with full-genome coverage (Tian *et al*, 2007) to characterize white light-inducible transcriptional changes in wild-type (WT) and various photoreceptor knockout strains. Earlier studies have shown that *Neurospora* can display dramatic transcriptional changes within 5 min or less (Crosthwaite *et al*, 1995; Linden *et al*, 1997). Therefore, we sampled most intensively within the first 30 min, namely at 0, 5, 10, 15, 30, 60, 90, 120, 180 and 240 min after lights-on. To eliminate intrinsic systemic variation between microarrays and have a direct comparison between experiments, we adopted an unsupervised hierarchical clustering approach (Eisen *et al*, 1998) to identify *bona fide* light-responsive genes (see Materials and methods for details). An assumption made is that genes that are truly responsive to light should behave more similar to each other across sequential time points and different strains. After clustering all of the data across 90 microarrays, a subcluster containing 314 genes (Supplementary Table I), equal to 5.6% of the total detectable transcripts, became evident with a robust and coherent light-responsive pattern. This ratio is slightly higher but close to a prior estimate (3%) from spotted cDNA microarrays covering 1343 genes (Lewis *et al*, 2002). Within this subcluster of genes, several divisions were merged and re-grouped as either early or late light-responsive genes based on visual inspection (ELRGs and LLRGs cover 92% of the total identified light-responsive genes, Supplementary Tables II and III). As shown in Figure 1A and B, lanes 1–4, light-induced transcriptional changes are extremely consistent in strains reported earlier to have normal light responses, including single- and double-knockout strains of *phy-1* and *phy-2* (lanes 2–4) (Froehlich *et al*, 2005) (The few genes showing altered expression in phytochrome gene knockouts are the subject of a separate study). In the *frq*^{7} strain, a long-period clock mutant, we observed a generally higher amplitude of induction for both types of light responses (lane 5, Supplementary Figure 1), presumably due to the positive feedback loop between FRQ and WC-1 level (Lee *et al*, 2000).

As predicted, both early and late light responses are severely impaired in *wc-1/wc-2* single- and double-knockout strains (lanes 6, 7 and 8). To enhance the robustness of clustering and to serve as a reference dataset for visual comparison, we combined microarray data from a WT strain in constant darkness (DD, lane 9). Overall, although quantitatively expanding the number of light-regulated genes by about eight-fold, these data are qualitatively consistent with what is known about *Neurospora* photobiology in general, with waves of ELRGs followed by LLRGs (Sommer *et al*, 1989; Linden *et al*, 1997; Lewis *et al*, 2002). To facilitate a direct comparison between WT and *wc* knockout strains, we plotted the microarray readouts of WT, Δ*wc-1*, Δ*wc-2* and WT (DD) side by side in another dimension. As shown in Figure 2A, the ELRGs peak between 15 and 45 min after onset of light, whereas LLRGs peak later between 45 and 90 min (Figure 2B). Reflecting photoadaptation, both types of light responses return to their basal level of expression after 4 h of constant light (Figure 2A and B, DD versus LL240 time point in the WT strain). The microarray data from Δ*wc-1*, Δ*wc-2* and the WT strain in constant darkness (Figure 2A and B) show no induction, suggesting that there is no increase in gene expression due to development or other changes over the 4-h time course. The distinct kinetics of induction that distinguishes the early and late light responses suggested to us that they are separately regulated, and that there might be common molecular mechanisms underlying the timing control of each response.

In addition to the visual inspection, the same coherent light-responsive clusters, one early and one late, were identified as statistically significant by an unbiased, scale-free bootstrapping analysis covering 53 genes (Shimodaira, 2004) (Supplementary Figure 2, see Supplementary data for details), supporting the clustering outcome as a valid estimate of regulatory similarity. To add further statistical verification to our list of light-responsive genes, we re-analysed our data with the SAM (significance analysis of microarrays) package (Tusher *et al*, 2001). Using 10% false discovery rate (FDR) as a cutoff, SAM identified 152 genes (Supplementary Table VI, see Materials and methods for details) that show at least a 2.2-fold increase after light treatment, whereas none of the light-repressed genes could be identified with the same FDR cutoff. Interestingly, the clustering approach identified twice as many target genes (314 versus 152) while covering 93% of the statistically significant genes and additionally found no evidence for a light-repressed subcluster. If a split in a cluster produces a nearly homogeneous pattern with regard to a unique property (i.e. light responsive) and encompasses most known targets (Supplementary Table IV, e.g. see Bell-Pedersen *et al* (1996); Linden *et al* (1997); Lewis *et al* (2002)) and statistically significant genes (Supplementary Table VI), such a subcluster of candidate light-induced genes should be considered interesting and valid (Murray *et al*, 2004).

Most but not all light-induced transcriptional responses are absent in strains lacking WC-1

Although most if not all light responses are impaired in the *wc* knockout strains (as shown in Figure 2A and B), residual light responses revealed in the Δ*wc-1* background (Figure 1B, lane 6) are interesting, suggesting that *Neurospora* might not be completely blind in the absence of WC-1. To validate these residual light responses, we performed quantitative RT–QPCR analysis with RNA samples from three independent biological replicates. After testing the induction of six candidate genes identified by the microarray (NCU01555.2, NCU02765.2, NCU04415.2, NCU04510.2, NCU04605.2 and NCU05490.2), we could detect a consistent, low increase of NCU02765.2 in the Δ*wc-1* background (Figures 2C, $P = 0.02$, as determined by paired *t*-test). NCU02765.2 encodes a small (116 aa) conserved, nonessential protein of unknown functions (data not shown). However, other genes examined showed variable responses to light stimulus (data not shown). Induction of *frq* is shown in Figure 2D as an internal control for our sample

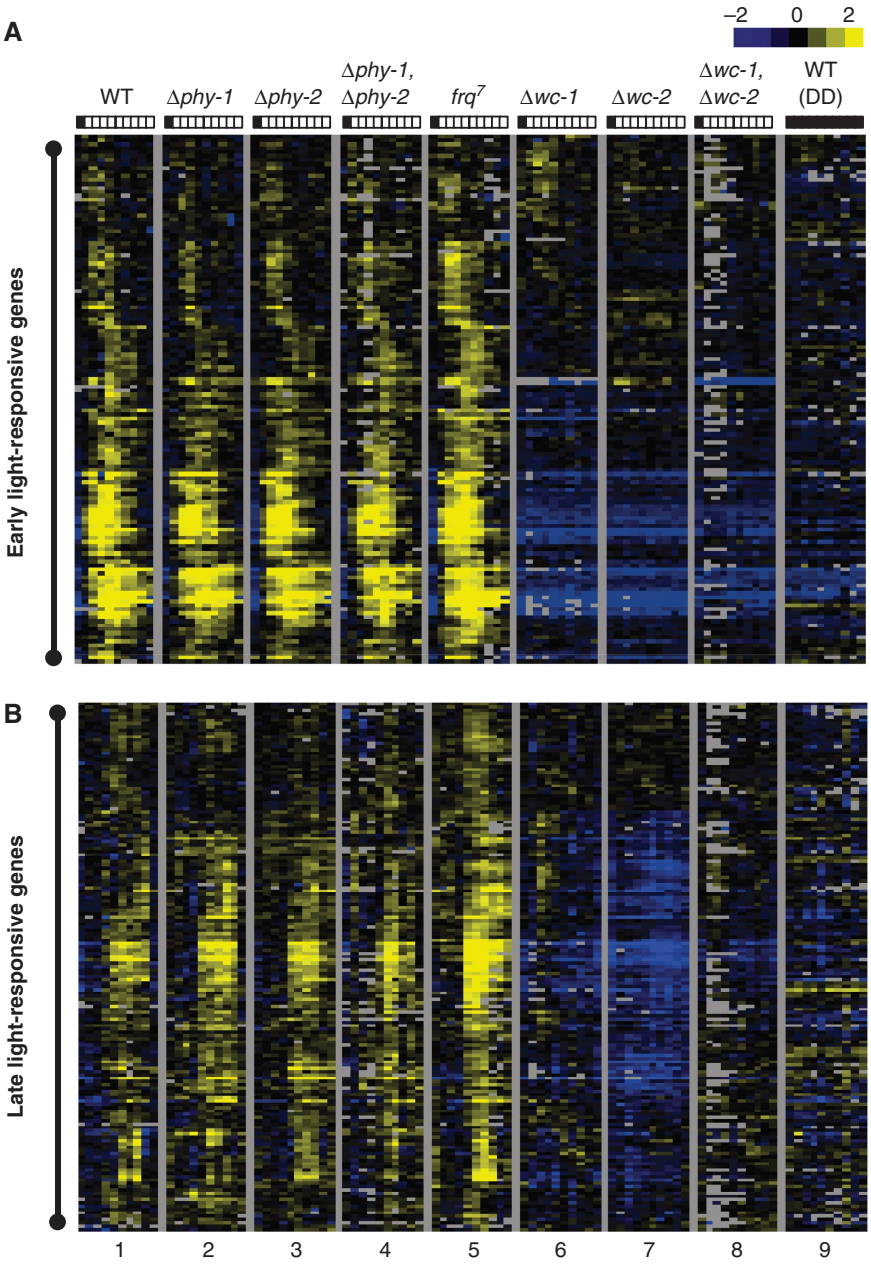

Figure 1 Unsupervised hierarchical clustering of light-inducible responses identifies early and late light-responsive genes. (**A**) Clustering of early light-responsive genes. (**B**) Clustering of late light-responsive genes. Experimental strains: Lane 1, 74A (WT); Lane 2, Δ*phy-1*; Lane 3, Δ*phy-2*; Lane 4, Δ*phy-1,* Δ*phy-2*; Lane 5, *frq*^{7}; Lane 6, Δ*wc-1*; Lane 7, Δ*wc-2*; Lane 8, Δ*wc-1,* Δ*wc-2*; Lane 9, 74A (DD). In each lane, from left to right, the individual columns correspond to light treatment lasting for 0, 5, 10, 15, 30, 45, 60, 90, 120, 240 min, respectively. For each row, the data were centred so that each measurement reflects transcript abundance relative to the mean expression across all 90 microarrays (a common reference RNA design). *Yellow squares* indicate transcripts with increased expression; *Blue squares* indicate transcripts with decreased expression; *Black squares* indicate transcripts with expression levels close to mean value; *Gray squares* represent missing data.

preparation. These residual light responses appear to be relatively weak and transient in contrast to the conventional light responses but are surprising nonetheless given the central role of the WC-1 photoreceptor in *Neurospora* light responses.

Light-sensing machinery in Neurospora co-regulates genes with similar biological functions

Co-regulation of transcript levels as shown by many microarray analyses is often seen among genes in a metabolic or developmental pathway and can be used to predict the functions of earlier unstudied genes (Eisen *et al*, 1998; Harmer *et al*, 2000; Kim *et al*, 2001; Kasuga *et al*, 2005). In this context, it is informative that enrichment of genes in specific developmental pathways is seen among the light-responsive genes of *Neurospora*. For instance, four genes having almost identical kinetics of induction (Figure 3A) encode enzymes that execute consecutive steps in the carotenoid biosynthesis pathway (Figure 3B). Particularly, NCU09306.2, previously unknown but predicted to be a gene related to β-carotene 15,15′-dioxygenase, has been shown recently to have a role in torulene cleavage, the last

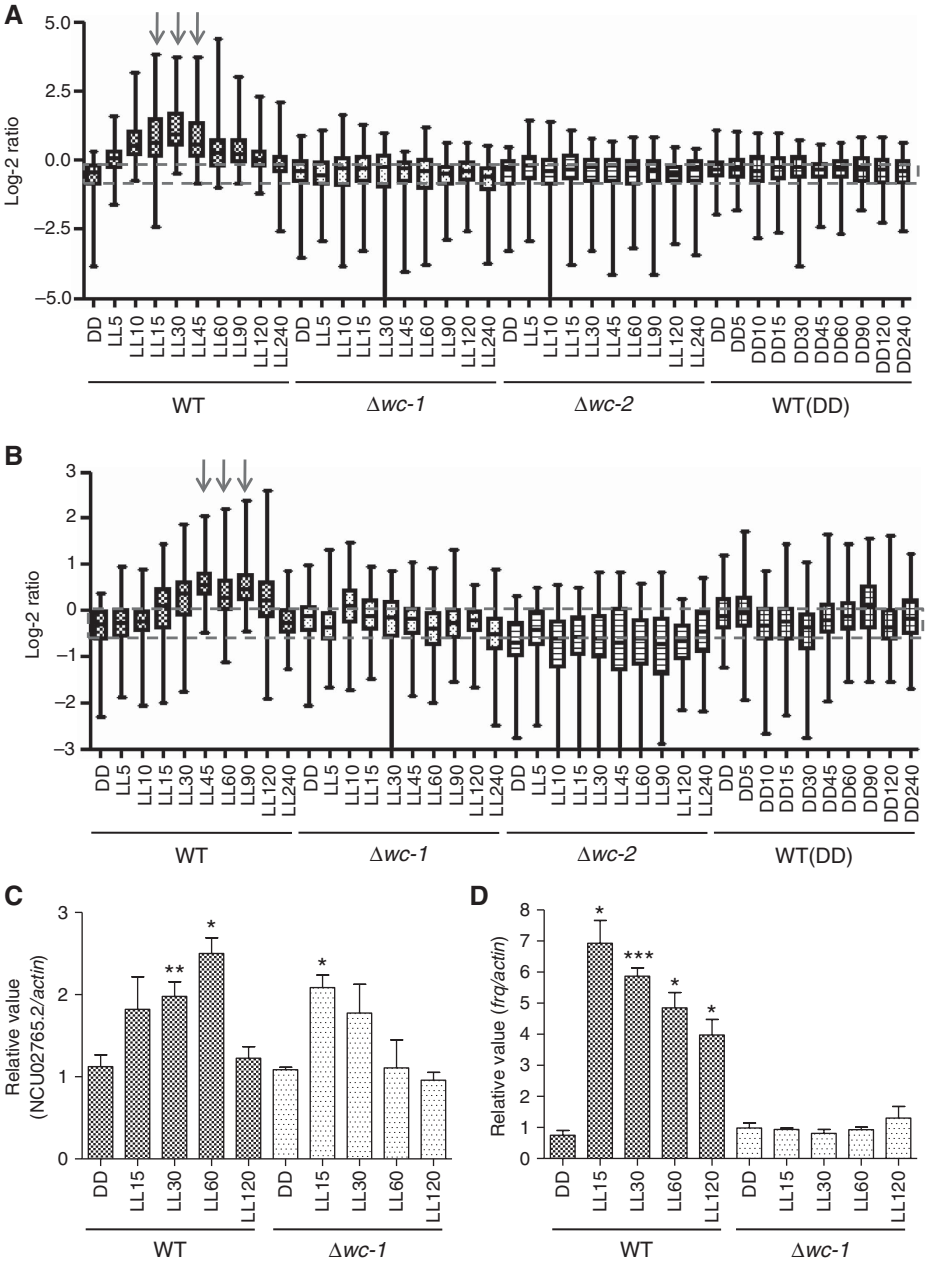

Figure 2 Most but not all light-induced transcriptional responses are absent in strains lacking WC-1. (**A**) Relative microarray readouts of early light-responsive genes. (**B**) Relative microarray readouts of late light-responsive genes in WT, Δ*wc-1*, Δ*wc-2* and WT (DD). Median value is shown as a horizontal bar with the 25–75% range are indicated as a box and extreme values are indicated as an extended line. Red arrows indicate where peaks of induction are. Red dashed lines enclose the 25–75% range of the WT readout in the first DD time point to facilitate a visual comparison for light induction. (**C**, **D**) RT–QPCR analysis of light induction of NCU02765.2 and *frq* in WT and Δ*wc-1*. The results obtained by three independent replicate experiments are shown. Columns represent mean values ± standard error; asterisks indicate statistical significance when compared with the DD time point, as determined by paired *t*-test, $*P<0.05$, $**P<0.01$, $***P<0.001$. A full-colour version of this figure is available at *The EMBO Journal* Online.

step of carotenoid biosynthesis in *Neurospora*, and named *cao-2* (carotenoid oxygenase-2) (Saelices *et al*, 2007). This is consistent with the notion that information from co-regulated light responses can be used to predict the roles for genes of unknown function. The induction kinetics of each light-responsive gene in WT is included in Supplementary Table VII.

Approximately half of the genes identified as light responsive remain unclassified as to function. Large-scale analysis of putative functions using FunCatDB (http://mips.gsf.de/proj/funcatDB/search_main_frame.html) indicates that there is a clear correspondence between the timing of induction and the underlying nature of the biological processes (Table I), such that some functional groups of genes are highly enriched. For instance, ELRGs involved in the synthesis of photoprotective pigments (the lipid, fatty acid and isoprenoid metabolism category) comprise 7.1% of the early light-responsive genes but only 2.7% of genes in the genome. This enrichment has a probability of only 8e−03 of occurring by chance, calculated based on hypergeometric distribution (Ruepp *et al*, 2004). Similarly, genes involved in the synthesis

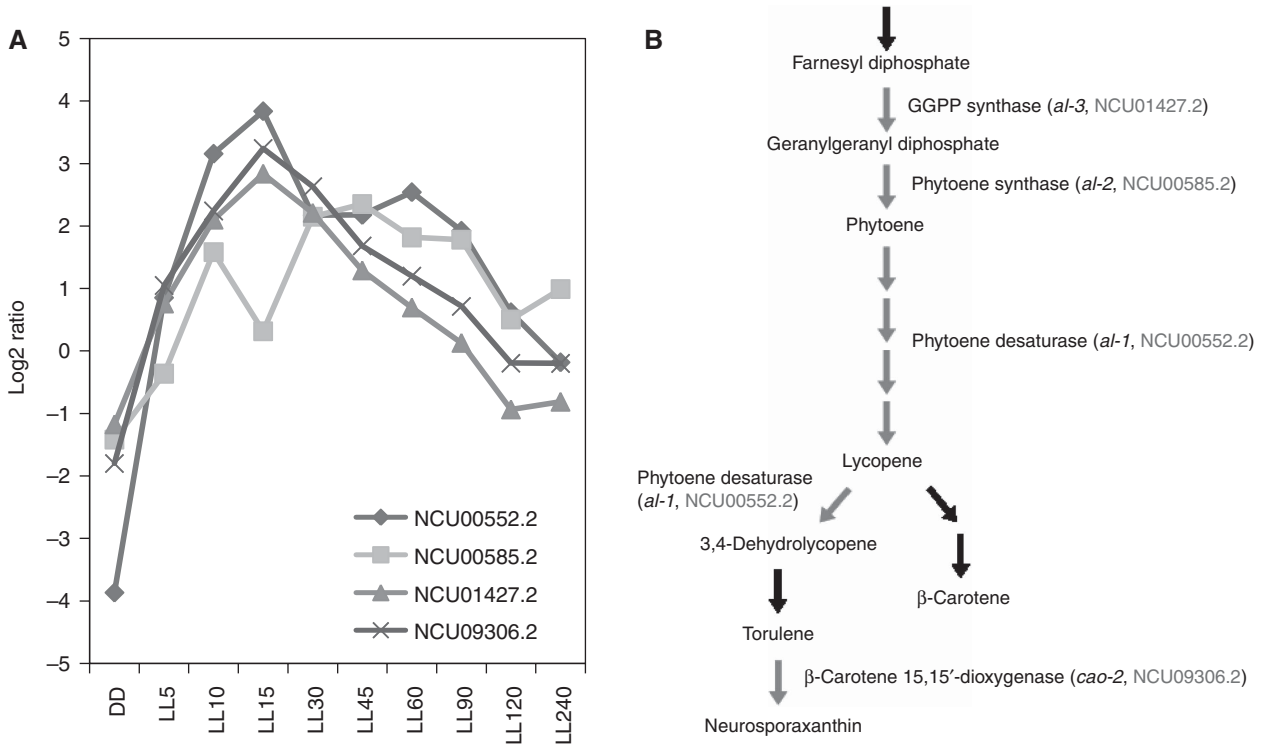

Figure 3 Light-sensing machinery in *Neurospora* co-regulates genes with similar biological functions. (**A**) Microarray readouts of four early light-responsive genes involved in the carotenoid biosynthesis pathway. Representative data from the WT strain are used for plotting. (**B**) The enzyme pathway of carotenoid biosynthesis in *Neurospora crassa*. The figure is modified from The *Neurospora* Compendium (Perkins *et al*, 2001). NCU numbers of light-regulated genes are shown in red. A full-colour version of this figure is available at *The EMBO Journal* Online.

Table I Functional analysis of ELRGs and LLRGs using FunCatDB

Functional category[a]	This study gene matches	Genome matches (%)	*P*-value[b]
Early light-responsive genes (ELRGs)			
Lipid, fatty acid and isoprenoid metabolism	7.1% (9/127)	2.7	8e−03
Biosynthesis of vitamins, cofactors, and prosthetic groups	4.7% (6/127)	0.8	4.6e−04
Secondary metabolism	4.7% (6/127)	1.3	5.9e−04
DNA processing	6.3% (8/127)	1.9	3e−03
Cellular signalling	5.5% (7/127)	1.8	7.9e−03
Osmotic and salt stress response	1.6 (2/127)	0.1	4.6e−03
Photoperception and response	1.6% (2/127)	0.04	1e−03
Circadian rhythm	1.6% (2/127)	0.1	6e−03
Unclassified protein	36% (49/127)	57	1
Late light-responsive genes (LLRGs)			
C-compound and carbohydrate metabolism	20% (31/157)	5.4	1.9e−10
Oxidation of fatty acids	1.9% (3/157)	0.2	1.7e−03
Oxygen and radical detoxification	2.5% (4/157)	0.2	1.4e−04
Unclassified protein	50% (78/157)	57	1

[a]MIPS Functional Catalogue Database (FunCatDB, http://mips.gsf.de/proj/funcatDB/search_main_frame.html).
[b]Functional categories selected to present here were based on *P*-value (<1e−02).

of vitamins, cofactors and prosthetic groups (4.7%), secondary metabolism (4.7%), DNA processing (such as DNA repair genes, 6.3%), cellular signalling (5.5%) and several key pathways implicated in cellular sensing and response to external stimuli (such as *os-2*, *wc-1*, *vvd* and *frq*) are over-represented relative to their percentage in the genome as well. Genes highly enriched among the late light-response group are those involved in carbohydrate metabolism (20%), oxidation of fatty acids (1.9%) and components involved in oxygen/radical detoxification reaction (2.5%, including *catalase-1*, *catalase-2*, *catalase-4* and *superoxide dismutase*). In summary, gene within distinct functional categories shows specific time-dependent regulation, illustrating a temporal sequence of important cellular events for *Neurospora* in response to the daily light stimulus.

SUB-1, an early light-responsive TF, is involved in regulating some early and most late light responses

Given the finding that the light-responsive TF WC-1 (Froehlich *et al*, 2002; He *et al*, 2002) is the primary component in mediating light signalling and acts with kinetics consistent with the early light responses, we predicted that it might initiate a regulatory cascade leading to the activation of late responses. Although such a cascade could take many

Table II Light-responsive transcription factors in *Neurospora crassa*

NCU no.	Class	Comment	Ratio[a]
NCU 02356.2	GATA zinc finger TF	*wc-1*	2/6
NCU 01154.2	GATA zinc finger TF	*sub-1*	
NCU 02713.2	C2H2 zinc finger TF	*csp-1*[b]	2/43
NCU 04179.2	C2H2 zinc finger TF	*sah-1*	
NCU 06407.2	$Zn(II)_2Cys_6$ fungal binuclear TF	*vad-3*	2/77
NCU 03643.2	$Zn(II)_2Cys_6$ fungal binuclear TF	Cutinase TF-1β	

[a]The ratio indicates the number of light-responsive transcription factors versus total number of transcription factors within the same family ([10]).
[b]The identity of *csp-1* was recently reported (Lambreghts *et al*, 2009).

forms, the simplest would be through a transcriptional hierarchy. Our analysis identified six light-responsive TFs, including WC-1 (Table II). We verified their light induction and WC-1 dependence by RT–QPCR analysis, using RNA samples from three independent biological replicates (Supplementary Figure 3). To investigate the function of these TFs in response to light, we used additional microarray analysis to follow the light-induced transcriptional changes in the strains bearing a gene-replacement knockout of each TF (Colot *et al*, 2006) (Figure 4A and B, lanes 2–6). Given that the light-responsive targets were known from the initial microarray analysis, only five sequential time points were followed for each knockout strain ranging from 0, 15, 30, 60 to 120 min after onset of light. Lane 1 in Figure 4A and B represents an independent biological replicate of WT, serving as an internal control for the microarray measurements.

Interestingly, as shown in Figure 4A and B, the regulation of most ELRGs and LLRGs appears to be more or less unchanged in the different TF knockout strains with the exception of Δ*sub-1* (lane 2). In the Δ*sub-1* strain, some early and most late light responses are severely impaired across sequential time points, suggesting that light-responsive SUB-1 might be involved in regulating light responses as WC-1. For other TF knockout strains, the variations in their late light responses (Figure 4B, lanes 3–6) did not signal a consistent pattern in the same manner as Δ*sub-1* as showed by SAM analysis (Supplementary Figure 6).

Identification of a full set of *bona fide* light-responsive genes also provides a unique opportunity to re-explore the function of another putative photoreceptor, NOP-1 (Bieszke *et al*, 2007), at the transcriptional level. As shown in Figure 4A and B (lanes 8), there appeared to be no systematic changes in the regulation of ELRGs and LLRGs, suggesting that the biological function of NOP-1 might not be in the modulation of light-induced transcriptional changes, and thus remains to be determined. Interestingly but not surprisingly, we confirmed the photoadaptation defects in the Δ*vvd* strain (Heintzen *et al*, 2001; Schwerdtfeger and Linden, 2001, 2003; Shrode *et al*, 2001) for both ELRGs and LLRGs (Figure 4A and B, lane 7). Clearly, the timing of induction is not altered for either early or late light responses in Δ*vvd*, suggesting VVD is not directly involved in the initial regulation of light responses. Instead, once transcription is turned on by the light-activated WCC, VVD can serve as a universal brake to repress both types of light responses under prolonged light exposure. The data from the Δ*vvd* strain is not only consistent with earlier observations on a small number of light-responsive targets but also provides a completely independent validation of the genes we identified as light responsive in Figure 1.

SUB-1 is required for efficient transduction of light signals to some ELRGs and most LLRGs under WCC control

To gain further insight into the light function of SUB-1, we choose two ELRGs, NCU00309.2 and NCU009535.2, and two LLRGs, NCU01107.2 and NCU06597.2, to validate the impaired light responses in the Δ*sub-1* strain. The early light-responsive gene *wc-1*, whose light induction has no obvious difference between WT and Δ*sub-1* as predicted by our microarray analysis, was monitored as quality control for our light treatment and RT–QPCR analysis. As shown in Figure 5A, *wc-1* is properly induced by 4–5-fold in both strains, indicating that *sub-1* is not required for light activation of *wc-1* transcript. In contrast, four candidate genes revealed by microarray analysis as either early or late light responsive in WT but defective in Δ*sub-1*, all reproducibly show a marked impairment in their light responses in the absence of *sub-1* (Figure 5B–E), confirming the microarray measurements. However, with the exception of NCU00309.2, a slight but significant increase of transcript in response to light could still be observed in Δ*sub-1* for genes NCU09535.2, NCU01107.2 and NCU06597.2 (Figure 5C–E), indicating that *sub-1* is not the sole regulator of these genes, and/or may work to modulate the action of other regulator(s). Also worth mentioning, the Δ*sub-1* strain, unlike Δ*wc-1*, appeared to have a normal accumulation of carotenoid in constant light (data not shown).

The circadian clock is not altered in the absence of sub-1

For many genes, light responses and circadian regulation or function are linked. To explore possible clock functions of these light-responsive TFs, we introduced the dominant RAS allele, *ras-1*^{bd} (Belden *et al*, 2007a), into the individual TF knockout strains by genetic crossing. As shown in Supplementary Figure 4, the only arrhythmic strain is the knockout of *wc-1*, in agreement with earlier data (Crosthwaite *et al*, 1997). Other TF knockout strains all display a largely normal clock phenotype ($n \geqslant 5$), indicating that these TFs are not required for the circadian clock, although there may be some small perturbations in clock period and phase.

An LLRE contributes to mediating late light responses in vivo

To search for distinct *cis*-acting regulatory motifs important for the induction of light responses, we used *SCOPE* (Carlson *et al*, 2007) to screen the entire intergenic regions for both early and late light-responsive genes. Briefly, *SCOPE* uses three algorithms to identify sequence motifs: *BEAM* (Carlson *et al*, 2006a) finds nondegenerate motifs (e.g. ACGCGT), *PRISM* (Carlson *et al*, 2006b) finds degenerate motifs (e.g. ACSCGW) and *SPACER* (Chakravarty *et al*, 2007) finds bipartite motifs (e.g. ACGnnnnnnnCGW). The results from all three algorithms are merged and the best scoring motifs are presented in rank order. *Sig value* in *SCOPE* is a measure of how likely it is that the consensus sequence in question could have been over-represented as observed by chance alone. For a detailed discussion of the *Sig value* see van Helden *et al*

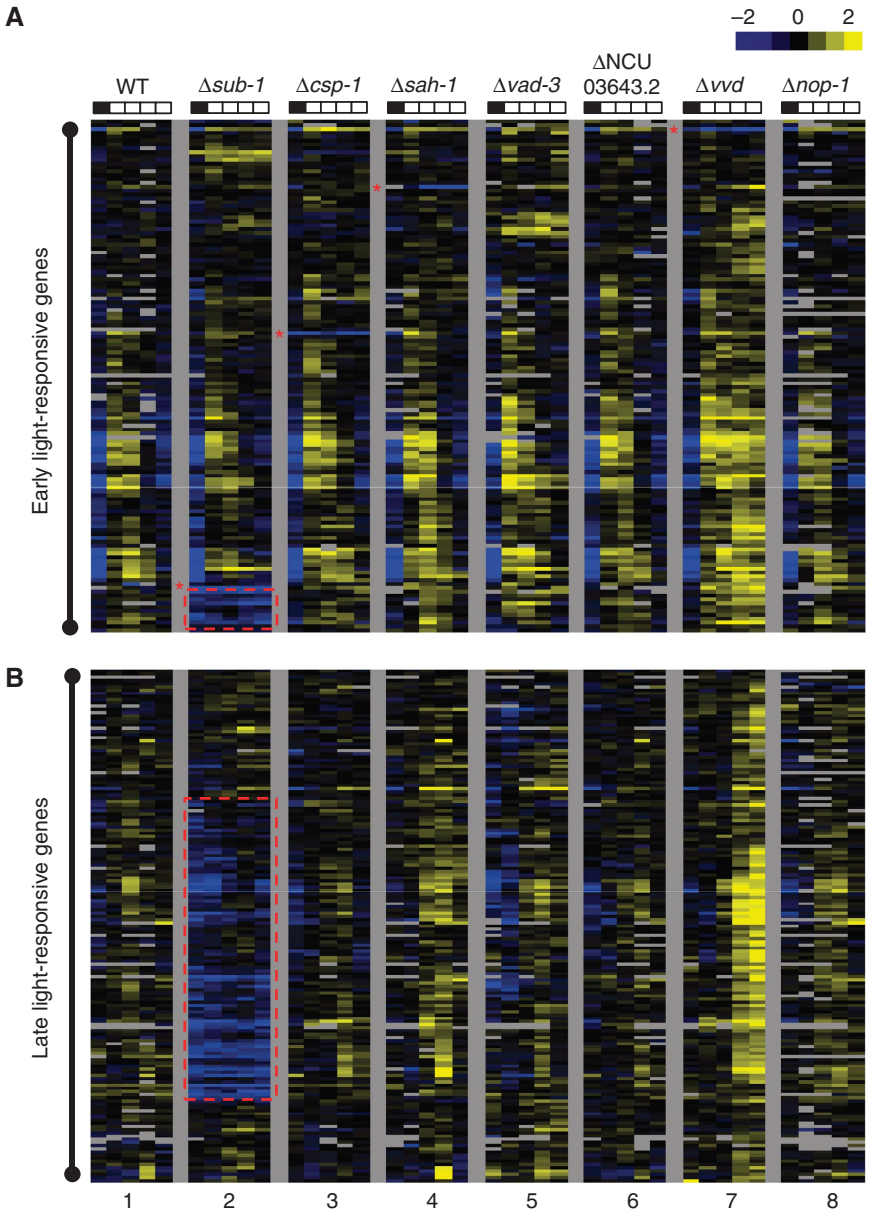

Figure 4 SUB-1, an early light-responsive TF, is involved in regulating some early and most late light responses. (**A**) Comparison of early light-responsive genes. (**B**) Comparison of late light-responsive genes. Lane 1, 74A (WT); Lane 2, Δ*sub-1* (NCU01154.2); Lane 3, Δ*csp-1* (NCU02713.2); Lane 4, Δ*sah-1* (NCU04179.2); Lane 5, Δ*vad-3* (NCU06407.2); Lane 6, ΔNCU03643.2; Lane7, Δ*vvd*; Lane 8, Δ*nop-1*. For each lane, from left to right, the individual columns correspond to light treatment for 0, 15, 30, 60, 120 min, respectively. For each row, the data were centred across different columns before clustering. Square colours are as described in Figure 1. Red asterisk to the left indicates the row corresponding to the knocked out gene in the respective knockout strain. Vertical red dashes identify clusters of genes whose light induction is abrogated in the Δ*sub-1* strains.

(1998) and Chakravarty *et al* (2007). For both types of light responses, the top five motifs identified and ranked by *SCOPE* are shown in Figure 6A and B. The number one motif identified from early light responses (Figure 6C, total 1282 counts for 123 genes with 97.6% coverage, *Sig value* = 114.1) is related to the core consensus sequence of the LRE that is bound by the WCC and has been shown to be necessary and sufficient for mediating light induction of the clock gene *frq in vivo* (Froehlich *et al*, 2002). Although *SCOPE* identified the core sequence, it is likely that this core is paired with another in close proximity to be effective, as seen in the *frq* promoter. This result supports the accuracy of motif prediction by *SCOPE*, although it is clear that additional context or sequence information, not detected by SCOPE, is needed to govern ELRGs. The distribution of this ELRE core motif among all early light-responsive genes is shown in Supplementary Figure 5A. In terms of regulating the late light responses, one of the top putative LLREs seemed particularly interesting (Figure 6D, ranked as number two in Figure 6B). Although this element is associated with only 154 genes (43.5% coverage), its relatively constant position (mostly within 500 bp upstream from the promoter of late light-responsive genes, Supplementary Figure 5B) and infrequent occurrence (~1.8 counts per gene) suggested

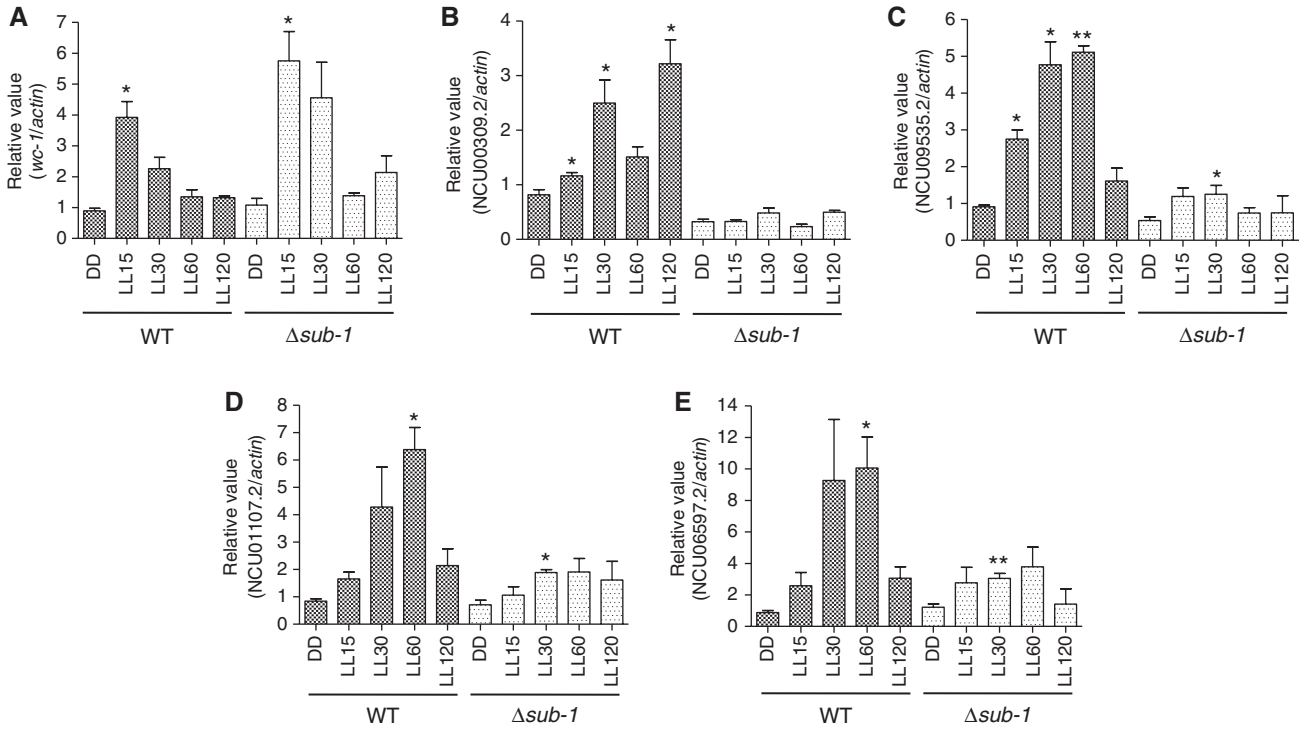

Figure 5 SUB-1 is required for efficient transduction of light signals to some ELRGs and most LLRGs under WCC control. (**A**) RT–QPCR analysis of light induction of *wc-1* in WT and Δ*sub-1*. (**B**, **C**) RT–QPCR analysis of light induction of two early light-responsive genes, NCU00309.2 and NCU09535.2 in WT and Δ*sub-1*. (**D**, **E**) RT–QPCR analysis of light induction of two late light-responsive genes, NCU01107.2 and NCU06597.2 in WT and Δ*sub-1*. The results obtained by three independent biological replicate experiments are shown here. Columns represent mean values ± standard error; asterisks indicate statistical significance when compared with DD time point, as determined by paired *t*-test, $*P<0.05$, $**P<0.01$.

functionality. For other over-represented putative ELREs and LLREs (Figure 6A and B), the detailed information can be obtained, along with the list of early and late light-responsive genes (Supplementary Tables II and III) at *SCOPE* website: http://genie.dartmouth.edu/scope/.

The promoter sequences from two verified late light-responsive genes, NCU01107.2 (Figure 5D) and NCU06597.2 (Figure 5E), were used to test the light function of this putative LLRE *in vivo*. As revealed by *SCOPE*, there are two overlapping LLRE motifs in each of the promoter sequences, each being 1 bp apart on opposite strands (Figure 6E and F). The intergenic regions upstream of the NCU01107.2 (622 bp) and NCU06597.2 (1140 bp) translation start site were fused to a luciferase reporter (Gooch *et al*, 2008) and integrated into the *his-3* locus. The reporter precisely recapitulates the endogenous late light-responsive pattern of expression as shown in Figure 6G and H (compared with their endogenous light induction in Figure 5D and E), with a peak at LL60. We used the expression of *actin*, which is not altered by light, as an internal reference for comparison (bottom panel, Figure 6G and H). Site-directed mutagenesis (Figure 6E and F) was used to change the sequences of the LLRE driving luciferase, and this construct was examined for function after transformation and integration at *his-3*. In these transformants, the amplitude of light induction driven by the promoters with mutated LLRE is decreased by about 50% compared with the promoters with WT LLRE. The data suggest that this LLRE is indeed a functional motif and is required for full induction of late light responses *in vivo*. However, a consistent light response was still observed from the mutated promoters, and this coupled with the fact that only 43.5% of late light-responsive genes contain this LLRE, which suggests that additional *cis*-regulatory motifs remain to be discovered for regulating late light responses.

Light enhances the direct binding of the WCC to the sub-1 promoter

To establish a direct molecular connection between WCC and the induction of *sub-1*, we asked whether the WCC can directly bind to the *sub-1* promoter in response to a light stimulus. As shown by an earlier study (Froehlich *et al*, 2002), a promoter sequence with adjacent repeats of the LRE might serve as a good indicator for where the WCC binds. As revealed by *SCOPE*, there are 11 putative core ELREs within 3000 bp upstream of the *sub-1* translation start site, with six appearing within a 26-bp region between −454 and −429 bp upstream of the *sub-1* start codon (Figure 7A). Therefore, we chose this region as a candidate for a direct binding site of the WCC.

Chromatin immunoprecipitation (ChIP) analysis using antibodies against WC-2 has been successfully applied to study the binding of the WCC to the promoter of several ELRGs *in vivo* (He and Liu, 2005; Belden *et al*, 2007b), so ChIP was performed to examine the direct binding of WCC to the *sub-1* promoter. As shown in Figure 7B (semiquantitative PCR) and Figure 7C (QPCR analysis), 15 min of light significantly enhances the direct binding of WCC to the *sub-1* promoter compared with DD time point. The interaction is

A

Top 5 results from *SCOPE*			
Consensus sequence	Count	Sig value	Coverage
bvgatcb	1282	114.1	97.6%
rtncacta	175	30.2	58.5%
aacnrttgt	48	16.0	28.5%
atcgrnnnsba	183	12.0	66.7%
cacta	589	10.7	86.2%

B

Top 5 results from *SCOPE*			
Consensus sequence	Count	Sig value	Coverage
Taata	2001	181.1	74.0%
rtgayrtca	122	117.7	43.5%
taanwhndnnwata	458	107.9	16.9%
atannnntaa	765	98.5	38.3%
atannnnnnntaa	748	94.8	40.9%

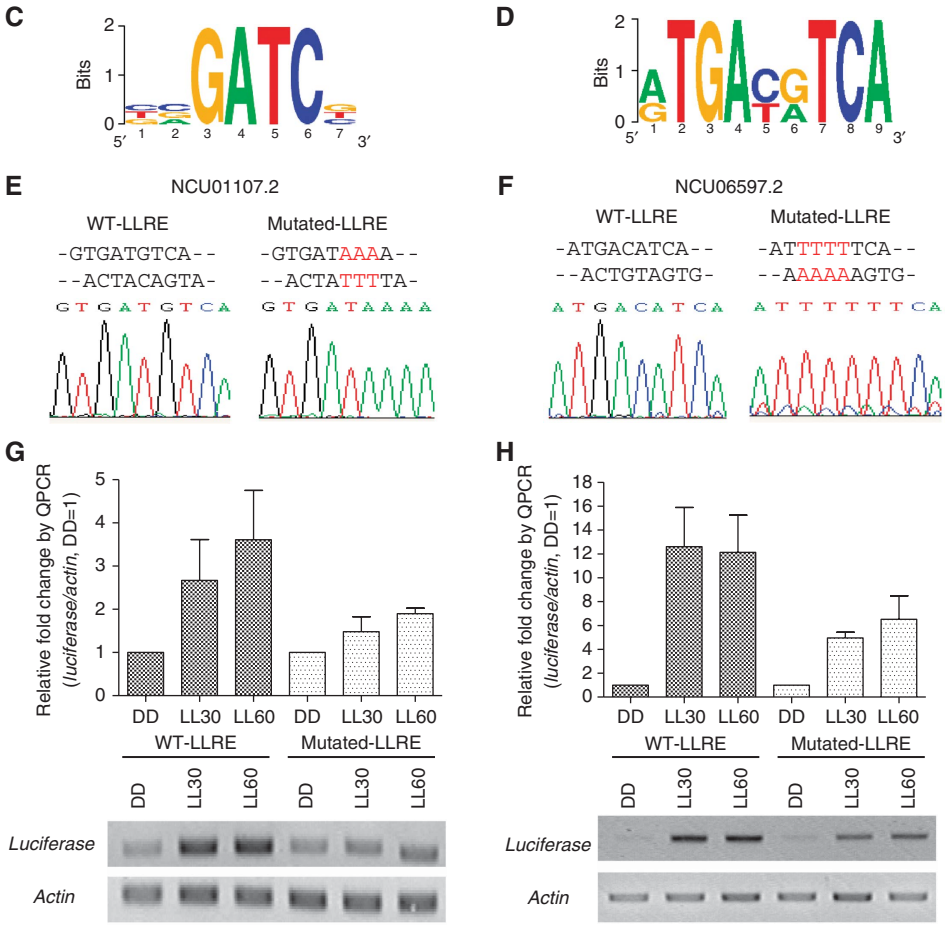

Figure 6 An LLRE contributes to mediating late light responses *in vivo*. (**A**, **B**) Top 5 ELREs and LLREs as predicted by *SCOPE*. (**C**, **D**) Sequence logo of a selected ELRE and LLRE. (**E**, **F**) Sequencing data of the WT LLRE versus a mutated LLRE. The mutated nucleotides are shown in red. (**G**, **H**) RT–QPCR analysis of light induction from a luciferase-containing transcript using the promoter sequence from NCU01107.2 and NCU06597.2 with either WT or mutated LLRE. Upper panel: data from RT–QPCR. Lower panel: data from semiquantitative RT–PCR. The results obtained by three independent replicate experiments are shown here. Columns represent mean values ± standard error.

eliminated after 60 min in constant light, presumably reflecting the ongoing photoadaptation process. As a positive control for the light treatment and ChIP sample preparation, we confirmed the direct interaction between the WCC and the proximal LRE (PLRE) on the *frq* promoter in response to light (Belden *et al*, 2007b) (Figure 7D and E).

Discussion

The transcriptional response of *Neurospora* to light has been described here on a genome-wide scale. Consistent with data from many labs (Dunlap and Loros, 2004, 2006; Corrochano, 2007; Herrera-Estrella and Horwitz, 2007), the majority of light responses depend on the WCC, which initiates a cascade of subsequent activities (Figure 8), including WC-1-dependent acetylation of histone H3 associated with the early light-responsive gene, *al-3* (Grimaldi *et al*, 2006). Once activated by light, WC-1 undergoes a light-induced conformational change and forms an L-WCC with WC-2 that activates early light responses by a direct binding to the ELRE of ELRGs, including *vvd* (He and Liu, 2005), *wc-1* (Froehlich *et al*, 2002; He *et al*, 2002) and, as shown here, *sub-1*. This happens rapidly after lights-on, certainly within minutes, at least for some genes (Crosthwaite *et al*, 1995). Late light responses, which peak about 30 min later or more, are shown here to be regulated in part by SUB-1, an early light-responsive TF. Without SUB-1, the amplitude of light induction appears to be severely impaired for some ELRGs and most LLRGs, supporting its important role in the *Neurospora* light signalling cascades. Interestingly, we found that the amplitude of light responses is tightly linked to the level of FRQ as

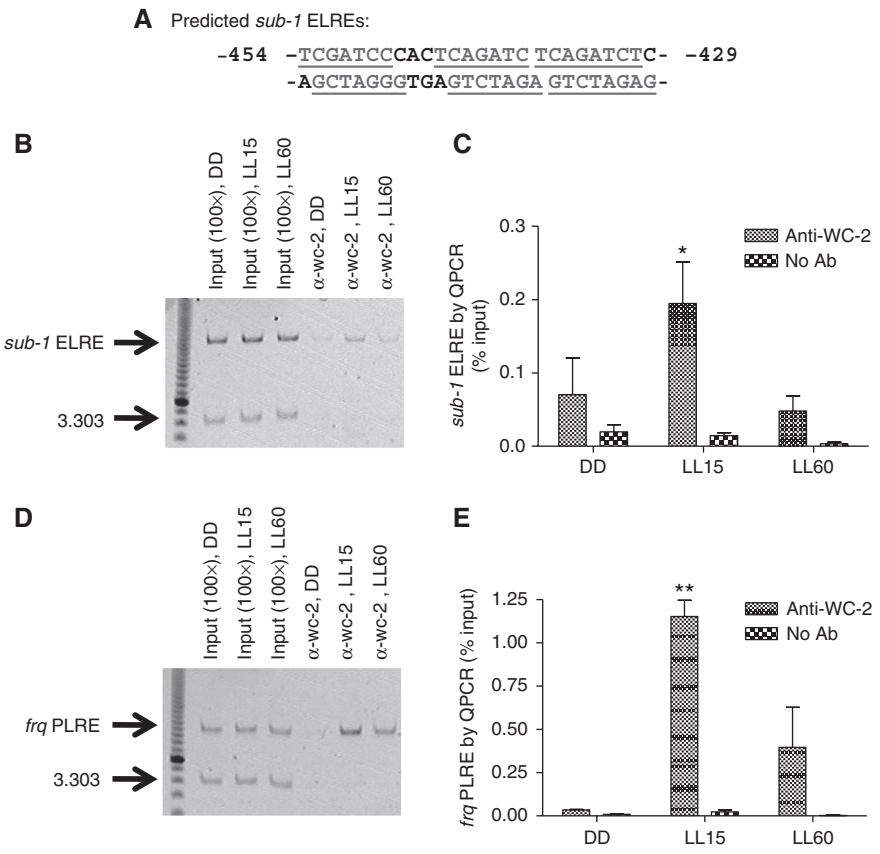

Figure 7 Light enhances the direct binding of the WCC to the *sub-1* promoter. (**A**) Six adjacent ELREs on the *sub-1* promoter (shown in red). (**B**, **C**) ChIP analysis of *sub-1* ELRE and (**D**, **E**) *frq* PLRE was performed with antibodies against WC-2. 3.303 indicates a primer pair targeting a noncoding region in the *Neuropsora* genome (Belden *et al*, 2007a). No Ab indicates no-antibody control. QPCR data were obtained for three independent replicate experiments. Columns represent mean values ± standard error. Asterisks indicate statistical significance when compared with the DD time point, as determined by paired *t*-test, $*P<0.05$, $**P<0.01$. A full-colour version of this figure is available at *The EMBO Journal* Online.

suggested in earlier study (Merrow *et al*, 2001). In the frq^7 strain, the amplitude of both early and late light responses was noticeably boosted. FRQ has been shown to positively regulate the amount of WC-1 posttranscriptionally (Lee *et al*, 2000; Schafmeier *et al*, 2006), and these data provide additional molecular evidence that the light function of WC-1 is indeed under the control of the clock, possibly through direct interaction with FRQ. In addition, we observed photoadaptation defects in the Δ*vvd* strain for both early and late light responses, suggesting that there might be a common underlying mechanism used by VVD to serve as a universal brake on light effects. One possible explanation is that VVD, after light activation, might temporarily inhibit the function of WCC through some posttranslational modification, presumably phosphorylation (Heintzen *et al*, 2001; Schwerdtfeger and Linden, 2001; He and Liu, 2005). Therefore, when VVD is absent, WCC constitutively activates ELRGs, including *sub-1*. In turn, increased SUB-1 together with other, unknown, activators continuously activates LLRGs, resulting in photoadaptation defects on a genome-wide scale.

These data also confirm the existence of residual, albeit low amplitude, light responses in the absence of WC-1 in *Neurospora*. This has been a topic of some confusion with an initial result later linked to the use of *wc* strains that were still partially functional mutants (Dragovic *et al*, 2002; Heintzen and Liu, 2007). Studies using gene replacements of each *wc* gene (Collett *et al*, 2002; Lee *et al*, 2003) showed complete loss of light-induced gene expression for specific individual genes. This genome-wide microarray analysis has confirmed the total loss of light responsivity in earlier studied genes but did identify a few novel genes of unknown function whose expression responded to light in strains lacking WC-1 but not in WC-2. We are pursuing the nature of this response, including the determination of whether it is actually mediated by a photoreceptor as distinct from a physical process. But, notably, in the absence of a WC-1 homologue, residual light responses have been reported in *Trichoderma* (Rosales-Saavedra *et al*, 2006) and are a possible explanation, although not the only one, for light responses in *Phycomyces* (Idnurm *et al*, 2006). Consistent with the findings here, these residual light responses appear to be comparatively weak in most cases. Therefore, the physiological significance of these residual light responses remains to be determined. It can be noted that, even without a specified photoreceptor, endogenous reactive oxygen species generated by a photon stimulus might serve as an alternative input to pass on light signals to downstream targets (Schroeder and Johnson, 1995; Bouvier *et al*, 1998; Yoshida and Hasunuma, 2004; Iigusa *et al*, 2005). Such an ancillary pathway might become more influential when the primary light-sensing machinery is absent.

A novel and important factor in light responses, SUB-1, was identified in this study. Preliminary data (not shown)

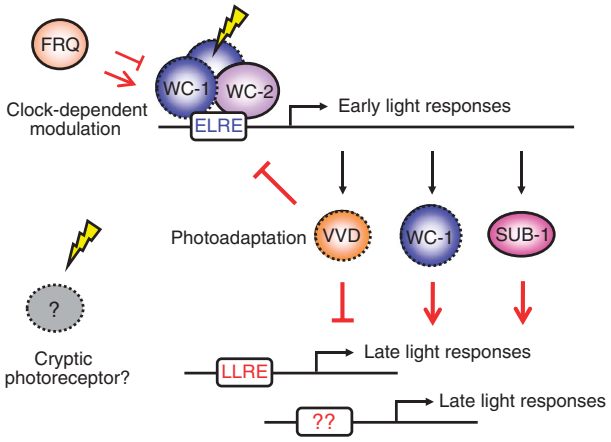

Figure 8 Model of a hierarchical light-sensing cascade in *Neurospora crassa*. Our results reveal an ordered sequence of molecular events resulting in light-regulated gene expression in *Neurospora*. The induction of early light responses is controlled primarily by WCC through direct binding to the ELRE on the promoter of ELRGs, including *vvd*, *wc-1* and *sub-1*. In turn, SUB-1 is required for efficient transduction of light signals to some ELRGs and most LLRGs. Meanwhile, in contrast to the ELRE, a novel LLRE is shown to be required for the full induction of late light responses *in vivo*. In addition to the modulation of induction amplitude by the clock protein FRQ, both ELRGs and LLRGs in constant light are subject to the repression mediated by VVD. Intriguingly, some residual light responses can still be observed in the absence of WC-1. Components capable of sensing light directly through a chromophore are marked with dashed lines in the figure.

suggested that simple expression of SUB-1 by itself in the dark was not sufficient to induce late light responses, suggesting that SUB-1 was activated or modified in a light-dependent manner, or was interacting with a light-regulated factor(s), leading to downstream activation of late light responses. As shown here, even without SUB-1, other factors can still activate several LLRGs although with a much lower amplitude of induction. Given the fact that late light responses share many regulatory features with early light responses (e.g. modulation by both FRQ and VVD; complete dependence upon functional WCC), WCC becomes a likely candidate for the missing factor. However, attempts to show a physical interaction between SUB-1 and WCC by co-immunoprecipitation were not successful (data not shown), indicating their interaction is weak or transient or direct physical interaction is not required for mediating late light responses. Both the substantial reduction in gene activation in response to light when the LLRE is mutated and severe loss of the late light response in Δ*sub-1* strains mutually support SUB-1 as a light-responsive TF interacting with this LLRE. Nevertheless, attempts to show a direct interaction *in vivo* using a ChIP assay with V5-tagged SUB-1 were inconclusive. Clearly, both SUB-1 and the LLRE contribute to the late light response, although the entire relationship between SUB-1 and the LLRE is not yet fully understood.

It should be noted that the culture conditions and experimental design may have altered the set of light-responsive genes identified. For instance, we failed to identify two known light-inducible genes, *ccg-1* (Arpaia *et al*, 1995) and *fluffy* (Belden *et al*, 2007a), as well as a few light-responsive targets reported in a separate microarray study that used 2% sucrose as the major carbon source (Lewis *et al*, 2002). The function of CCG-1 is still unknown, but its expression is strongly repressed in the presence of glucose (McNally and Free, 1988; Xie *et al*, 2004). Therefore, it is not surprising that the light induction of *ccg-1* was impaired as well under our high glucose condition (2% glucose). However, because most light responsive genes actually have a higher amplitude of induction under high glucose condition (2% compared with 0.1% glucose medium, data not shown), medium with 2% glucose was used for this study to enhance the sensitivity of the microarray detection. Meanwhile, several light-responsive genes have also been shown to have altered induction in the presence of the *ras-1*^{bd} allele that has commonly been present in strains analysed for photoresponses (Dunlap and Loros, 2005; Liu and Bell-Pedersen, 2006). For instance, light induction of the TF *fluffy* is significantly reduced in the WT strain 74A (Belden *et al*, 2007a) when compared with strains carrying the dominant RAS allele *ras-1*^{bd}. To reflect the native light responses in *Neurospora*, we used strains without the *ras-1*^{bd} mutation in this study. A recent study focusing on genetic network models of the circadian clock in *Neurospora* (Dong *et al*, 2008) report light-responsive genes from microarray analysis using a *ras-1*^{bd} strain grown in galactose/Fries-based medium. Response to light was followed between 20 min and 24 h in constant light although total growth time was not controlled. After 24 h of light treatment, 60% of their total detectable transcripts were reported to be potentially light responsive. The involvement of RAS signalling pathways in both light input and clock output further highlights the complexity underlying the regulatory networks of the *Neurospora* light-sensing cascade.

SUB-1 is a GATA type zinc finger TF, first identified as a mutant resulting in the development of submerged protoperithecia (Colot *et al*, 2006). There are only six GATA zinc finger family members (out of 176 TFs) in the *Neurospora* genome (Borkovich *et al*, 2004). In addition to SUB-1, WC-1 and WC-2 are members in this family and are both essential components in controlling *Neurospora* light responses. Therefore, although some biological functions have been associated with the other three GATA members (Fu and Marzluf, 1990; Zhou and Marzluf, 1999; Feng *et al*, 2000) and none were light induced, their involvement with light responses might be worth of re-evaluating. Notably, unlike other zinc finger family members, GATA factors are found exclusively in eukaryotic organisms, which might provide insight into the evolution and conservation of light signalling components in the fungal and possibly other kingdoms.

Are there additional photoreceptors or light-inducible TFs beyond WC-1, VVD and SUB-1 involved in controlling transcriptional responses to light in *Neurospora*? On the basis of our profiling data, the influence from additional TFs and putative photoreceptors appears to be minor at most. Their biological function in response to the light stimulus might either not relate to the modulation of transcription or could become evident at specific developmental stages or under different growth conditions. In a recent study, the *nop-1* gene was reported not to be light responsive, as additionally found here, but to have a regulatory function late in development (Bieszke *et al*, 2007). Thus, in the case of *nop*-1, sample collection time was critical in uncovering function. This principle may hold true for the light responsive TFs identified in this study.

Materials and methods

Strains

The WT strain used here is OR74A. All single-knockout strains came from the *Neurospora* knockout project (Colot *et al*, 2006) and have been deposited in the Fungal Genetics Stock Center, Kansas City (www.fgsc.net). Double-knockout strains, 559-4 (*wc-1*::hph; *wc-2*::hph) and 560-8 (*phy-1*::hph; *phy-2*::hph), were generated by crossing single-knockout strains and confirmed by Southern blot. 521-2 (WT, frq^7) was generated by crossing 585-70 (*bd*, frq^7) with 74A to remove the band ($ras\text{-}1^{bd}$) mutation (Belden *et al*, 2007a). Strain 324-8 ($ras\text{-}1^{bd}$) was used for the ChIP assay. NCU strain numbers are from *Neurospora* annotation (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html).

Culture conditions and light treatment

Frozen conidia were inoculated onto a minimal slant (Davis and De Serres, 1970) one week in advance to generate fresh conidia. On day 0, conidia were suspended in sterile water for quantification. To form a mycelial layer, 1×10^7 of conidia or similar amount of mass (e.g. no asexual sporulation in Δ*csp-1*) were inoculated into a 10-cm Petri dish with 20 ml *Bird* medium (Metzenberg, 2004) containing 2% glucose. After 24 h of incubation in darkness at 25°C, a mycelia plug was cut with a No. 4 cork borer (8 mm diameter) and transferred into a 125-ml flask with 50 ml *Bird* medium containing 2% glucose. All procedures were performed under a low red light environment to avoid any possible light-stimulating effects (Aronson *et al*, 1994).

After another 24 h of culture with constant shaking (125 r.p.m.) in darkness (DD) at 25°C, the flasks were moved to a shaker at 25°C with a continuous white light stimulus (LL), covering a wide range of the spectrum from 400 to 700 nm (cool white fluorescent light bulb, General Electric F20T12-CW, 40–50 μmole photons/m^2/s), and then harvested before (DD) and after 5 (LL5), 10 (LL10), 15 (LL15), 30 (LL30), 45 (LL45), 60 (LL60), 90 (LL90), 120 (LL120) or 240 (LL240) minutes of white light treatment. Using vacuum filtration, mycelia were harvested and immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Microarray probe sequence and layout format

Detailed information can be found online at Home of the Filamentous Fungal Microarray Database (http://www.yale.edu/townsend/Links/ffdatabase/downloads.html).

Image and data analysis

The slides were scanned with a GMS 418 microarray scanner (Affymetrix). After griding with ScanAlyze 2.51, the raw data were uploaded and normalized with GeneTraffic software (Stratagene). Spots of insufficient quality were flagged by visual inspection and signal to background noise ratio. Because of the fact that some light-inducible genes have almost no transcription in the dark, only spots with a fluorescent intensity more than one-fold (at least over 200) in the sample channel and more than two-fold (at least over 400) in the reference channel over local/average background were selected for further analysis. Genes missing more than 25% of their measurements from 90 microarrays were all excluded. Under the culture conditions used, we obtained reliable measurements for 5588 spots equivalent to about 54% of the genes in *Neurospora*. Genes with absolute values of a log-2 ratio greater than 0.5 on at least two arrays while having more than four-fold changes (max–min) across 90 microarrays were all included for final clustering and SAM analysis. In total, 2864 out of 10 372 spots passed these criteria. Unsupervised hierarchical clustering was done using Cluster 3.0 (Eisen *et al*, 1998) with the method of average linkage after centering with mean value. Graphical representations of clustering results were generated using TreeView 1.0.13. Complete datasets of all 135 arrays are available on Gene Expression Omnibus (reference number for access: GSE8932, http://www.ncbi.nlm.nih.gov/projects/geo).

Sample preparation for microarray, microarray hybridization, quantitative RT–PCR, semiquantitative RT–PCR, site-directed mutagenesis, ChIP assay, bootstrapping and SAM analysis

These methods are described in Supplementary data available at *The EMBO Journal* Online (http://www.embojournal.org).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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