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The synchronization and adaptation of *Neurospora crassa* circadian and conidiation rhythms to short light-dark cycles

Running title: Adaptation of Neurospora conidiation to LD cycles

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ABSTRACT Circadian clocks control the physiological and behavioral daily rhythms to adapt to the changing environment with a period of ~24 h. However, the influence and mechanism of extreme light-dark cycles on the circadian clock remain unclear. We show that, in the fungus *Neurospora crassa* under short LD cycles, both the growth rate and the ratio of microconidia production contributes to adaptation in LD12:12 (light for 12 h and dark for 12 h, periodically). Mathematical modeling and experiments demonstrate that in short LD cycles, the expression of the core clock protein FREQUENCY is entrained to the LD cycles when LD>3:3 while it free runs when $T \leq$ LD3:3. We investigated the changes in circadian/diurnal rhythms under a series of different LD conditions, and the results showed that conidial rhythmicity can be adapted to the short LD cycles. We further demonstrate that the existence of unknown blue light photoreceptor(s) and the circadian clock might promote the conidiation rhythms that resonate with the environment. A high-intensity light induced the expression of a set of downstream genes involved in various metabolic pathways. The ubiquitin E3 ligase FWD-1 and the previously described CRY-dependent oscillator system were implicated in regulating conidiation under short LD conditions.

KEYWORDS *Neurospora crassa*; circadian clock; FREQUENCY (FRQ); conidiation; light/dark cycle

The Earth rotates with a period of approximately 24 h, which results in the periodic change in of many environmental factors. Circadian clocks are the inner mechanisms that allow organisms to adjust their physiology and behavior to the daily cycling of environmental factors, e.g., light and temperature (Bell-Pederen *et al*. 2005).

In certain ranges, non-24h light/dark cycles can entrain the circadian rhythms, and the flexibility of this entrainability dramatically varies among different organisms. Within the range of entrainment which is also called the limit of stable entrainment, the rhythms change in accordance with the non-24 T-cycles and show stable phase angles; beyond the ranges, the circadian clocks free-run (Refinetti 2004; Madrid *et al*. 1998; Boulos *et al*. 2002; Abraham *et al*. 2010; Jewett *et al*. 1994). The entrainment ranges have been characterized in a number of species including human, hamster and Neurospora (Ralph and Menaker 1988; Nsa *et al*. 2015; Dong *et al*. 2008; Dunlap *et al*. 2004; Binkley 1990; Czeisler *et al*. 1999). In the wild-type filamentous fungus *Neurospora crassa*, the conidiation banding rhythms are consistent with a range of T-cycles that include LD6:6, LD12:12 and LD14:14, which illustrates the masking effects (Nsa *et al*. 2015). These observations have been extended to conidiation rhythms in LD3:3 and LD9:9 (Dong *et al*. 2008).However, under extremely short T-cycles that exceed the thresholds, the rhythms free run in certain species, including Neurospora, *Canavalia ensiformis* and sparrow (Dunlap *et al*. 2004; Binkley 1990).

It is very challenging for the endogenous circadian rhythms to be entrained to non-24 h LD cycles compared to the rest-activity rhythms (Czeisler *et al*. 1999; Lewis and Lobban 1957). Under such conditions, the endogenous rhythmicities may desynchronize with the behavioral rhythmicities, which leads to inadaptation or disorders that have been observed in a number of checked organisms (Czeisler *et al*. 1999; Ouyang *et al*. 1998; Highkin and Hanson 1954; Hut and Beersma 2011).

Neurospora is an important model organism for circadian clock study; it exhibits overt clock phenotype in its conidiation. On the molecular level, the core circadian clock system of Neurospora is composed of two positive elements: White Collar 1 (WC-1) and WC-2 and one negative element: FREQUENCY (FRQ). The FRQ/WC-based circadian oscillator (FWO) dictates the circadian rhythms at molecular and physiological levels (Hurley *et al*. 2015; Baker *et al*. 2012). WC-1 harbors three PER-ARNT-SIM (PAS) domains; it is responsible for

light responses of circadian clock as a blue light photoreceptor (Froehlich *et al*. 2002; He *et al*. 2002).

Neurospora has several additional validated or putative photosensing-associated factors, including VVD, CRY, NOP-1, PHY-1 and PHY-2 (Schwerdtfeger and Linden 2001; Corrochano 2007). VIVID (VVD), another PAS domain-containing protein, is a flavin-binding blue-light photoreceptor that regulates the light responses and temperature compensation of the Neurospora circadian clock (Zoltowski *et al*. 2007; Heintzen *et al*. 2001). Interestingly, *acry-dependent oscillator gate-1* (*cog-1*) mutation led to conidiation rhythmicity in constant light in Neurospora, suggesting a role for *cog-1* in regulating light sensing in the circadian clock. The *cog-1* related oscillator is called the CRY-dependent oscillator (CDO) system since it requires the blue-light photoreceptor CRYPTOCHROME (CRY) (Nsa *et al*. 2015).

In this work, we investigated the influence of short light-dark cycling conditions on the circadian clock in Neurospora, and compared the effects of the different light-dark cycles on growth, microconidia production, conidiation rhythmicity and gene expression. The findings of this work would shed new light on the knowledge of circadian clocks under conditions with extreme T-cycles.

Materials and Methods

Media, growth conditions and transformation procedure

The *Neurospora crassa 301-5* (*ras-1*^{bd}, *a*) strain was used as the wild-type strain in this work. The 2% LCM media contained 1Vogel's medium and 0.17% arginine, with 2% glucose. The race tube solid media consisted of 1Vogel's medium containing 2% glucose, 50 ng of biotin/ml, and 1.5% agar.

The 315-13 (*fwd-1*^{RIP}, his-3) strain, a strain in which the *fwd-1* was disrupted, was obtained from Dr Yi Liu's lab and described previously (He *et al*. 2003). The *vvd*^{KO} strain was obtained from FGSC. All of the strains information are listed in supplemental Table S1.

Conidia were inoculated to seed mycelial mats in petri dishes, and disks were cut from these and used for 50-ml liquid cultures under certain LD conditions. At designated time points the cultures were harvested by filtration, frozen in liquid nitrogen, and ground in liquid

nitrogen. Protein extraction, quantification and western blot analysis were conducted as described elsewhere (Görl *et al*. 2001). For western blotting, equal amounts of total protein (50 μg) were loaded in each protein lane of SDS-PAGE (7.5%) gels containing a ratio of 37.5:1 acrylamide/bisacrylamide.

An incubator (Percival Scientific, USA) was used to simulate different LD cycles, the white light intensity during light was 5000 lux or 1000 lux as indicated. The red light (λ= 660nm) was generated by an LED lamp, and the intensity was 5000 lux or 1000 lux as indicated. The blue light (λ= 470 nm) was generated by an LED lamp, and the intensity was 5000 lux or 1000 lux as indicated**.**

The counting of microconidia was conducted on a hemocytometer under an optical microscope. Basically, since the shape of microconidia is nearly round and they usually contain a single nucleus, we consider those hyphae with length/width ratio <1.5 as a subjective criteria as microconidia.

Race tube assay

The conidiation banding profiles were assayed on race tubes under standard conditions (Nsa *et al*. 2015). The growth front was marked every 24 h under a red safe light in all light conditions in this work. All race tube experiments were carried out at room temperature (25°C).

Protein analysis

Protein extraction, quantification, western blot analysis was performed as previously described (Guo *et al*. 2010).

Dynamic modeling

See supplemental materials for details.

RNA-seq and analysis

The strains subjected to RNA-seq were grown and the RNA samples were isolated from duplicates. All of the Pearson's Correlation values between each duplicates were >0.99. Tophat was used as the aligner to map the reads to the reference genome [N. crassa OR74A (NC12)]. The genes showing ≥2-fold increase were selected for further analysis. RNA-seq data sets are available at the GEO database (GSE108814). See supplemental materials for more details.

Statistical analysis

Data are mean ± SE or mean ± SD where indicated. n≥3. Student's *t* test was used for all statistical

133 analyses. * represents the p-value of the statistical tests is less than the significance level of 0.05 ($p \leq 0.05$);
134 ** represents $p \leq 0.01$ and *** represents $p \leq 0.001$.

135 ***Data availability***

136 All strains and reagents are available upon request. The RNA-seq data sets are available at the
137 GEO database (GSE108814). File S1 contains Table S1, Table S1, Figures S1-S5 and
138 supplemental methods and protocols for dynamic modeling and RNA sequencing. Table S1
139 in File S1 lists all Neurospora strains. Table S2 in File S1 lists the parameters of the
140 mathematical model. File S2 contains Table S3 that provides the up-regulated genes in *Δcry,*
141 *Δvvd, Δwc-1* in 5000 lux. File S3 contains Table S4 that lists the KEGG pathway of genes
142 differentially expressed in *Δcry, Δvvd, Δwc-1* in 5000 lux light and 1000 lux light. Figure
143 S1 in File S1 shows the conidiation rhythms of FGSC4200 strain. Figure S2 in File S1
144 shows the FRQ protein levels under different LD routines. Figure S3 in File S1 provides
145 the scheme for the mathematical model of *Neurospora* circadian rhythm. Figure S4 in File
146 S1 shows the impact of light sensors on the adaptation to short LD cycles. Figure S5 in File
147 S1shows the conidiation rhythms of *Neurospora* strains in short LD cycles. Figure S6 in
148 File S1 shows the growth phenotype and conidiation rhythms of indicated strains.
149

150 **Results**

151 ***The synchronization and resonance of Neurospora rhythms under LD routines***

152 To address how the short LD cycles affect the rhythms, we conducted race tube assays with
153 the wild-type strain (*301-5, bd)*, under the following series of short LD cycling conditions
154 including LD6:6, LD4:4, LD3:3, LD2:2, LD1:1, LD45min:45min and LD30min:30min,
155 which consisted of symmetric light and dark phases. Additionally, we tested Neurospora
156 growth in LD65min:25min, an asymmetric condition, to mimic the light/dark condition on
157 orbital flight. The results show that under LD6:6, LD4:4, LD3:3, LD2:2, the conidiation
158 displayed overt rhythms with periods that coincided with the LD cycles. However, when the
159 cycles were shorter, under even shorter cycles, the entrained conidiation rhythms were
160 abolished (Figure 1A).

161 The arrhythmicity of Neurospora under extremely short LD cycles (e.g., LD1:1) could be
162 explained by two possibilities: 1) the conidiation rhythms were abolished; 2) the bands were

too compact and ambiguous to detect. To validate, we used the non-band strain FGSC4200 which grows very fast in race tube. In constant dark FGSC 4200 shows mild conidiation rhythms (Belden *et al*. 2007), while in LD1:1 it showed weak but recognizable conidiation rhythms (Figure S1 in File S1). These data suggest that although the free-running circadian period of Neurospora is approximately 22 h, the conidiation rhythm can be induced by extremely short LD cycles.

The circadian clock can adjust to fit with the cycling environment, especially the light. We compared the growth rate of two *frq* mutants frq^2 and frq^7, which possess shorter and longer free-running periods in constant dark, respectively (Gardner and Feldman 1981). The race tube assay results reveal that in LD9:9 the growth rate of frq^2 was faster while in LD13.5:13.5 frq^7 was faster (Figure 1, B and C), suggesting a resonance of Neurospora circadian period to the environment.

However, when we compared the growth rate of the wild-type strain under various LD cycles, we got unexpected results showing that Neurospora grew much faster in constant dark than that in constant light or most LD conditions. Moreover, the growth rate in shorter LD cycles was not necessarily slower than LD12:12. For instance, the growth in DD, LD6:6, LD3:3 and LD1:1 was significantly faster than that in LD12:12. The growth under LD65min:25min was also significantly faster than LD12:12 (Figure 1D).

Neurospora microconidia are small uninucleate spores that serve as male gametesor as asexual reproductive structures (Maheshwari 1999). When we counted the ratio of microconidia under LD12:12 and short LD cycles, respectively, and the results show that the ratio of microconidia in LD12:12 was significantly higher than those in other LD conditions (Figure 1E).

Expression of the FRQ protein in different LD cycles

As the circadian clock is involved in controlling the conidiation rhythmicity, we asked whether the molecular rhythmicity of the FRQ protein is subject to changes in response to the length of the LD cycles. As one of the core factors in Neurospora circadian clock, FRQ acts as the negative component in the transcription/translational feedback loop (Baker *et al.,* 2012). The western blotting results show that under LD12:12 and LD6:6, the expression of the FRQ

protein and the phosphorylation profile displayed coincident rhythms with the periods of LD cycles. However, under LD3:3 and shorter cycles, the FRQ levels seemed to free run but we also observed some saw-tooth shaped fluctuations under LD3:3 (Figure 2, A-F and Figure S2 in File S1).

Mathematical modeling suggested that under LD12:12 and LD6:6, the concentration of FRQ protein matches the LD cycles (Figure 2G and Figure S3 in File S1 and Table S2 in file S1; supplemental protocol and parameters for modeling in File S1), instead, but underLD2:2 the FRQ levels show a free running pattern with some saw-tooth shaped fluctuations. The simulation results show that the mRNA levels have a direct response to light, but the protein curves are smoother, especially for high-frequency light signals. Therefore, the post-translational regulatory and translocation processes may perform a buffering function or high-frequency filtering function. Furthermore, the numerical data also predicted that the direct response of *frq* mRNA to the light stimuli is lower under shorter LD cycles (Figure 2G), which may be due to the light adaptation module (VVD-WCC loop) and the core negative feedback loop.

FWO is not required for adaptation of conidiation rhythms to short LD cycles in high-intensity light

In the Neurospora circadian oscillator, WC-1 and WC-2 are the two positive elements in which WC-1 function as a blue light sensor (He *et al*. 2002). We asked whether WCC proteins are responsible for conidiation rhythmicity under LD cycles.

We grew the $wc\text{-}1^{RIP}$, $wc\text{-}2^{KO}$and wcc^{DKO} and frq^{10}strains in race tubes under different LD cycles and observed condition rhythms for all of these mutants exhibit conidiation rhythms under LD12:12, LD6:6, LD3:3andLD2:2, but not in DD and LD45min:45min (Figure 3). The strains were exposed to a light intensity of 5000 lux, during the light periods. These data suggest that under short LD conditions, the conidiation rhythms cycle to the environment even without a functional circadian clock. We have also shown that in WT, the FRQ protein oscillation is masked under LD cycles ($6 \leq T < 24$), but free runs when $T < 6$ (Figure 2 and Figure S2 in File S1). Taken together, these results suggest that the FWO system is not required for the conidiation banding rhythms induced by the cycling environment.

Conidiation rhythmsof photosensor-related mutants under short LD regimes

First, we investigated the impacts of individual photosensing-associated genes on the conidiation rhythms under short LD cycles in Δ*cry*; Δ*vvd*; Δ*phy-1*; Δ*phy-2*; Δ*nop-1*and in *cog-1*, a newly identified mutant showing circadian conidiation rhythmicity under constant light (Nsa *et al*., 2015). WC-1, VVD and CRY are blue light sensors while PHY-1 and PHY-2 are potential red light receptors (Corrochano 2007; Froehlich *et al*. 2005; Chen *et al*. 2009). Unexpectedly, all of these strains showed conidiation rhythms with periods in accordance with the LD cycling periods, under conditions of LD12:12, LD6:6, LD3:3 and LD2:2 (Figure 4, A-D and Figure S4 in File S1). Together with the fact that the Δ*wc-1* strain can be entrained at LD2:2, these results suggest that either there exists additional photosensor(s), or the lack of an individual photosensor is not sufficient to abolish the adaptation of conidiation to short LD cycling cues.

With this in mind, we investigated the conidiation rhythms in strains bearing multiple mutations/deletions of light-sensor or related genes, which included Δ*cry*, Δ*vvd*; Δ*cry*, Δ*wc-1*; Δ*vvd*, *cog-1*; Δ*wc-1*, *cog-1*; Δ*vvd*, Δ*wc-1*, *cog-1*; Δ*cry*, Δ*vvd*, *cog-1*; Δ*cry*, Δ*vvd*, Δ*wc-1*; Δ*cry*, Δ*wc-1*, *cog-1* and Δ*cry*, Δ*vvd*, Δ*wc-1*, *cog-1*. Unexpectedly again, all of these strains still showed conidiation rhythms in short LD cycles (Figure 4, E and F).

We also observed these strains under~5000 lux red and blue light. In blue:dark (BD) cycles, most of the tested strains exhibit conidiation rhythms. In contrast, under red light, all the examined strains showed no entrained rhythms in LD6:6, LD3:3 and LD2:2 (Figure S5, A-G in File S1). Red light can also be sensed by some fungi, e.g., *Aspergillus nidulans*, *Trichoderma atroviride* and Neurospora, which is important for the asexual/sexual transition during development, hyphal growth and DNA stability (Corrochano 2007; Froehlich *et al*. 2005; Chen *et* al. 2009). Therefore, these data suggest that the conidiation rhythmicity is controlled predominantly by the blue light sensors and not the red light sensors.

The temperature increase that accompanied the switch-on of the lighting in the incubator, which might also have affected the conidiation. To rule out this possibility, we measured conidiation rhythms under temperature cycles (25.5℃6h: 24.5℃6h) as the range of incubator temperature is ~±0.5^{o}C in our experiments, as previously described (Nsa *et al*. 2015). The results showed no synchronization in all tested strains (Figure S5H in File S1), demonstrating this temperature variation is not sufficient for synchronization. Taken together, these results

show that all of these tested light sensors and associated factors were not critical for the conidiation rhythms. Instead, an unidentified blue light photoreceptor might be involved in controlling the conidiation rhythms.

The effects of different photosensing-associated genes on the production of carotenoid in all of these strains in constant light were also analyzed. As previously has shown, the strains containing *vvd* mutations, including Δ*vvd*; Δ*vvd*, *cog-1*; Δ*vvd*, Δ*cry* and Δ*vvd*, Δ*cry*, *cog-1* displayed bright orange color in their mycelia. In contrast, the mycelia color of strains Δ*vvd*; Δ*wc-1*, *cog-1*; Δ*cry*, Δ*vvd*, Δ*wc-1* and Δ*vvd*, Δ*cry*, Δ*wc-1*, *cog-1* were not bright orange. Interestingly, the *fwd-1*^{RIP}strain showed bright orange color similar to that of Δ*vvd* (Figure S6 in File S1).

In contrast to our results, Nsa *et al* showed that the Δ*wc-1* strain exhibit no obvious rhythms under a number of T-cycling conditions including LD6:6, LD9:9, LD12:12 and LD14:14 (Nsa *et al*. 2015). However, we used a white light intensity (~5000 lux) that was much higher than that used by Nsa et al (1200 lux), which may account for the inconsistency. In support of this view, when we grew the clock mutants in LD conditions and the light intensity was approximately 1000 lux, and conidiation rhythmicity was abolished at the tested LD conditions (Figure 5, A and B and Figure S5 in File S1 and Figure S6 in File S1). Conversely, the conidiation rhythms in clock gene mutants, *wc-1*^{RIP}, *wcc*^{DKO}, *frq*^{10} and Δ*wc-2*, were present in 5000 lux blue light but were absent in 1000 lux (Figure S6 in File S1). These data suggest that while clock components might act to promote the function of a potential photoreceptor to elicit conidiation rhythms, the response of clock mutants to a high-intensity but not low-intensity light, suggests that higher-intensity light might overcome the repression effect caused by the loss of clock components (Figure 5, C-E).

It is intriguing to note that in BD cycles (blue light: 1000 lux), the strains *cog-1*; Δ*wc-1*, *cog-1* and Δ*vvd*, Δ*wc-1*, *cog-1* exhibited biomodal patterns on the first day (Figure S5F in File S1), suggesting that the CDO pathway might play a role in mediating the synchronization of conidiation rhythmicity (Figure S5 in File S1).

Implication of FWD-1 in regulating the conidiation rhythms under short LD cycles

To probe the possible mechanisms regulating the adaptation to LD cycles, we also checked

the conidiation rhythms of several random mutants, among them the *fwd-1*^{RIP}strainthat lacks the functional *fwd-1* gene which encodes FWD-1, the Neurospora homolog of *Drosophila* Slimb, a ubiquitin E3 ligase. Neurospora FWD-1 has been implicated in the regulation of FRQ ubiquitination and turn over (He *et al*. 2003; Larrondo *et al*. 2015).

The *fwd-1*^{RIP}strain exhibited no overt conidiation bands under LD3:3 and shorter LD cycles (Figure 4 and Figure 5 and Figure S5 in File S1). The mutants lacking tested photosensors showed normal responses to short LD cycles, suggesting that the phenotype of *fwd-1*^{RIP} is not based on the impacts of FWD-1 on these genes, i.e., additional photosensor(s) controlled by FWD-1 might be implicated in regulating the conidiation rhythms under short LD cycles. Alternatively, a potential factor connecting the specific photosensor to conidiation and, that is controlled by FWD-1, might be implicated (Figure 5, C and D).

Transcriptomic analysis of genes induced by high-intensity light

The above data suggest that high-intensity light influences conidiation rhythms, thus we conducted RNA-seq analysis to assess the changes of gene expression in response to high-intensity light.

We compared the transcriptomic changes of the strain Δ*cry*, Δ*vvd*, Δ*wc-1*, which contains no known blue light photoreceptors, after exposure for 45min to either 1000 lux or 5000 lux white light (Fig. 6A). The results showed that upon light exposure, a variety of genes involved in metabolism and gene expression were up-regulated (folds ≥ 1.5). For the genes exclusively induced by 5000-lux light, one putative motif (GAXGA)was identified by the XXmotif online tool (http://xxmotif.genzentrum.lmu.de/), which is present in 56 promoter areas of the 73 genes (Figure 6C). Light of 5000-lux induced the exclusive expression of 73 specific genes, many of which were enriched in fatty acid metabolism, ribosome biogenesis, etc. (Figure 6, C and D and Table S3 in File S2 and S4 in File S3). The induced expression of three representative genes (NCU00298, NCU00992 and NCU09771) were confirmed in Δ*cry*, Δ*vvd*, Δ*wc-1*; Δ*phy-1*, Δ*phy-2* and *Δnop-1* by qRT-PCR (Figure 6, E-G). Although the pathways of ribosome biogenesis and propanoate metabolism were induced in both 1000-lux and 5000-lux light (Table S4 in File S3), the genes involved were different, suggesting that higher-intensity light plays specific roles in regulating the expression certain genes and metabolism.

Taken together, these data confirm that high-intensity light induces the expression of a

313 set of genes in clock mutants that might represent the targets for studying the potential
314 unidentified photoreceptor(s).
315

316 ## Discussion

317 Within a certain range, the cyclical environmental changes (*T*-cycles) can induce a rhythm
318 with the same periodicity as the environment even when these are non-24-h cycles, which
319 means *T*-cycles mask the endogenous circadian periodicity. This range varies in different
320 organisms (Jud *et al*. 2005; Aschoff 1960). In Neurospora, we show that under a number of
321 short LD conditions, including even very short cycles, such as LD1:1, induce conidiation
322 banding rhythms with periods that change along with the environmental periods. Under
323 constant darkness, Neurospora conidiation rhythms display an ~22 h period which reflects the
324 endogenous circadian period. Our study shows that under almost all of the short LD cycles,
325 the conidiation rhythms were induced by the *T*-cycles. The conidiation rhythms under short
326 *T*-cycles are not endogenous as they disappeared in constant dark; instead, they are the
327 hourglass-type rhythms.

328 FRQ is one of the central circadian clock components and the rhythmic changes in FRQ
329 levels and phosphorylation patterns represent the endogenous circadian rhythms (Baker *et al*.
330 2012). When we further looked at the expression of the core circadian protein FRQ under
331 short LD cycles, we found that similar to the conidiation rhythms, the period of FRQ changed
332 in accordance to the LD cycles when $T >$ LD3:3. In contrast, under LD cycles wit $T \leq$ LD3:3,
333 FRQ level no longer oscillated with the *T* cycles, and instead exhibit periods close to 24h, i.e.,
334 the masking effects faded while the FRQ rhythm free ran. The inconsistency between
335 conidiation rhythms and FRQ rhythms might reflect the decoupling between the intrinsic and
336 extrinsic rhythms under the extremely short LD cycles. The dynamic modeling results showed
337 the superimposition of endogenous and the light-induced rhythms, which can also be
338 observed in the western blot results of FRQ protein under very short LD cycles ($T \leq$LD3:3)
339 (Figure 2 and Figure S2 in File S1).

340 In many species, it has been demonstrated that non-24h T-cycles decrease their fitness
341 (Hut *et al*., 2011), suggesting the adaptation of the endogenous rhythm of an organism to

342 external cycling cues is critical for the development and growth. We show that the growth rate
343 of Neurospora was slower under LD12:12 compared to many other conditions. However, the
344 proportion of microconidia produced in LD12:12 prevailed over those in other conditions.

345 Faster growth of an organism does not necessarily mean optimal fitness to the
346 environment (Hut *et al*. 2011). Instead, the capability to produce variable and fertile progenies
347 may be more crucial. Neurospora microconidia function predominantly as spermatid
348 (Maheshwari 1999), suggesting that the LD12:12 condition might be optimal for sexual
349 reproduction. It is likely that in nature, the circadian clock renders Neurospora better prepared
350 to use its sexual reproduction strategies to cope with constantly changing environmental
351 stresses. Compared to the findings in some other organisms, these results reflect that different
352 organisms may employ different strategies for adaptation and propagation (Vaze and Sharma
353 2013). In addition, the dramatic differences in growth under LL vs DD, LD45min:45min vs
354 LD65min:25min indicate that the growth is influenced by the daily length of lighting in
355 Neurospora, which might be similar to the seasonal physiology responses in different
356 kingdoms (Arendt 1988; Tan *et al*. 2004).

357 In fungi, a number of developmental and physiological processes are affected by light
358 (Lauter 1996; Linden *et al*. 1997). In Neurospora, the most prominent light response is the
359 light-regulated biosynthesis of the photo protective mycelia carotenoids (Schrott 1980;
360 Harding and Turner 1981), phototropism of perithecial beaks, protoperithecia development,
361 promotion of conidia, light entrainment of the circadian clock and the formation of spores and
362 phototropism (Lauter *et al*. 1997; Ballario *et al*. 1996).Almost all Neurospora light responses
363 described so far are only known to be triggered by blue light. Transcriptomic analysis showed
364 that expression of some genes can be triggered by light in the *wc-1* null strain, suggesting the
365 existence of additional photoreceptors (Chen *et al*. 2009). Surprisingly, we show that upon
366 removal of several photosensors, the conidiation rhythms still exist in short LD cycles. In
367 addition, the strains bearing *cog-1* mutation can still be entrained (Figure 3 and Figure 4 and
368 Figure S4 and Figure S5 in File S1). Together with the results of cog-1 in BD3:3 (Figure S5F
369 in File S1), these data suggest that the conidiation rhythms in short LD cycles are independent
370 of either FWO or CDO although the CDO system affects the conidiation rhythms in short LD
371 cycles.

In Neurospora, in addition to WC-1, there are several additional confirmed or putative photoreceptors in Neurospora including VVD, CRY, PHY1/2 and NOP-1 (Chen *et al*. 2009; Chen *et al*. 2010a; Chen and Loros 2009). VVD is a small LOV domain-containing blue-light photoreceptor protein that affects photo adaptation for many light-responsive genes (Zoltowski *et al*. 2007; Schwerdtfeger and Linden 2003). As has been known, some light sensors can regulate other light sensors at transcriptional or post-transcriptional levels. For instance, in the light, WCC regulates the expression of VVD in Neurospora. Conversely, VVD inhibits WCC in a light-dependent fashion though it is not essential for clock function. Furthermore, VVD has been shown to physically interact with WCC (Zoltowski *et al*. 2007; Chen *et al*. 2010a; Malzahn *et al*. 2010; Gin *et al*. 2013; Shrode *et al*. 2001). Cryptochrome is a putative blue light sensor and its function remains elusive (Froehlich *et al*. 2005; Chen *et al*. 2009). PHY1/2 are putative red light receptors and NOP-1 is a putative green light receptor (Chen *et al*. 2010b).

It has been claimed that WCC and VVD are responsible for most light-dependent physiological processes, if not all. However, the clock mutants showed synchronization to 5000 lux white light but not 1000 lux white light (Nsa *et al*. 2015) (Figure 3 and Figure 5). Moreover, the conidiation rhythmicity only occurs in blue light but not red light (Figure S5 in File S1).

Collectively, these data suggest the presence of an unidentified blue photoreceptor which induces conidiation rhythmicity. Interestingly, the synchronization of conidiation rhythms is independent of the FWO system; instead, the circadian clock might promote the conidiation responses (Figure 5, C and D).

Under 1000 lux white light, clock mutants displayed no conidiation rhythms, suggesting that the circadian clock functions to maintain or promote conidiation responses to short LD cycles. These data also suggest that the circadian clock might help Neurospora to promptly adjust its conidiation according to changeable light condition in the natural environment; for instance, caused by cloud movement or shadows being cast in the daytime. Under high-intensity light, even the clock mutants showed conidiation rhythms, suggesting the unidentified photoreceptor(s) might only sense very strong light, which can confer the condition rhythmicity in clock mutants.

Light initiates adaptation in a comprehensive set of metabolic pathways (Tisch and Schmoll 2010). In this work, we found that a subset of metabolic pathways was affected upon higher light exposure relative to lower light, suggesting that additional photoreceptor(s) than those that are known (e.g., WC-1, VVD and CRY) play a role in regulating light-induced metabolic adaptation.

FWD-1 is another regulator of conidiation rhythms in short LD cycles. In LD cycles (6h≤*T*<12h), the depletion of FWD-1 showed no overt impacts on conidiation rhythms, suggesting that FWD-1 does not affect the NFLO pathways. However, in shorter LD cycles (*T*<6h), the depletion of FWD-1 caused abolishment of conidiation rhythmicity, suggesting that FWD-1 controls NFLO pathways under such conditions. FWD-1 might function to affect the responsible photoreceptor(s) or sensitize transducers that couple specific light sensors and conidiation. Therefore, a lack of FWD-1 leads to abolishment of conidiation rhythms responding to the very short LD cycles.

The light conditions in certain extreme environments, e.g., space, dramatically differ from that on Earth. In low orbit, the LD cycles are very short (90-120-minute) with only 30% of the time in light and the remainder of the time in the dark (Stampi 1994). The present data also demonstrate that the circadian rhythms and fitness is impaired in simulated LD cycles with a 90-min period (Figure 1).

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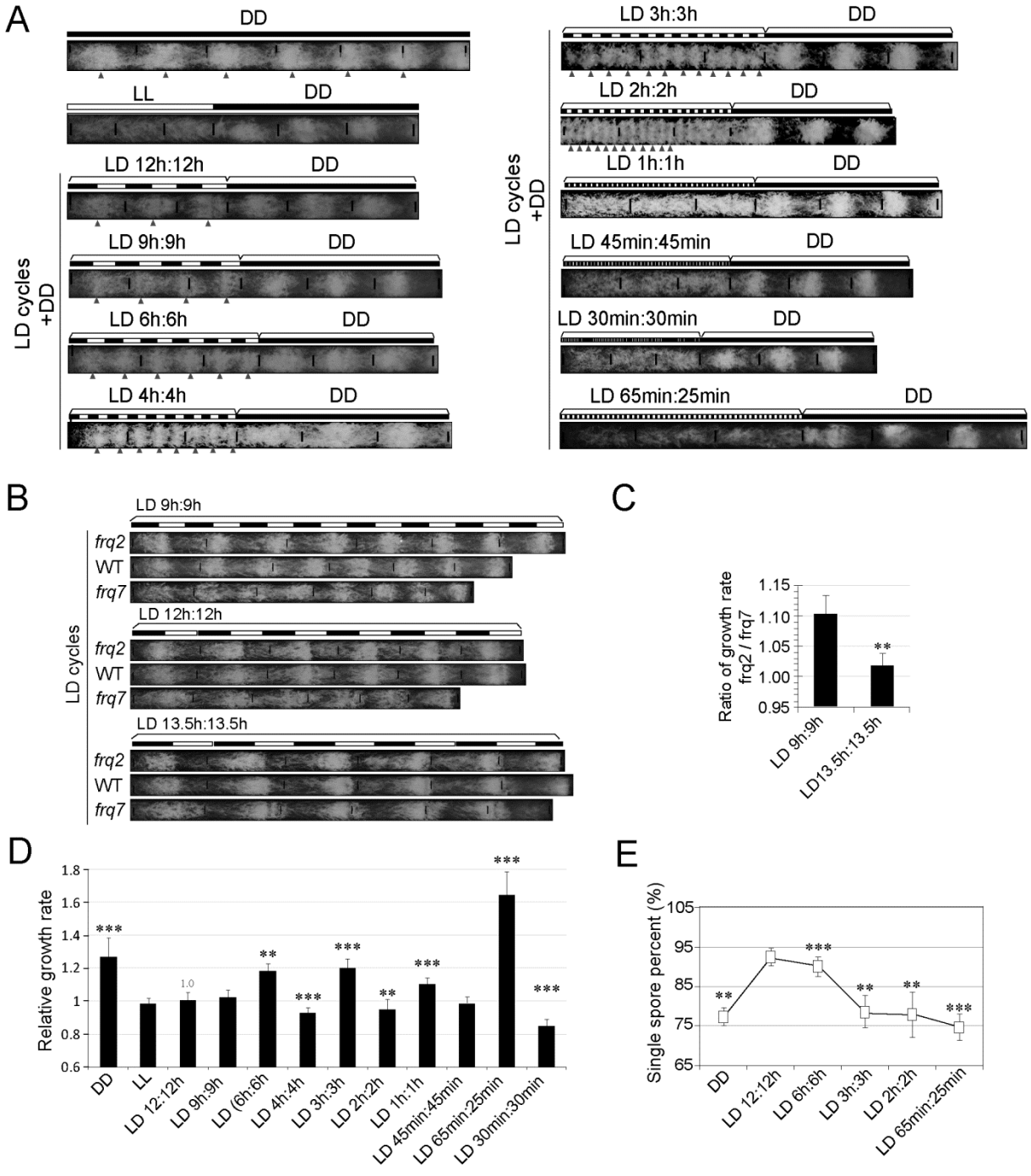
543 FIG 1

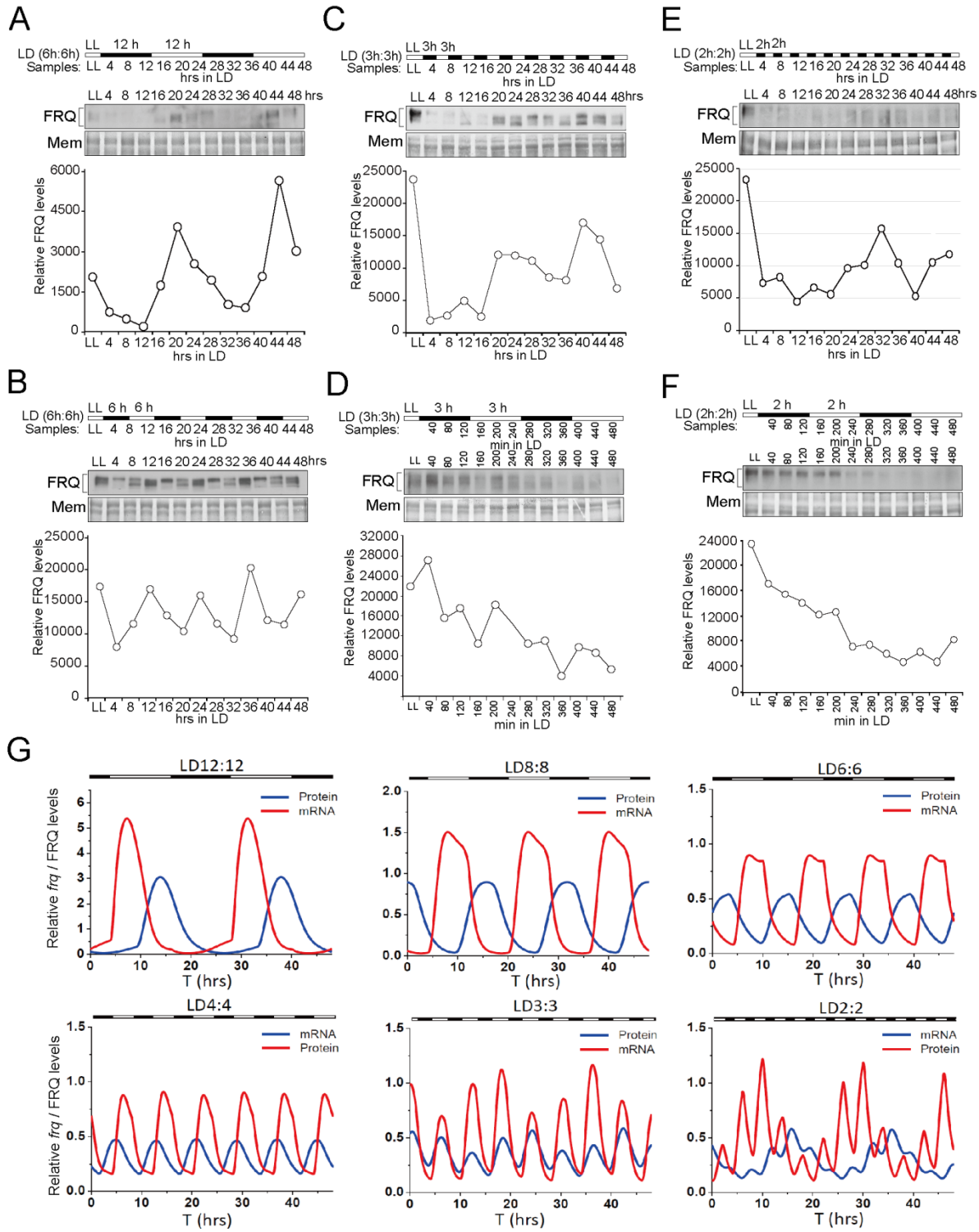


544

545 **Figure 1** Conidiation rhythms, growth and adaptation of *Neurospora* in short LD cycles. A.
546 Conidiation rhythms of *Neurospora* under a series of LD cycles. The black and white bars
547 denote different LD regimes. Triangles denote the conidiation bands. B. Growth rate of
548 *Neurospora* under a series of LD cycles. Data are mean ± SD, n=3. Represent results (n≥3) are
549 shown. C. Ratio of the growth rates between *frq2* and *frq7*. Data are mean ± SD, n=3. D.
550 Growth rates of indicated strains. The growth in LD12:12 was normalized to 1.0. Data are mean
551 ± SE, n=3. E. Proportion of microconidia produced under different LD cycles. Data are mean ±
552 SE, n=3.The light intensity was 5000 lux.

553

554 FIG 2

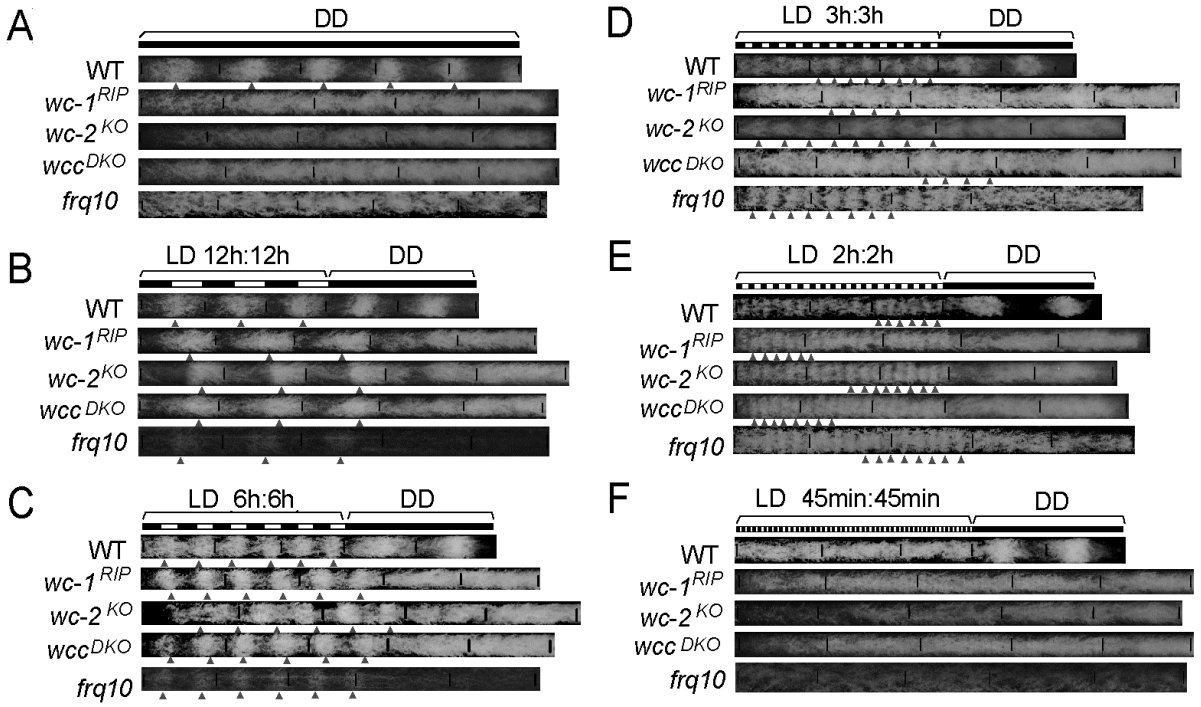


555

556 **Figure 2** The FRQ protein levels under different LD routines. The FRQ protein levels are shown
557 by western blot analysis. The LD routines are LD12:12 for two cycles (A), LD6:6 for 48 h (B),
558 LD3:3 for 48 h (C), LD3:3for two cycles (D), LD2:2 for 48 h (E) and LD2:2 for two cycles (F),
559 respectively. The black and white bars denote different LD regimes. Membranes (Mem) stained
560 with Amido Black were used as loading control. The densitometric analysis of FRQ levels is
561 shown at the bottom of each panel. Representative data of triplicates are shown. See more

562 data in Fig. S1. The light intensity was 5000 lux.(G) Dynamic modeling results showing the time
563 series of *frq* mRNA and FRQ protein under different light-dark cycles. Both the *frq* mRNA and
564 FRQ protein can be entrained to 12:12 LD cycles, 8:8 LD cycles, 6:6 LD cycles and 4:4 LD
565 cycles. As the frequency of the light signal increases to 3:3 cycles, the *frq* mRNA and FRQ
566 protein still exhibit the oscillation with a period of approximately 6h. If the frequency of the light
567 signal is further increased to 2:2 cycles, although the *frq* mRNA can respond to the induction by
568 the light, the FRQ levels show the free running pattern with some fluctuations.
569

570 FIG 3

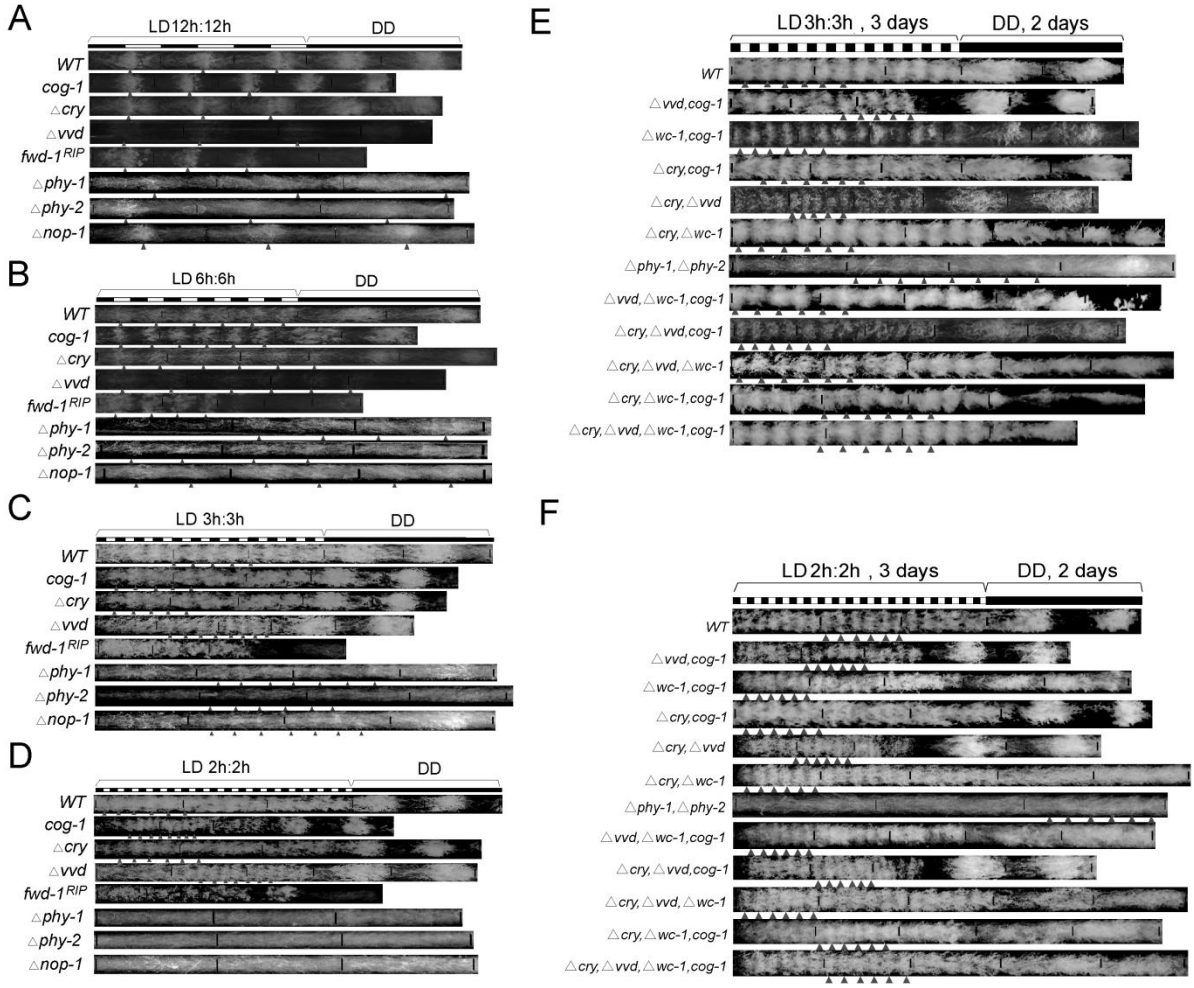


571
572

573 **Figure 3** The conidiation rhythms of *Neurospora* strains in short LD cycles. The conditions were
574 DD (A), LD12:12 (B), LD6:6 (C), LD3:3 (D), LD2:2 (E) and LD45min:45min (F). Represent
575 results (n≥3) are shown. Triangles denote the conidiation bands. The strains were grown under
576 white light and the light intensity was 5000 lux.

577
578

579 FIG4.



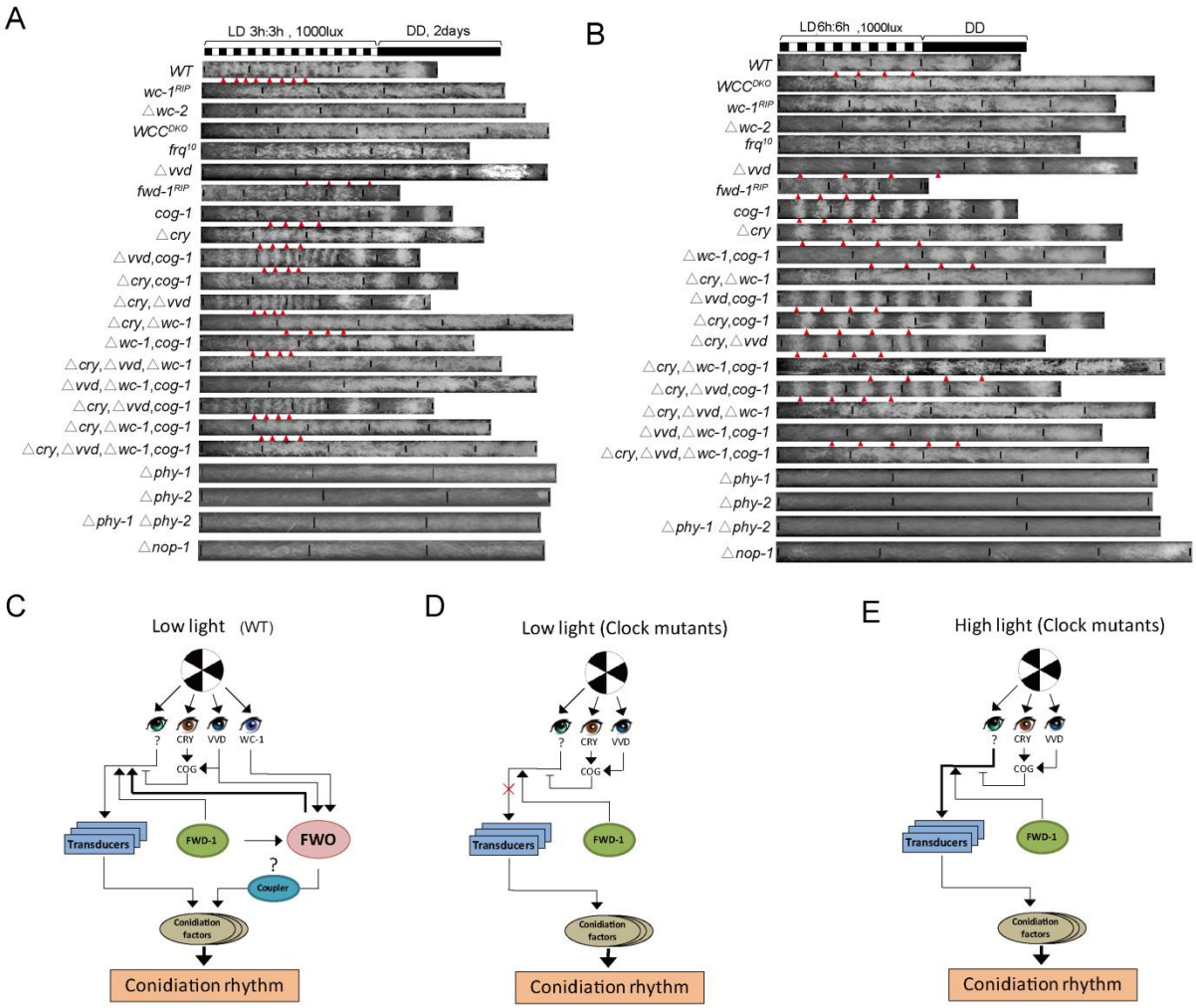
580

581 **Figure 4**. Impact of light sensors on the adaptation to short LD cycles. Race tube results of
582 indicated strains under indicated LD cycles. Represent results (n≥3) are shown. Triangles
583 denote the conidiation bands. The strains were grown under white light and the light intensity
584 was 5000 lux.

585

586

587 FIG5



588

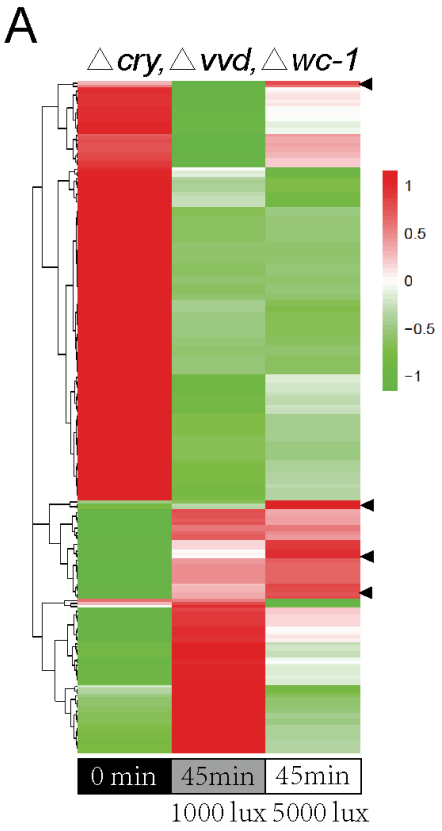
589

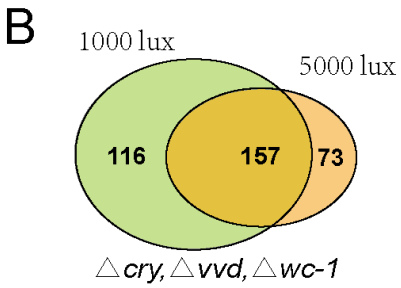
590 **Figure 5.** Conidiation rhythms in low white light (1000 lux). A/B. Race tube results of conidiation
591 rhythms of indicated strains in LD3:3 (A) and LD6:6 (B). The white light intensity was 1000 lux.
592 Triangles denote the conidiation bands. C-E. Schematics of the control of conidiation rhythms
593 under short LD cycles of WT in low-intensity light (C) and clock mutants in low-intensity light (D)
594 and high-intensity light (E). WC-1, VVD and additional photoreceptor(s)are implicated in the
595 regulation of the conidiation rhythms. In 6 h≤ T ≤ 24 h, the FWO system is entrained, and the
596 FRQ rhythms are not endogenous as they cannot be maintained in constant dark. The question
597 masks denote unidentified photoreceptor and putative coupler linking FWO and conidiation,
598 respectively. Transducers denote the factors linking photoreceptor and conidiation.

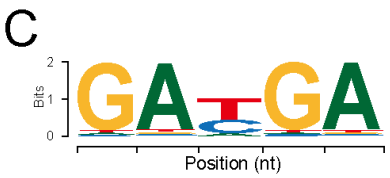
599

600

FIG 6





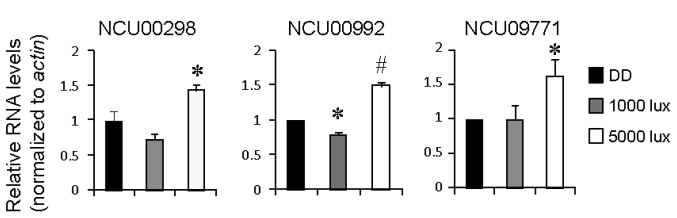


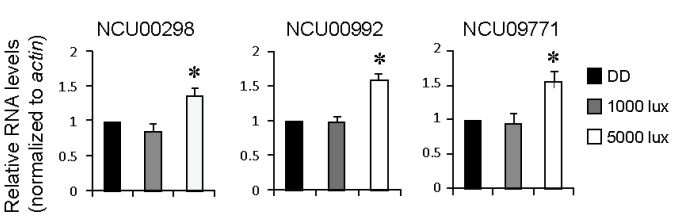
D

Induced exclusively in 1000 lux	
Term	P-value
Ribosome biogenesis in eukaryotes	0.006
Pentose and glucuronate interconversions	0.021

Induced in both 1000 lux and 5000 lux	
Term	P-value
Ribosome biogenesis in eukaryotes	0.000
Purine metabolism	0.000
RNA polymerase	0.001
Pentose and glucuronate interconversions	0.004
Biosynthesis of secondary metabolites	0.012
Pyrimidine metabolism	0.020
Biosynthesis of antibiotics	0.045
Propanoate metabolism	0.047

Induced exclusively in 5000 lux	
Term	P-value
Fatty acid biosynthesis	0.000
Ribosome biogenesis in eukaryotes	0.001
Fatty acid metabolism	0.001
Propanoate metabolism	0.011
Metabolic pathways	0.013





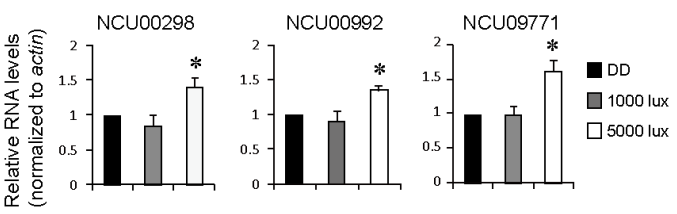
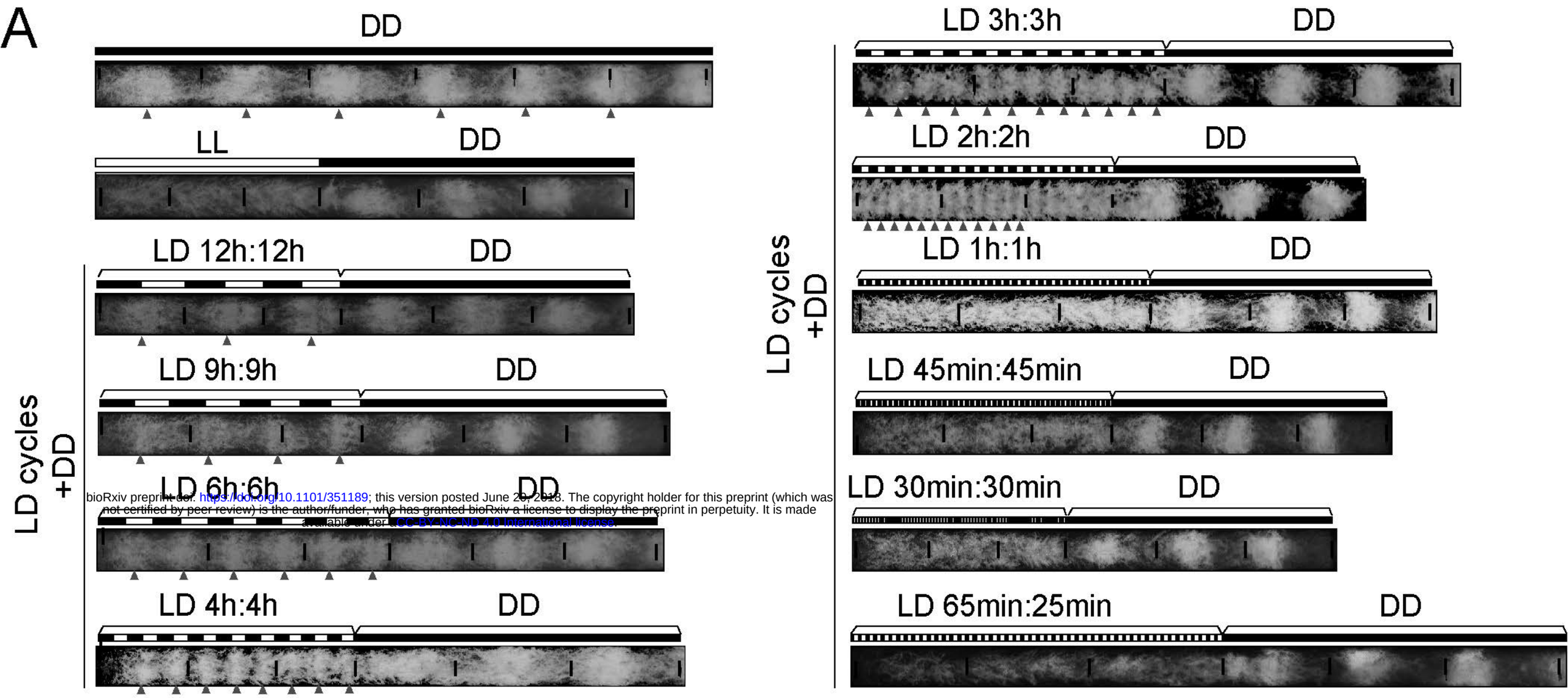
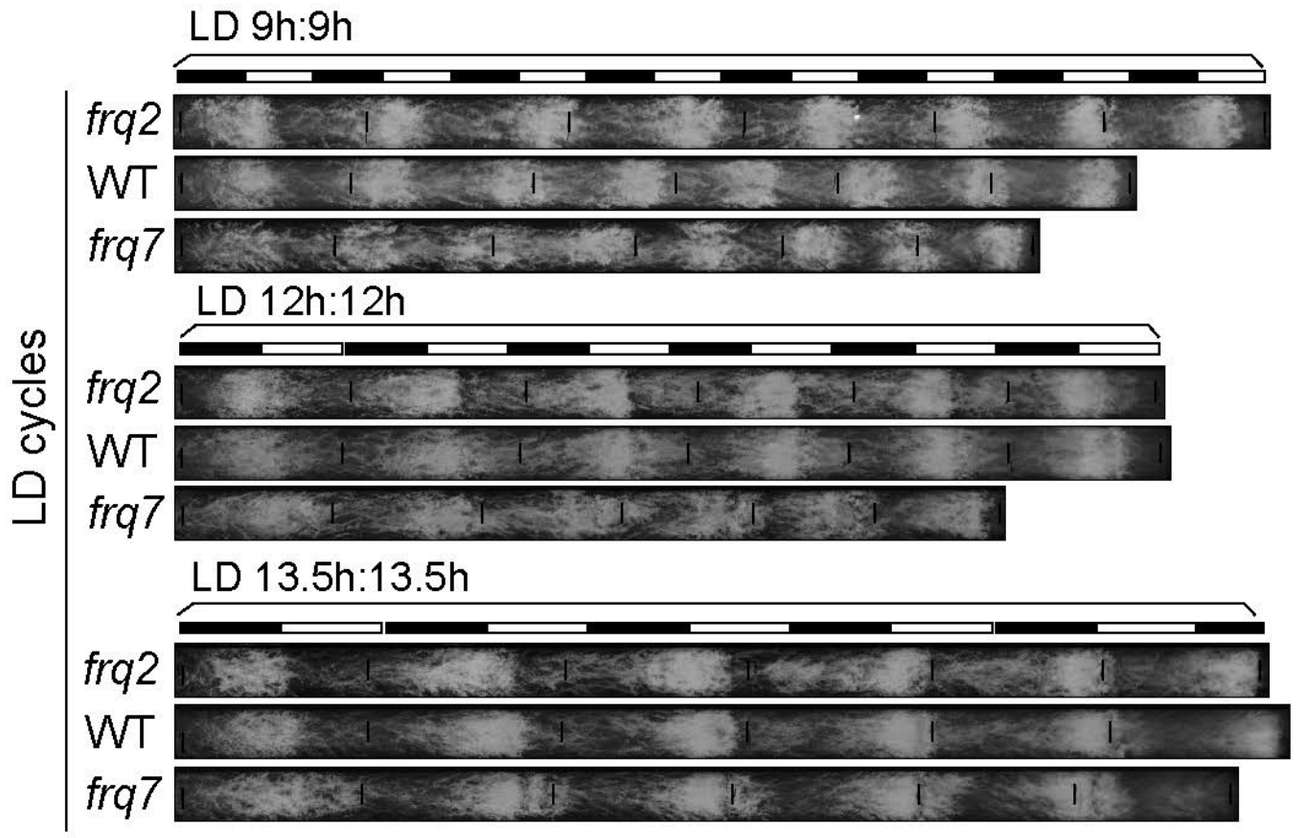


Figure 6 Characterization of genes showing light responses in high-intensity white light. A. Heat map of the differentially expressed genes in Δ*vvd,* Δ*cry,* Δ*wc-1* at DD24, 1000-lux white light and 5000-lux white light. The heat map was created according to the RNA-seq data. B. Venn diagram showing the genes up-regulated in 1000 lux and 5000 lux relative to DD24, respectively. C. Sequence logo of a predicted motif. D. Distribution of affected KEGG pathways according to the up-regulated genes revealed from RNA-seq. E-G. qRT-PCR validation of three genes specifically induced by 5000-lux light in Δ*vvd,* Δ*cry,* Δ*wc-1* (E), Δ*phy-1*, Δ*phy-2* (F) and Δ*nop-1* (G) strains. The gene expression was normalized to *actin*. The levels at DD24 was normalized to 1.0. Data are mean ± SE, n=3.

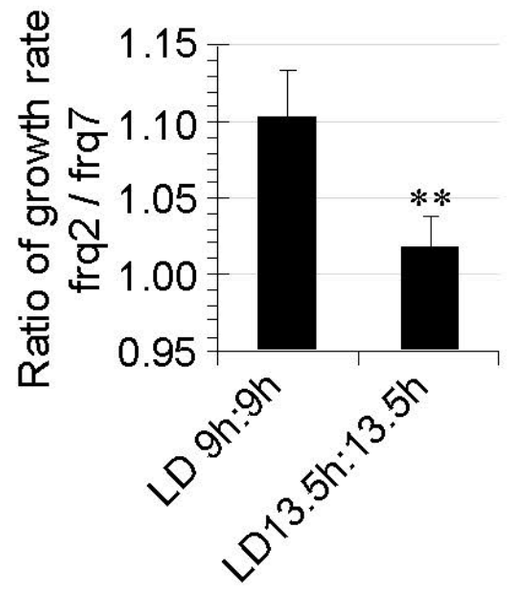
A



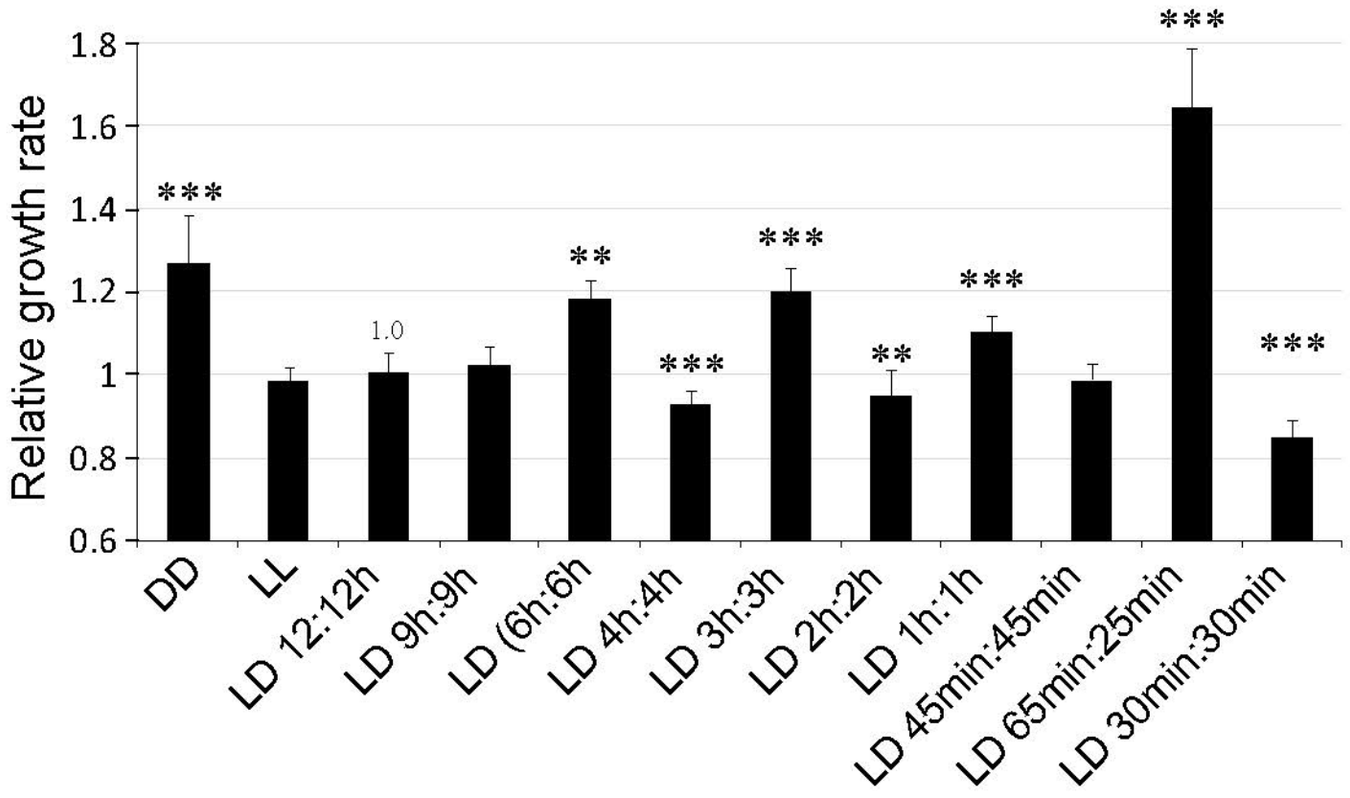
B



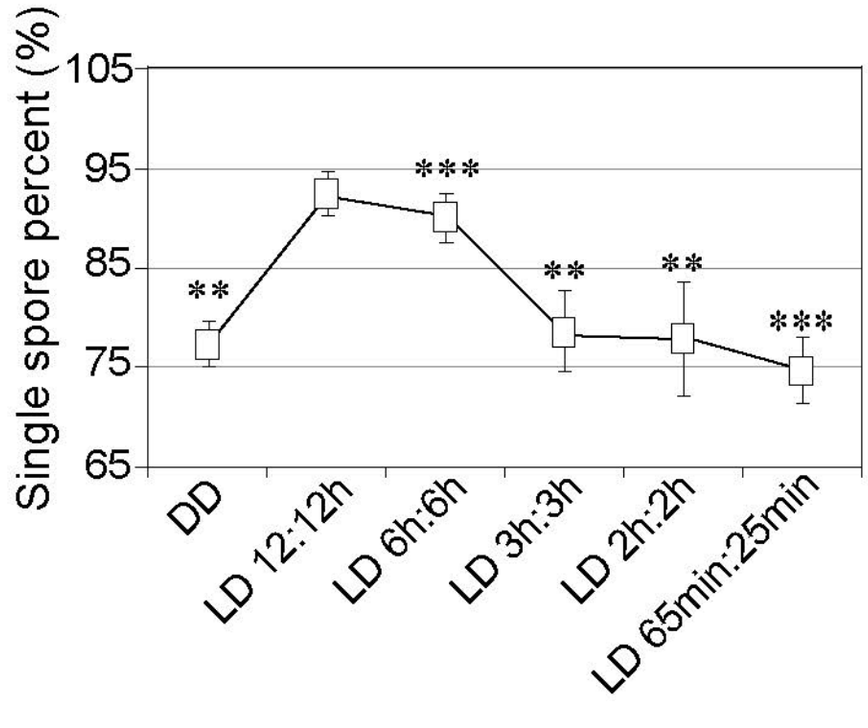
C

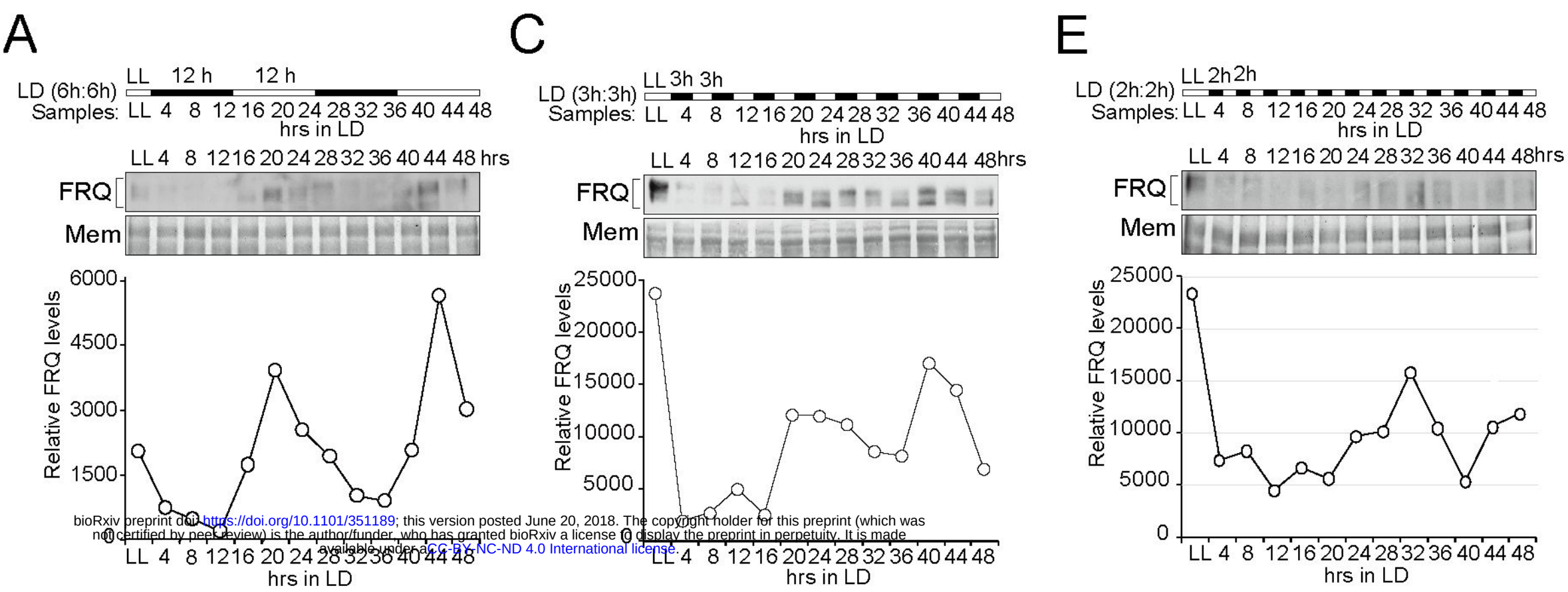


D

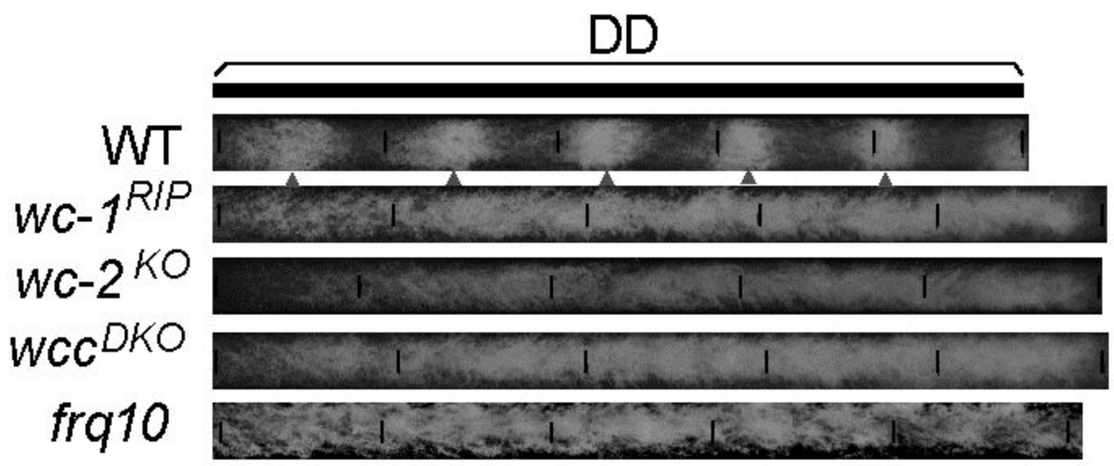


E

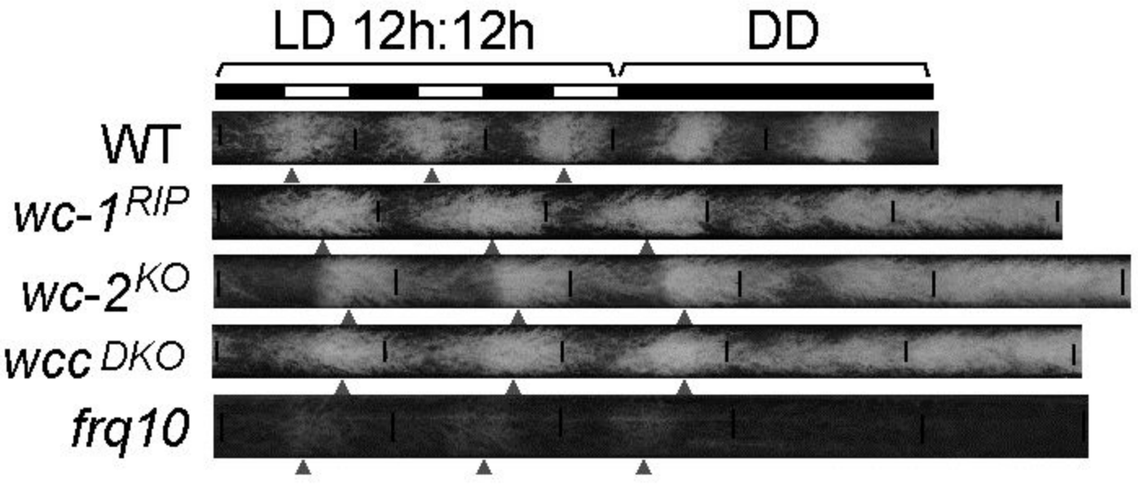




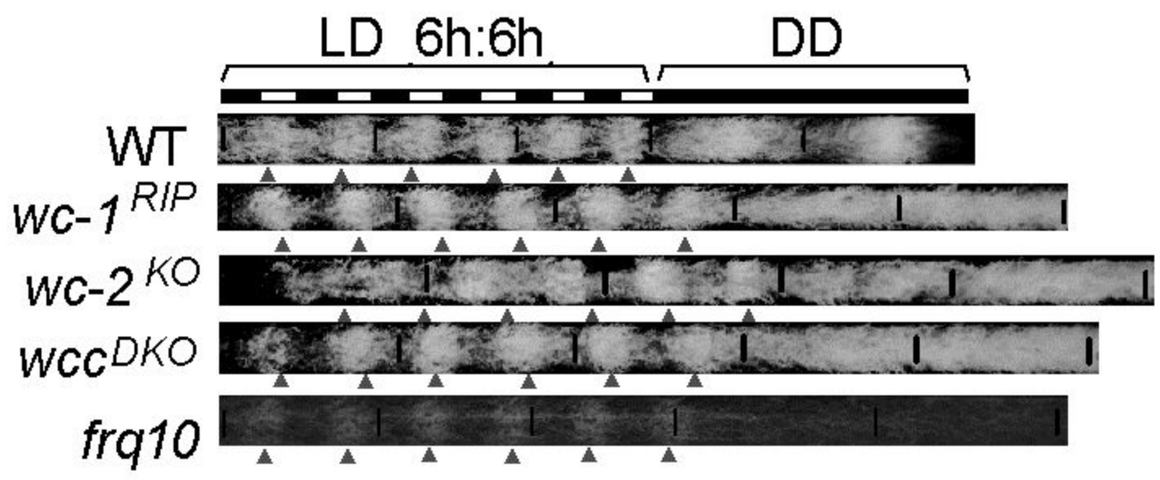
A


DD
WT
wc-1RIP
wc-2 KO
wcc DKO
frq10

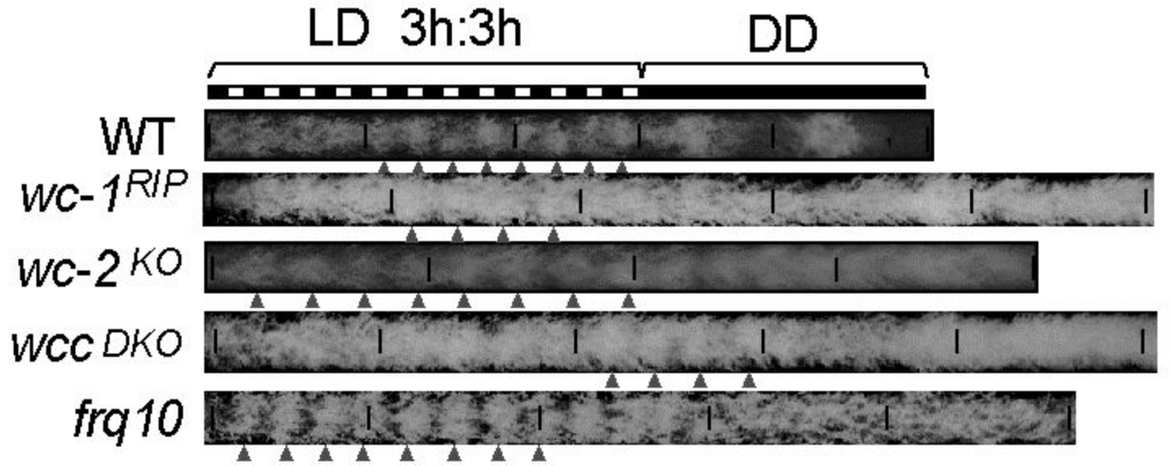
B


LD 12h:12h
DD
WT
wc-1RIP
wc-2KO
wcc DKO
frq10

C


LD 6h:6h
DD
WT
wc-1 RIP
wc-2 KO
wccDKO
frq10

D


LD 3h:3h
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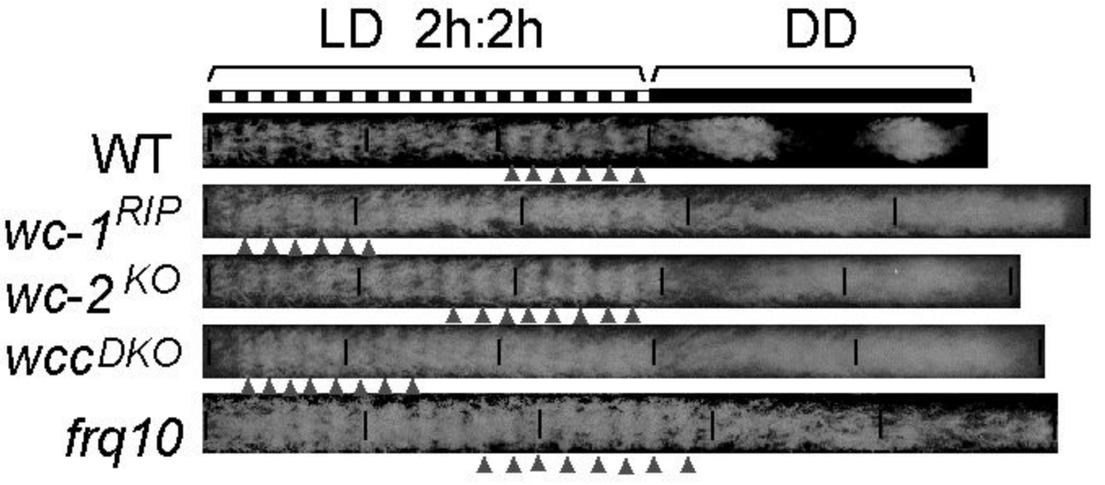
wc-1RIP
wc-2 KO
wcc DKO
frq10

E


LD 2h:2h
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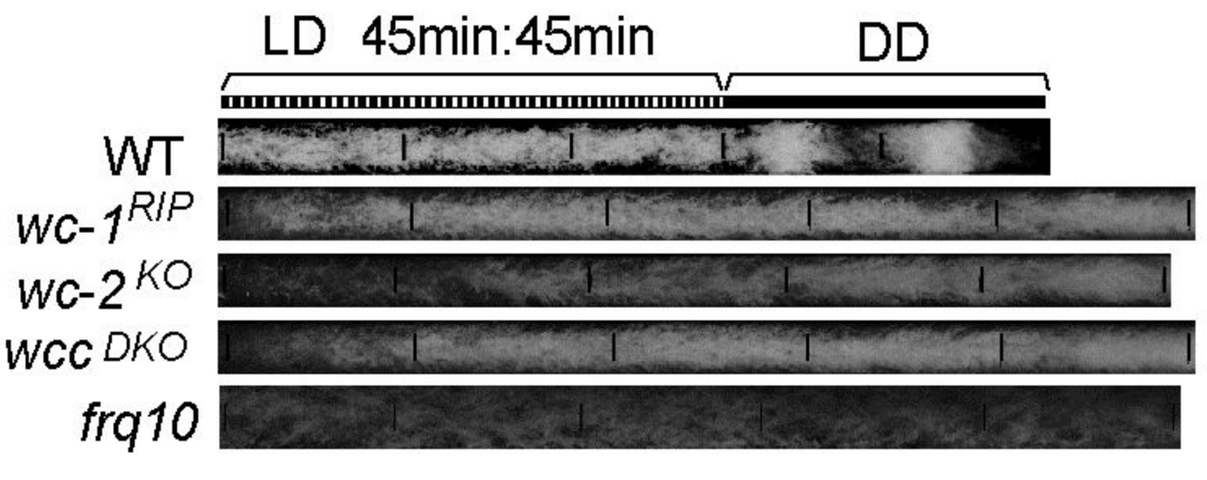
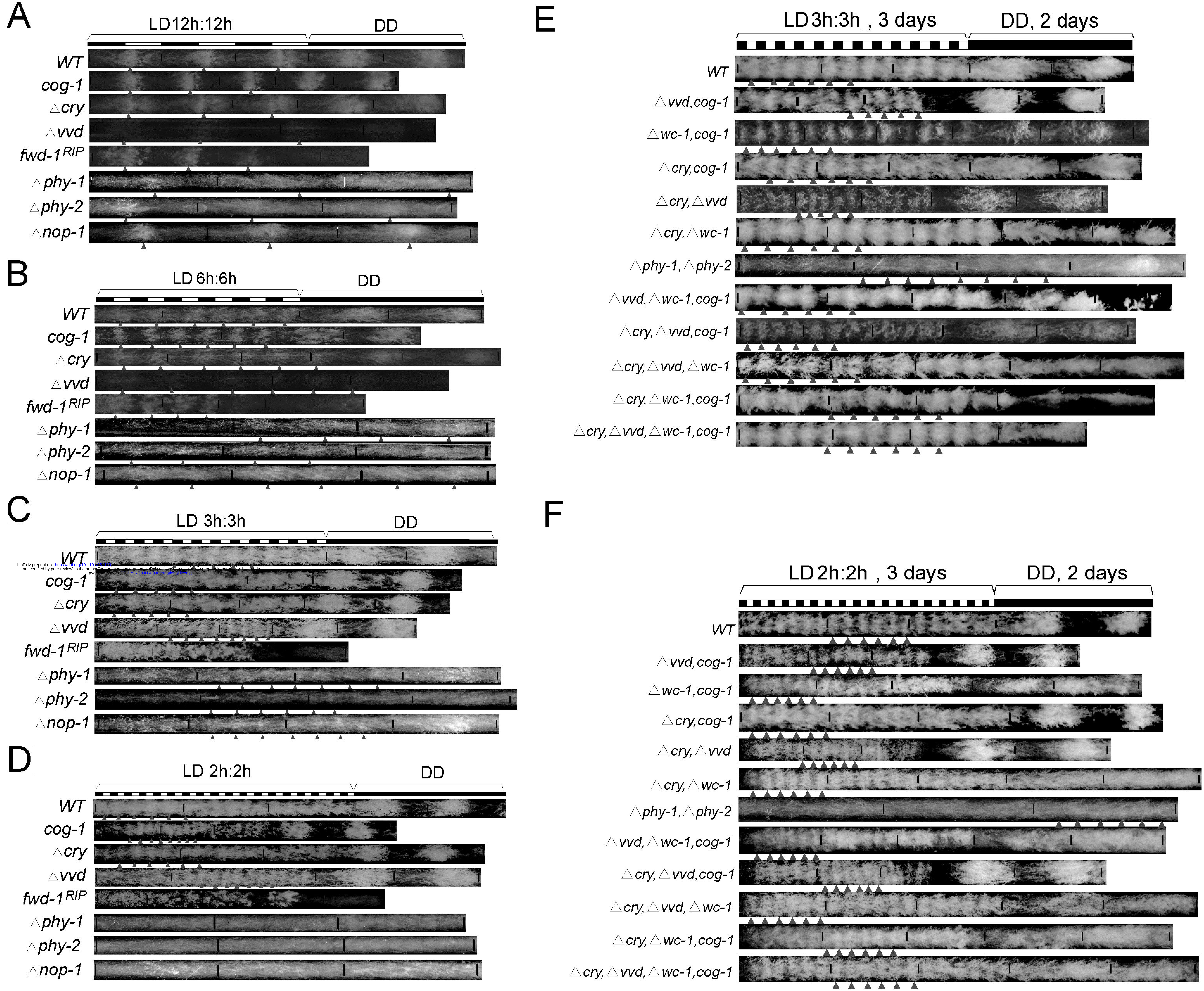
WT
wc-1RIP
wc-2 KO
wccDKO
frq10

F


LD 45min:45min
DD
WT
wc-1RIP
wc-2 KO
wcc DKO
frq10


A
LD 12h:12h
DD
WT
cog-1
△cry
△vvd
fwd-1RIP
△phy-1
△phy-2
△nop-1
B
LD 6h:6h
DD
WT
cog-1
△cry
△vvd
fwd-1RIP
△phy-1
△phy-2
△nop-1
C
LD 3h:3h
DD
WT
cog-1
△cry
△vvd
fwd-1RIP
△phy-1
△phy-2
△nop-1
D
LD 2h:2h
DD
WT
cog-1
△cry
△vvd
fwd-1RIP
△phy-1
△phy-2
△nop-1
E
LD 3h:3h , 3 days
DD, 2 days
WT
△vvd,cog-1
△wc-1,cog-1
△cry,cog-1
△cry,△vvd
△cry,△wc-1
△phy-1,△phy-2
△vvd,△wc-1,cog-1
△cry,△vvd,cog-1
△cry,△vvd,△wc-1
△cry,△wc-1,cog-1
△cry,△vvd,△wc-1,cog-1
F
LD 2h:2h , 3 days
DD, 2 days
WT
△vvd,cog-1
△wc-1,cog-1
△cry,cog-1
△cry,△vvd
△cry,△wc-1
△phy-1,△phy-2
△vvd,△wc-1,cog-1
△cry,△vvd,cog-1
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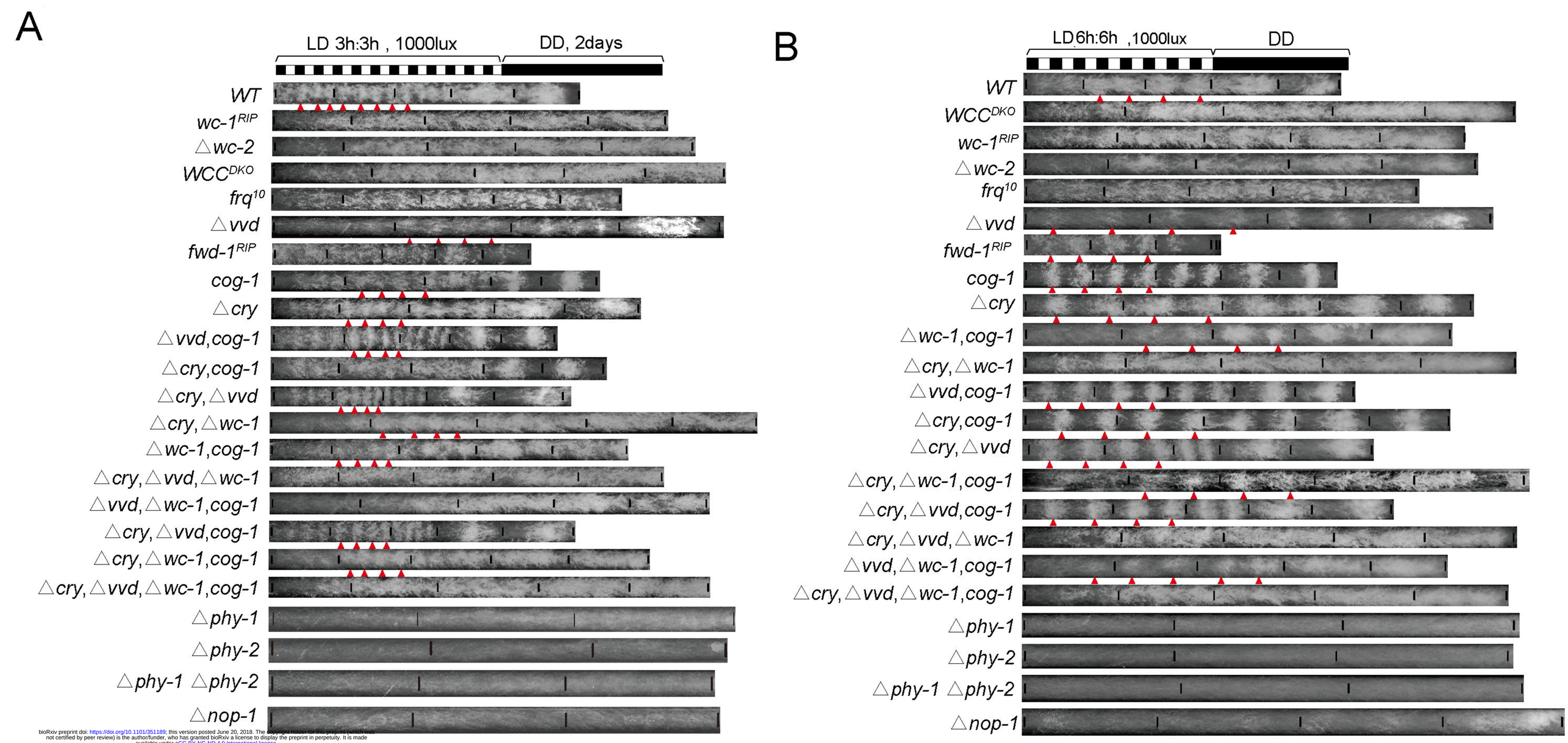
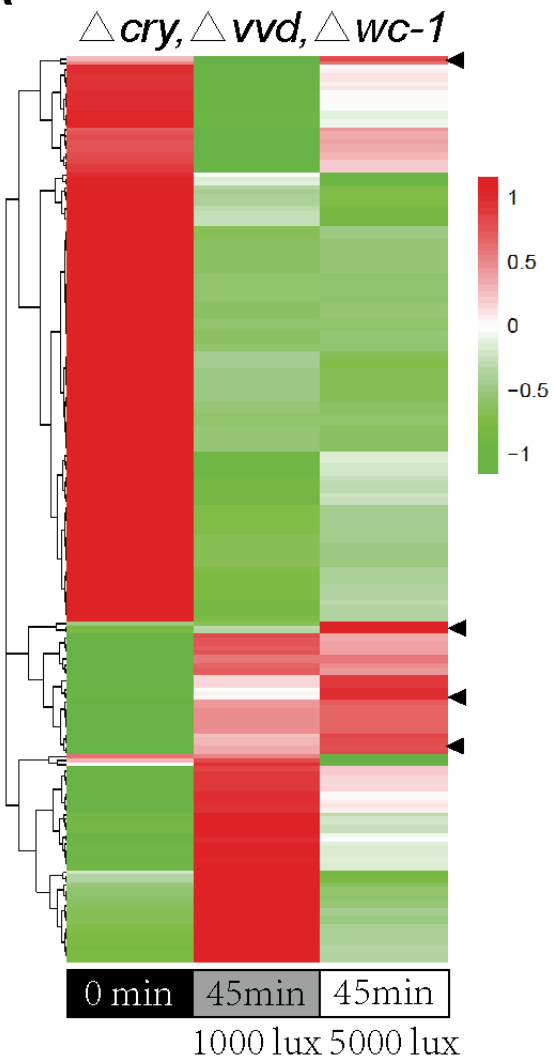
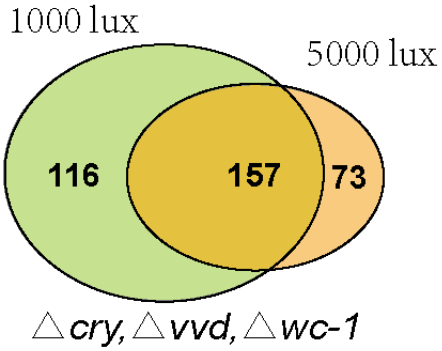


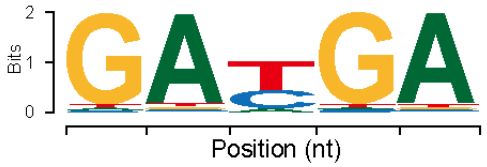
A



B



C



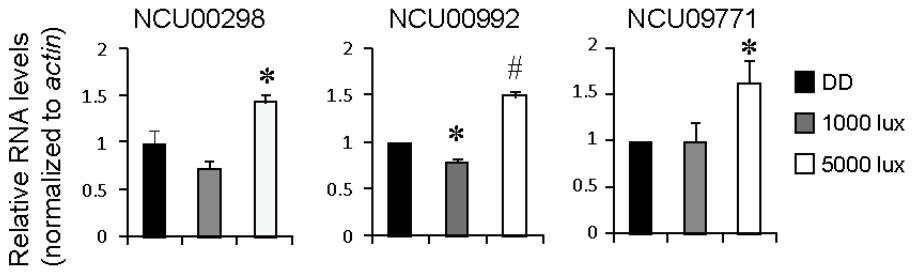
D

Induced exclusively in 1000 lux	
Term	**P-value**
Ribosome biogenesis in eukaryotes	0.006
Pentose and glucuronate interconversions	0.021

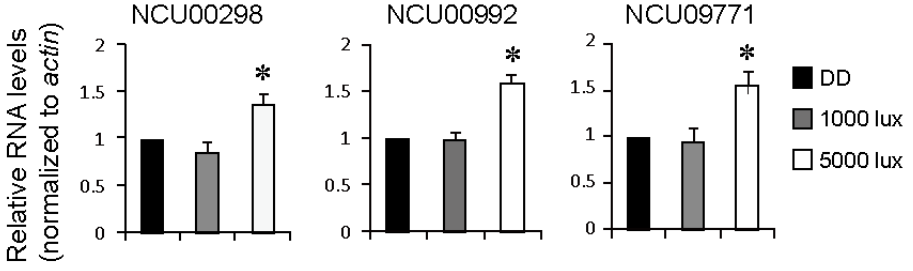
Induced in both 1000 lux and 5000 lux	
Term	**P-value**
Ribosome biogenesis in eukaryotes	0.000
Purine metabolism	0.000
RNA polymerase	0.001
Pentose and glucuronate interconversions	0.004
Biosynthesis of secondary metabolites	0.012
Pyrimidine metabolism	0.020
Biosynthesis of antibiotics	0.045
Propanoate metabolism	0.047

Induced exclusively in 5000 lux	
Term	**P-value**
Fatty acid biosynthesis	0.000
Ribosome biogenesis in eukaryotes	0.001
Fatty acid metabolism	0.001
Propanoate metabolism	0.011
Metabolic pathways	0.013

E



F



G