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Review

Light input and processing in the circadian clock of *Neurospora*

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ABSTRACT

Circadian clocks are endogenous oscillators that use zeitgebers as environmental cues to synchronise with the exogenous day–night cycle. The role of light as a zeitgeber has been investigated intensively to date. In *Neurospora crassa* the transcription factor White Collar Complex (WCC) is directly activated by light, which resets the clock. In addition, a hierarchical cascade of transcription factors activates the light-induced expression of hundreds of genes. Disturbance of the clock during the day through changes in light intensity should be prevented to ensure efficient synchronisation. This can be achieved by desensitisation to the ambient light (photoadaptation). Photoadaptation in *Neurospora* is dependent on the blue light receptor Vivid (VVD), which accumulates immediately after light activation and rapidly silences the expression of WCC-dependent genes. Recent studies have elucidated the molecular mechanism of VVD-mediated photoadaptation. Here we review the increasing knowledge about light-dependent gene expression and photoadaptation in *Neurospora* and discuss their relevance for synchronisation of the circadian clock.

1. Introduction

Life on earth depends on light, primarily because photosynthesis constitutes the fundament of the food chain and defines the composition of the atmosphere. In addition, for the vast majority of life forms light provides environmental information, which is for example used as a zeitgeber for circadian clocks. Zeitgebers are used to synchronise the endogenous clock with the geophysical cycle of day and night, a process called entrainment. To entrain circadian clocks, light activates photoreceptors and information is integrated via signal transduction pathways into the central oscillating unit, which in eukaryotes is constituted by a transcriptional and translational autoregulatory feedback loop [1–5]. In the ascomycete *Neurospora crassa* – an intensively studied model organism for circadian clocks – light resetting of the clock is achieved via activation of the blue light receptor White Collar-1 (WC-1), which is a subunit of the heterodimeric clock transcription factor White Collar Complex (WCC) [6]. Thus, in *Neurospora* a core clock component directly provides an input pathway by integrating blue light information into the circadian system and guarantees the synchronisation of the endogenous clock to the 24 h day–night cycle. Further examples for physiological processes that are regulated by responses to blue light are the production of carotenoids and the development of conidia, all of which are also clock-controlled processes [7–12]. Current genome-wide analyses revealed that blue light directly or indirectly controls expression of hundreds of genes in *Neurospora* via the WCC. It could be shown that light-responsive gene expression is activated by a hierarchical transcription factor cascade [13,14].

Sequencing of the *Neurospora* genome revealed several genes encoding putative light receptors [15], suggesting that light regulates various physiological processes in filamentous fungi. In addition to the previously characterised blue light receptors WC-1 and VVD [6,16,17], genes encoding orthologs of phytochromes (*phy-1* and *phy-2*), cryptochromes (*cry*) and rhodopsin (*nop-1*) were identified [18–20]. Cryptochrome appears to be involved in light-entrainment of the circadian clock [19]. In addition, cryptochrome, phytochrome and rhodopsin were found to be involved in the photoactivation of the expression of *con-10*, a gene involved in the formation of asexual spores in *Neurospora* [12]. However, to date there is still poor knowledge about other physiological roles of these light receptors in *Neurospora*.

Contrary to that the function of WCC and VVD in blue light responses in *Neurospora* has been investigated in detail during the past 20 years. Recent studies provided new insights into the molecular mechanisms of WCC light-activation and photoadaptation, both of which are essential to guarantee a robust oscillation of the circadian clock in natural photocycles. This review discusses current knowledge of regulation of blue light-dependent gene expression in *N. crassa*, which is the fundament of synchronisation of the circadian clock to day-night circles.

2. LOV domains and blue light-dependent gene expression in *Neurospora*

All light responses in *Neurospora* that are known to date depend on blue light [21,22] and are achieved by two photoreceptors: WC-

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1 and VVD. There is, however, evidence for additional light input pathways by yet unidentified photoreceptors as shown by analysis of light induction in wc-1 (and wc-2) deletion strains [23]. WC-1 and VVD each contain a LOV (light–oxygen–voltage) domain, which is a member of the Per-ARNT-Sim (PAS) domain superfamily [6,24]. LOV domains have been first described to mediate blue-light signalling by phototropins, which function as photoreceptor kinases in plants [25]. In the following years, LOV domain containing proteins have been identified in bacteria, plants and fungi and function as reversible photo-switches, thereby regulating numerous blue light-dependent processes including chloroplast movement, stomatal opening, phototropism and resetting of circadian clocks [26,27]. LOV domains are functionally conserved, as indicated by rescue of function after replacement of fungal with plant LOV domains [28].

All LOV domains share a common tertiary fold that creates a binding pocket for a flavin chromophore, which is either flavin mononucleotide (FMN) of flavin adenine dinucleotide (FAD) [29]. Light irradiation of LOV domains induces the formation of a covalent cysteinyl-flavin-photoadduct, resulting in a conformational change of the protein [30]. By that means, blue light can mediate alterations in protein characteristics, in particular protein–protein interaction, by affecting the chemical state of the cofactor [29]. The adduct formation is fully reversible in darkness. LOV domains can be divided into two subgroups on the basis of the decay kinetics of their photoadduct. In some LOV domains, for example in the well-studied LOV2 in phototropins, it is converted back into the dark state within seconds. Hence, the corresponding LOV domains are able to undergo rapid photocycles. Other LOV domains, mainly in fungi and bacteria form quite stable photoadducts that need several hours to be converted. Recently, characterisation of the thermal reversion of the VVD–LOV domain of *N. crassa* revealed that deprotonation of the nitrogen at position 5 in the flavin ring, steric stability of the cysteinyl-flavin-bond and electronic effects are rate limiting for the recovery of the dark state. N5-deprotonation likely depends on pH and solvent accessibility. Moreover, substitution of several amino acids within the active centre revealed two regions in the VVD–LOV domain, which determine the decay kinetics of its photoadduct. In VVD the N5 proton of the bound FAD is protected against solvent exchange mainly by an isoleucine at position 85 [31]. Hence, the stability of the cysteinyl-flavin-photoadduct of LOV domains directly depends on the amino acid sequence.

Via microarray analyses, initial studies estimated that hundreds of genes are regulated by the WCC in *Neurospora* [32,33]. However, until recently only a few direct targets were known. A current analysis using full-genome covering microarrays indicated that more than 300 genes are expressed light-dependently, thereby confirming the former assumption [14]. Another study, using ChIP sequencing, revealed that WCC binds to at least 400 target sequences in the *Neurospora* genome after light induction, among them the previously identified WCC-controlled genes *frq*, *vvd* and *al-3* [13,34]. Since not all identified genes overlap in both studies, the total number of WCC-controlled genes might be even higher than 400.

Light inducible genes can be roughly divided into an early (transcript levels peak within 15–45 min) and a late type (transcript levels peak within 45–90 min). Among the first group of light inducible genes, comprising 45% of the hits [14], are the negative regulators of the clock, *frq* and *vvd* as well as the *albino* genes (*al-1*, *-2*, *-3*), which are responsible for light-induced synthesis of carotenoids [35,36]. The second group, making up 55% of light-inducible genes [14], includes the clock controlled genes *ccg-1* and *ccg-2* [37]. One major question was how the difference in the timing of expression was achieved. The recent genome-wide analyses indicated that several transcriptional regulators are among the early light induced genes (Fig. 1A and [13,14]). One of these transcription factor genes is *submerged protoperithecia-1* (*sub-1*), the product of which is essential for proper protoperithecia formation [38]. SUB-1-target genes were found to be expressed light-dependently and cover a large subset of the late light-inducible genes [14]. The recent analyses support a model in which light-dependent expression of 5–20% of the genome is controlled by a WCC-based, temporally regulated hierarchical system of transcription factors (Fig. 1B and [13,14]).

In the absence of light cues, WCC activity oscillates in a circadian manner and drives rhythmic expression of many targets including the clock protein FRQ. FRQ in turn negatively feeds back on its own expression by inhibiting WCC activity in a way that it mediates casein kinase-dependent hyperphosphorylation of the

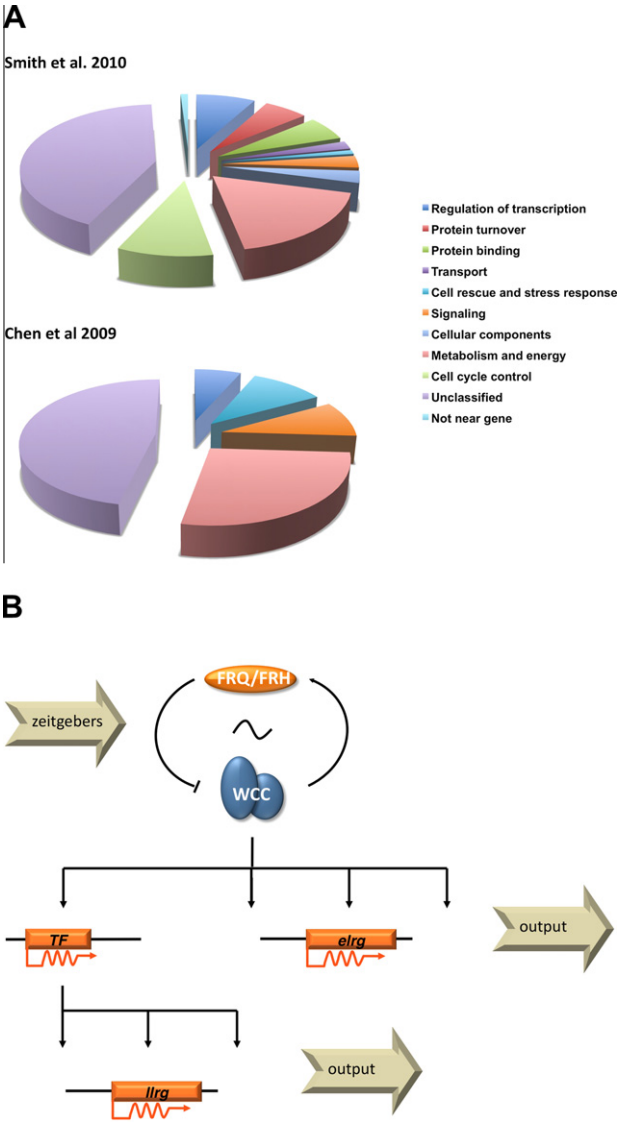

Fig. 1. (A) Light-induced gene expression in *Neurospora*. WCC-dependent and early light responsive genes adapted from Refs. [13,14]. Note that different algorithms have been used for classification of genes. (B) Simplified model for hierarchical light- and clock-controlled gene regulation in *Neurospora*. Light resets the circadian clock by activation of the WCC. Light-activated WCC enhances the expression of early light responsive genes (*elrg*). Among the *elrgs* are several transcription factors (TF), which activate the expression of late light responsive genes (*llrg*). *elrgs* and *llrgs* together are responsible for light- and/or clock-regulated alterations in physiological or metabolic processes (output). Further hierarchical steps are potentially generated by TFs among the *llrgs*.

transcription factor [39,40]. Phosphorylation of WCC is accompanied by dissociation of at least the WC-2 subunit from the DNA followed by CSW-1 dependent chromatin compaction [41]. FRQ is progressively phosphorylated over time and eventually degraded via the ubiquitin–proteasome pathway [42–44]. Consequently, FRQ levels oscillate in a circadian manner and regulate the daytime-specific WCC activity.

In addition to circadian regulation, which persists in constant darkness, the activity of WCC is negatively regulated in a light-dependent manner. The activation of light-induced gene expression is a transient process: After an initial burst of transcription, the levels of RNA decrease despite the presence of continuous light and reach a steady state level after 1–2 h [45]. This attenuation of light-induced gene expression is known as photoadaptation. In the adapted state, the expression of light-induced genes can be activated by application of light of higher intensity. The peak of expression, however, is lower than after initial induction, whereas the adapted state levels after the second light pulse are slightly increased [24,46,47] (see also Fig. 2, box).

Expression of blue light-dependent genes in *Neurospora* requires the WCC subunits WC-1 and WC-2, which are GATA-type zinc finger transcription factors [48,49]. WC-1 and WC-2 dimerise via their PAS domains to constitute the transcription factor complex [50–52]. Formation of the complex is crucial for the stability of WC-1, which is the limiting factor of the WCC due to its low levels compared to WC-2 [39,50,53]. When light activates WC-1, the WCC binds to light responsive elements (LREs) of promoters and drives gene expression [34,54]. WCC-dependent transcription is apparently accompanied by opening of the chromatin structure, which is reversed by the ATP-dependent remodelling enzyme Clockswitch-1 (CSW-1) [41]. Light activation of WCC results in a reduced mobility of the complex in electrophoretic mobility shift assays (EMSA). Consequently, distinct dark and light forms of WCC have been postulated [34,54,55]. Thus, light irradiation might either cause a substantial conformational change of WCC, allows oligomerisation of WCC protomers or promotes binding of additional factors. Many efforts have been made to elucidate the composition of the WCC in the light and dark state and it could be shown recently that blocking of the WC-1-LOV domain by a specific antibody prevents formation of the slower migrating, light specific form in an EMSA. Furthermore, yeast two-hybrid analysis revealed that the LOV domains of WC-1 interact in a light-dependent manner [56]. These results strongly imply an essential role of the WC-1-LOV domain in formation of the light form of WCC. Evidently, in the light state WCC is a dimer of two WCC protomers and dimerisation occurs via interaction of the light-activated LOV domains of the WC-1 subunits.

3. Photoadaptation: VVD enters the stage

In the beginning of this decade it has been shown that photoadaptation in *Neurospora* depends on the blue light receptor VVD, which is a small protein of 21 kDa containing a LOV domain with high similarity to that of WC-1 [16,17]. The LOV domain is preceded by an α-helix and a short extension which are, however, not essential for VVD-function [57]. *vvd* is expressed under control of the WCC and protein levels accumulate rapidly and transiently upon light irradiation [17]. In *vvd* loss-of-function mutants photoadaptation kinetics of light-induced genes is slow. Impaired photoadaptation manifests in enhanced pigmentation that is caused by an overexpression of light-inducible genes responsible for carotenoid production [17,58]. In addition to VVD, the transcriptional

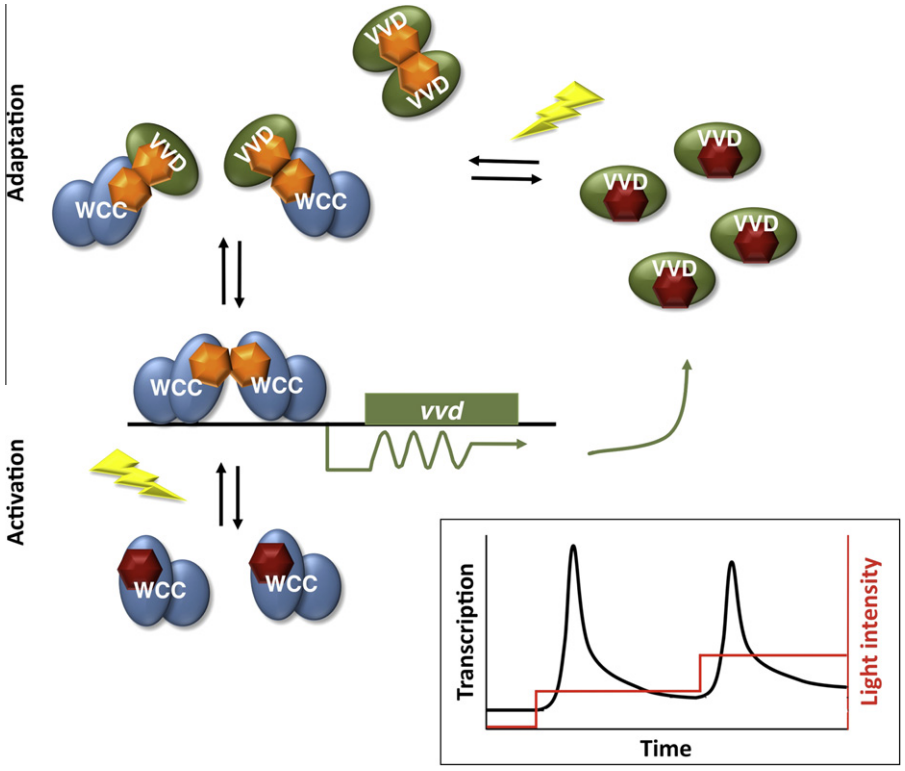

Fig. 2. Molecular mechanism of light-dependent activation of the WCC and photoadaptation. Light treatment of monomeric dark state WCC (red hexagons indicate dark state LOV domain) results in formation of the photoadduct (orange hexagons) and dimerisation. *vvd* transcription is activated and VVD protein accumulates. VVD forms a photoadduct and can either homodimerise or heterodimerise with WCC, thereby competing with the assembly of the WCC light complex. Box: WCC-dependent gene expression (black line) rapidly increases upon light induction. Transcription adapts to steady state levels after an initial peak. Increase of light intensity (red line) results in a second peak of transcription followed by adaptation of gene expression on slightly elevated levels.

corepressors RCO-1 and RCM-1, which are orthologues of Tup1 and Ssn6 from *Saccharomyces cerevisiae*, apparently play a role in regulating light sensitivity and photoadaptation in *Neurospora* [59]. Since the Tup1/Ssn6 corepressor complex inhibits multiple transcriptional activators in yeast by several modes of action [60], a possible function in modulating light responses in *Neurospora* could be a general inhibitory regulation of WCC-controlled gene expression.

Despite clear molecular evidence of its inhibitory function on light responses, the question arose what the physiological relevance of VVD could be. The onset of light induced gene expression (i.e., *al-1*, *frq*, *vvd*) is only marginally affected [24,47] and the circadian clock phenotype is almost comparable to wild type, yet a *vvd* loss-of-function mutant exhibits a slight phase delay of the clock [17]. However, by modulating photoadaptation of the WCC, VVD regulates the gating of light responses. Gating means that responsiveness of the WCC to light is modulated in a time-of-day specific manner. In particular, light-inducibility of WCC-controlled genes is higher in the subjective morning (CT 0, 6) than in the evening/night (CT 12, 18) [17]. To some degree, these observations are contrary to the findings of Merrow et al., who showed that light induction of *wc-1* and *al-1* is stronger during the subjective night than in subjective day [61]. Due to rising VVD levels, resetting of the clock by moderate fluctuations in light intensity during the day is prevented [17]. Moreover, VVD apparently sustains a daytime oscillator that is independent of the FRQ/WCC feedback loop. This oscillator keeps the circadian clock running during the light phase and thereby prevents constitutive resetting of the clock by light. By that means, the *Neurospora* clock keeps time during the day and is not simply reset by dusk [62].

Another function of VVD might be to enhance the robustness of the clock. The circadian system of *Neurospora* responds to light signals during the subjective night in a different manner dependent on the time of the light input. Light pulses perceived in the early night are interpreted as evening and result in a phase delay. Thus, circadian output, in particular conidiation, occurs later in the following cycle. Vice versa, administration of light pulses in the late night mimics dawn, which results in phase advance and conidiation takes place earlier [63]. In a *vvd* deficient strain the clock exhibits higher sensitivity to light pulses, resulting in increased phase responses. This effect could be reverted by overexpression of VVD from an inducible promoter [62]. These findings suggest that VVD is required to decrease the susceptibility of the *Neurospora* clock to disturbing light cues.

vvd transcription exhibits circadian oscillation after release into free running conditions (constant darkness) at least in the first period. Consequently, *vvd* levels anticipate dawn and are high at the first subjective morning, thereby muting the acute response to light at dawn [62]. In summary, VVD functions as a molecular brake that modulates light dependent processes in *Neurospora*. As a result, the circadian clock shows a variation in sensitivity to light throughout the day and is protected against disturbances by overshooting light responses during the photoperiod.

Beside its function in modulating light responses, VVD might also play a role in temperature compensation of the *Neurospora* clock phase. Although the period of the clock is well compensated against temperature fluctuations in *vvd* deletion mutants, the phase of conidiation is delayed at lower temperatures. Furthermore, VVD protein levels increase with falling temperatures, which results in a less active WCC [64]. To further elucidate the function of VVD in temperature compensation of clock output will be a future challenge.

In 2007 the crystal structure of VVD lacking 36 N-terminal amino acids (VVD-36) was resolved in its dark and light state [57]. In the dark, VVD-36 exhibits a compact, tightly packed form. When VVD-36 is exposed to light, the photoadduct is formed between the conserved Cys108 and the bound FAD. Photoadduct formation induces a conformational change in the N-terminal region of VVD-36. A mutant VVD-36 variant that contains a Cys to Ser exchange at position 71 is functionally inactive despite the fact that it can still undergo the photocycle, indicating that Cys71 is required for coupling of photoadduct formation to conformational change of the protein and thus signal transduction [57].

4. A molecular mechanism of photoadaptation

The molecular mechanism of WCC inactivation by VVD remained unclear for a long time. Biochemical analysis of subcellular distribution of WCC and VVD suggested that the proteins are located exclusively in the nucleus and cytoplasm, respectively [24,65]. Recent studies reinvestigated this issue by using live cell imaging of *Neurospora* strains expressing GFP-tagged versions of WCC subunits and VVD. It turned out that substantial amounts of WC-1–GFP and WC-2–GFP are located in the cytosol and that both proteins undergo rapid cycles of nucleo-cytoplasmic shuttling [66]. Moreover, it could be shown by fluorescence microscopy analysis of VVD–GFP localisation that the fusion protein is present in both compartments and is even enriched in the nucleus [56,67,68]. Furthermore, after formaldehyde crosslink of *Neurospora* cells and subsequent fractionation, large amounts of VVD were found in the nuclear fraction [56]. These findings indicate that the earlier proposed cytoplasmic localisation of VVD was probably based on a technical artefact, namely that due to its small size most of the 21 kDa protein diffused out of the nuclei during preparation. Thus VVD could directly affect the activity of WCC in the nucleus.

Recently, it has been reported that VVD forms instable dimers in a light-dependent manner [69]. This was not unexpected, due to the structural similarity of LOV domains to other members of the PAS domain superfamily that mediate protein–protein interaction [70]. Furthermore, it has been reported that LOV domains of the YtvA protein of *Bacillus subtilis* dimerise via a hydrophobic interface [71,72]. VVD dimerisation occurs via interaction of the light-activated LOV domains [69], suggesting that VVD and WCC could interact by a similar mechanism. However, attempts to prove interaction of both proteins by co-immunoprecipitation failed. The Dunlap/Loros lab recently performed a more sensitive approach introducing cross-linking followed by immunoprecipitation. The study revealed that VVD and a small amount of WCC might physically interact [67]. This finding has been confirmed by a study that was published by Heintzen and co-workers, who succeeded in co-immunoprecipitation of VVD and WCC even in the absence of cross-linkers [68]. At the same time, it could be shown in our group by yeast two-hybrid assays that the LOV domains of VVD and WC-1 form light-dependent heterodimers, similar to the previously shown homodimers of VVD [56,69]. Moreover, we could show that addition of VVD to light-irradiated nuclear extracts results in the disappearance of the slow migrating WCC form in EMSAs [56]. These observations strongly support the prediction that photoadaptation in *Neurospora* is mediated by light-dependent interaction of the VVD and WC-1-LOV domains. VVD and WC-1-LOV domains compete for interaction and replacement of a WCC protomer by VVD results in inactivation of the WCC. The observation that the light-dependent interaction of the LOV domains of VVD and WC-1 might be transient (as it is in VVD homodimers) could explain that co-immunoprecipitation of both proteins has not been observed previously.

Active WCC is rapidly degraded [66]. Inactivation of the light form of the WCC by VVD interaction prevents degradation of WCC. Since WCC has a slow photocycle of several hours [56], stabilization by VVD might be required to provide a time buffer so that the WCC can be converted back into the dark state and thus

maintaining a pool of light sensitive transcription factor. As a result the system is not completely blind to strong upshifts in light intensities, which is for example required for resetting the circadian clock.

VVD accumulates rapidly after light irradiation and VVD–WCC heterodimerisation competes with WCC–WCC homodimerisation. In contrast to other investigated light-dependent genes in *Neurospora*, the amount of VVD rises with increasing light over several orders of magnitude [56]. In the adapted state, WCC inhibition and VVD synthesis are in balance. The VVD-bound fraction of WCC can now be converted into its dark form. The slow decay of the photoadduct is the rate-limiting factor of conversion. A dramatic increase of the light intensity results in immediate activation of the dark-WCC pool and consequently to accumulation of more VVD protein. The current findings resulting in an improved insight into light-dependent gene expression and photoadaptation in *Neurospora* are summarised in Fig. 2.

5. The remains of the day: light memory keeps the clock running

Photoadaptation in *Neurospora* by VVD has been interpreted as being essential for gating of light input into the circadian clock [17]. Gating is considered to be important to restrict light synchronisation of the clock to a time around late night/early morning, thereby preventing "accidental" resetting by fluctuations in light intensity during the day, e.g. through changes between sunny and cloudy weather. However, *vvd* is also a clock controlled gene and its transcript levels rise in anticipation of the upcoming next day, which probably prevents an excessive induction of light-dependent genes by twilight in the early morning [62]. Furthermore, VVD protein is degraded in darkness with a half-life of 2–3 h [56]. Therefore, in natural day–night cycles VVD is present in considerable high amounts at all times, even during the night phase, where its levels gradually decline. Since the amount of VVD protein produced in the day-phase correlates with the light intensity, in the night the protein provides a memory of the preceding daylight [56]. This may be a mechanism to protect against spurious exposures to zeitgebers during the night, which are of considerable lower intensity compared to the sunlight of the day, e.g. from the moon or artificial light sources. Malzahn et al. have recently provided experimental evidence supporting this hypothesis: By performing racetube assays in natural day/night cycles the authors were able to show that moonlight was able to disrupt conidiation cycles in a *vvd* knockout strain, whereas it did not in a wild type [56]. Taken together, all functions of VVD that have been described to date contribute to a robust oscillation of the circadian clock of *Neurospora* in natural day–night cycles. The question remains if a similar mechanism does also exist in higher organisms. In mice, twilight does not influence the phase of entrainment in long photoperiods (12–18 h), suggesting an unknown adaptation mechanism in mammals [73]. Light application during the night, however, resulted in body-mass increase by shifting food intake behaviour into the light phase, when rodents usually rest [74]. Thus, low light application in the dark phase apparently is able to disturb the clock in mammals. Interestingly, there is a correlation between shift work and obesity in humans, underpinning the coincidence of shifted food intake and increased weight gaining [75].

6. Conclusions and perspectives

Attenuation of light-induced gene expression by physical interaction of LOV domains of inhibitory and activating photoreceptors is a novel and unconventional mechanism to downregulate light responses. In *Neurospora*, light adaptation is crucial for keeping the clock running in natural day/night cycles. This adaptation is achieved by competition of dimerisation of WC-1 LOV domains – and thus light activation of gene expression – by VVD. The concept of inhibitory LOV domains was recently introduced for phototropin 1 (Phot1) in higher plants [76]. Phototropins are receptor kinases that dimerise upon blue light irradiation and induce signal transduction cascades that mediate several processes like chloroplast accumulation, stomatal opening and phototropism. They contain two LOV domains, LOV1 and LOV2 [77]. LOV2 activation is essential for light signalling, whereas LOV1 is not required for that purpose. On the contrary, light activation of the LOV1 domain apparently attenuates signal transduction at high light intensities [76]. These findings incite to speculate about a photoadaptation mechanism of phototropins similar to that of the VVD/WCC system in *Neurospora* in a way that LOV1/LOV2 heterodimerization prevents receptor dimerization and thus intensity of downstream signalling. Further investigations might reveal if competitive interaction of inhibitory and activating LOV domains is a commonly used means to mediate desensitisation of photoreceptors to light.

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