PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Genética Molecular y Microbiología

TESIS DOCTORAL:

"FORWARD AND REVERSE GENETIC APPROACHES TO UNVEIL NOVEL REGULATORS OF THE *Neurospora crassa* CIRCADIAN CLOCK"

Por

FELIPE ANDRÉS MUÑOZ GUZMÁN

MARZO 2019

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Tesis presentada a la Pontificia Universidad Católica de Chile como parte de los requisitos para optar al grado de Doctor en Ciencias Biológicas mención Genética Molecular y Microbiología

Por

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MARZO 2019

ACKNOWLEDGEMENTS

I would like to thank all current and past members of the Larrondo lab for their continuous help, input and support. Among them, I would like to single out Alejandra Goity, Dra. Consuelo Olivares, Dr. Paulo Canessa, Dr. Francisco Salinas and Dr. Alejandro Montenegro, who helped both experimentally and intellectually in the development of the specific work described in this thesis. I am forever indebted to Valeria Caballero for her significant contribution to the generation of the reporter library. I would also like to thank all Committee members for their valuable input.

I would like to thank my advisor, Dr. Luis F. Larrondo, for his continuous support throughout this long journey and for his advice and guidance during this period. Further, I am very grateful to Professor Rafael Vicuña, for his help and support in my many years at this School, from my undergraduate work to my PhD thesis.

I would like to specially acknowledge Dr. Andrew Millar (University of Edinburgh) and all of the members of his lab, especially Dr. Tomasz Zielinski, for their help in the use of the BioDare platform and the mathematical analysis of the time series data.
Finally, I thank Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) for their financial support; in the form of a doctoral fellowship, grant 21110734, and also, Vicerrectoría de Investigación (PUC) and Dirección de Postgrado (FCB- PUC). In the latter, I particularly thank María Teresa Pino. The work herein presented was funded by the aforementioned grants and Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) 1171151 and 1131030, Howard Hughes Medical Institute International Research Scholar Program and Millennium Institute for Integrative Biology (iBio) from Iniciativa Científica Milenio-MINECON.

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LIST OF ABBREVIATIONS

KO: Knockout
*ccg*s: clock controlled genes
WT: Wild Type
TTFL: Transcriptional-Translational Feedback Loop
WC-1: White Collar 1
WC-2: White Collar 2
WCC: White Collar Complex
FRQ: Frequency
PTFL: Post-translational feedback loop
TF: Transcription Factor
bd: *band mutation*
luc: *luciferase gene*
LG: Linkage Group
BioDare: Biological Data Repository
RAE: Relative Amplitude Error
FFT-NLLS: Fast Fourier Transform Non Linear least Square
DD: Darkness, a
TR: Temperature Reset
TC: Temperature Compensation
Ck1: Casein kinase 1
ctm-6: clock timing modifier by 6 hours
PTM: Post-translational modifications
HygR: Hygromicine Resistance
VM: Vogel's Media
DNA: Desoxirribonucleic Acid
gDNA: genomic DNA
SNP: Single Nucleotide Point
RFLP: Restriction Fragment Length PC

PREFACE

Circadian rhythms are a widespread phenomenon present in almost all living organisms on this planet; these rhythms confer them with the capacity of coordinating cellular and organismal metabolism, physiology, and behavior to day/night cycles. The circadian core oscillator is composed of a transcriptional-translational negative feedback loop (TTFL), which is topologically conserved through different lineages as a common molecular circuit based on a positive element able to activate the expression of a negative one, which then represses the action of the former inhibiting its own expression. This system is capable of sustaining oscillations under several external perturbations, but compensation mechanisms to support this system remain unknown. Studies in mammals, Drosophila and Arabidopsis have revealed additional transcriptional inputs, sustaining the central clock by modulating the expression of its core components. In *Neurospora crassa*, the core-clock central circuit has been well characterized but the participation of other associated transcriptional components remains unknown. Considering all these, we proposed the following hypothesis:

"There are additional transcriptional regulators that are critical for proper circadian clock function in Neurospora crassa"

To validate this hypothesis, we focused on the aim of identifying new transcriptional regulators that affect circadian parameters using forward and reverse genetic approaches in *Neurospora crassa*. The first strategy was generating a collection of strains with a circadian luciferase reporter and bearing individual deletions (Knockout) of the transcription factors known in *Neurospora crassa*, to then identify through a *reverse genetic approach*, the possible transcriptional regulators capable of affecting the circadian oscillations in Neurospora. We found only a few Knockouts for transcription factors displaying circadian phenotypes, work that is detailed in Chapter 1.

Coincidentally, from the first genetic screening and following a *forward genetic approach*, we identified a novel spontaneous single nucleotide mutation in the *casein kinase 1a* locus that leads to a period defect of over 6 h. Interestingly, despite clear differences in core clock components origin, identity and sequence across phyla, their posttranslational modifications such as phosphorylation, appear to be largely controlled by the same conserved kinases and phosphatases, where CK1a plays a pivotal role in all eukaryotic models studied to date. Our results in this area are described in Chapter 2 of this thesis.

Thus, these results come to highlight that transcriptional mechanisms, albeit necessary for the proper expression of core-clock components, have little effect in modifying circadian properties, leading only to subtle phenotypes. In comparison, mutations that affect phosphorylations dynamics of the clock negative element, can

dramatically modify period, highlighting the role of posttranslational modifications in period determination.

Chapter 1

Global Search for novel Transcription Factors involved in the *Neurospora crassa* Circadian Clock.

Keywords: Circadian Clock – *Neurospora crassa* - Transcription Factors – Luciferase Reporter.

ABSTRACT

All organisms in which circadian clocks have been characterized exhibit a common molecular circuit, which is based on a positive element able to activate the expression of a negative one that then represses the action of the former, inhibiting its own expression. This system is capable of sustaining oscillations under several external perturbations, but the compensation mechanisms remain unknown. Studies in mammals, Drosophila and Arabidopsis have revealed some new transcriptional inputs, maintaining the central clock circuitry, by modulating the expression of several of the core-clock components. In *Neurospora crassa* the core-clock central circuit has been well characterized but the participation of other associated transcriptional components remain unknown. In this chapter, we are trying to identify these novel transcriptional networks controlling the clock in *N. crassa* through a reverse genetic screen based on luminescent circadian reporters and a Neurospora Knockout collection of transcription factors. Thus, we have defined a set of 8 transcriptional regulators that modulate circadian features in our experimental conditions, which fall in a broad range of functions or processes. Finally, we selected the KO strain for the transcription factor for *kpr-16* to defying its circadian behavior to environmental changes; this strain shown a remarkable period lengthening of ~2 hours and by literature are associated to temperature sensibility. We demonstrated that in absence of this transcription factor the Neurospora clock was unable to respond to temperature changes over the compensation temperature range, showing the further usefulness of this kind of approach.

INTRODUCTION

Circadian rhythms are a widespread phenomenon in almost all representative life forms on earth, conferring individuals the capacity of coordinating cellular and organismal metabolism, physiology, and behavior to day/night cycles (DUNLAP 1999; LOUDON 2012; DUNLAP and LOROS 2017). These rhythms exhibit periodic oscillation *circa* 24 hours, which can be sustained even in the absence of environmental signals. Yet, such rhythms can be entrained, that is, synchronized to external cues such as light and temperature (LAKIN-THOMAS and BRODY 2004; KUHLMAN *et al.* 2017; NARASIMAMURTHY and VIRSHUP 2017). At the cellular level these rhythms depend on a molecular oscillator, or pacemaker, which can be entrained by these external cues (through input pathways), and convey rhythmic information through output pathways to control gene expression and cellular and systemic signals. Thus, the output pathways coordinate the physiological changes orchestrated by the circadian core-oscillator, largely impacting the expression of clock-controlled genes (*ccgs*) (HARMER *et al.* 2001; WAGNER 2005; HURLEY *et al.* 2016; MONTENEGRO-MONTERO and LARRONDO 2016). The circadian core oscillator is composed of a transcriptional-translational negative feedback loop (TTFL), which is topologically conserved through different lineages, although it differs in the sequence composition of the core components: positive and negative elements (MERROW *et al.* 2010; ZHANG and KAY 2010; ZHENG and SEHGAL 2012). In the case of the model fungus *Neurospora crassa,* the positive element is a heterodimer formed by the transcription

factors White Collar 1 (WC-1) and White Collar 2 (WC-2) (White Collar Complex; WCC), capable of activating the transcription of the *frequency* gene, which encodes for the negative element (FRQ), feeds back to inhibit the activity of the positive element, there closing the loop (DUNLAP 1999; KING and TAKAHASHI 2000; ROSBASH 2009; RIPPERGER and BROWN 2010; MONTENEGRO-MONTERO *et al.* 2015). The changing levels and activities of these two components allow the entire system to oscillate (ARONSON *et al.* 1994a; ARONSON *et al.* 1994b; BRUNNER and KALDI 2008; NEISS *et al.* 2008). However, different aspects of how these clocks keep their oscillatory parameters like period or phase in a persistent manner, and the mechanisms underlying this process remain partially unresolved (RIPPERGER and BROWN 2010; BROWN *et al.* 2012; PARTCH *et al.* 2014; KRAMER 2015; LARRONDO *et al.* 2015; HURLEY *et al.* 2016).

The mechanisms involved in eukaryotic clock regulation implicate several layers of modulation, among them: chromatin remodeling, transcriptional control, antisense transcripts, post-transcriptional events, and alternative splicing (MONTENEGRO-MONTERO *et al.* 2015). In addition, negative clock elements appear to bear interesting characteristics such as particular codon bias, high degree of post-translational modifications (phosphorylation), and features of intrinsic disorder as opposed to defined structure (BRUNNER and SCHAFMEIER 2006; RIPPERGER and BROWN 2010; KOJIMA *et al.* 2011; HURLEY *et al.* 2014; HURLEY *et al.* 2016; MENDOZA-VIVEROS *et al.* 2017). Transcription regulation in the different clock models has been profusely addressed, leading to the identification of several transcription factors that participate in accessory transcriptional loops interlocked with the central core oscillator (RIPPERGER and BROWN 2010; ZHANG and

KAY 2010; ZHENG and SEHGAL 2012). Thus, in the case of *Drosophila melanogaster* the transcription factors PDP1 and VRI have been described to control the expression of the positive element CLK (HARDIN 2011), while in mammals the nuclear orphan receptors RORs and REV-ERVα/β modulate expression of the positive element BMAL (LOWREY and TAKAHASHI 2011). Moreover, in both systems there is evidence of an orthologous transcription factor involved in light-induced entrainment in both systems, CWO in flies and DEC1/2 in mammals (HONMA *et al.* 2002; KADENER *et al.* 2007; ROSSNER *et al.* 2008), further supporting the resemblance between such circadian clocks.

On the other hand, in the plant model *Arabidopsis thaliana*, the transcriptional regulations involved in the clockworks are widely studied and mapped, showing the participation of several networks to control different times of the day (NAGEL and KAY 2012; RONALD and DAVIS 2017). Interestingly, although these proteins have a similar impact over their respective circadian oscillators, except few exceptions, they do not share sequence homology, strengthening the idea of multiple independent origins of circadian systems, yet maintaining a conserved architecture (Figure 1.1).

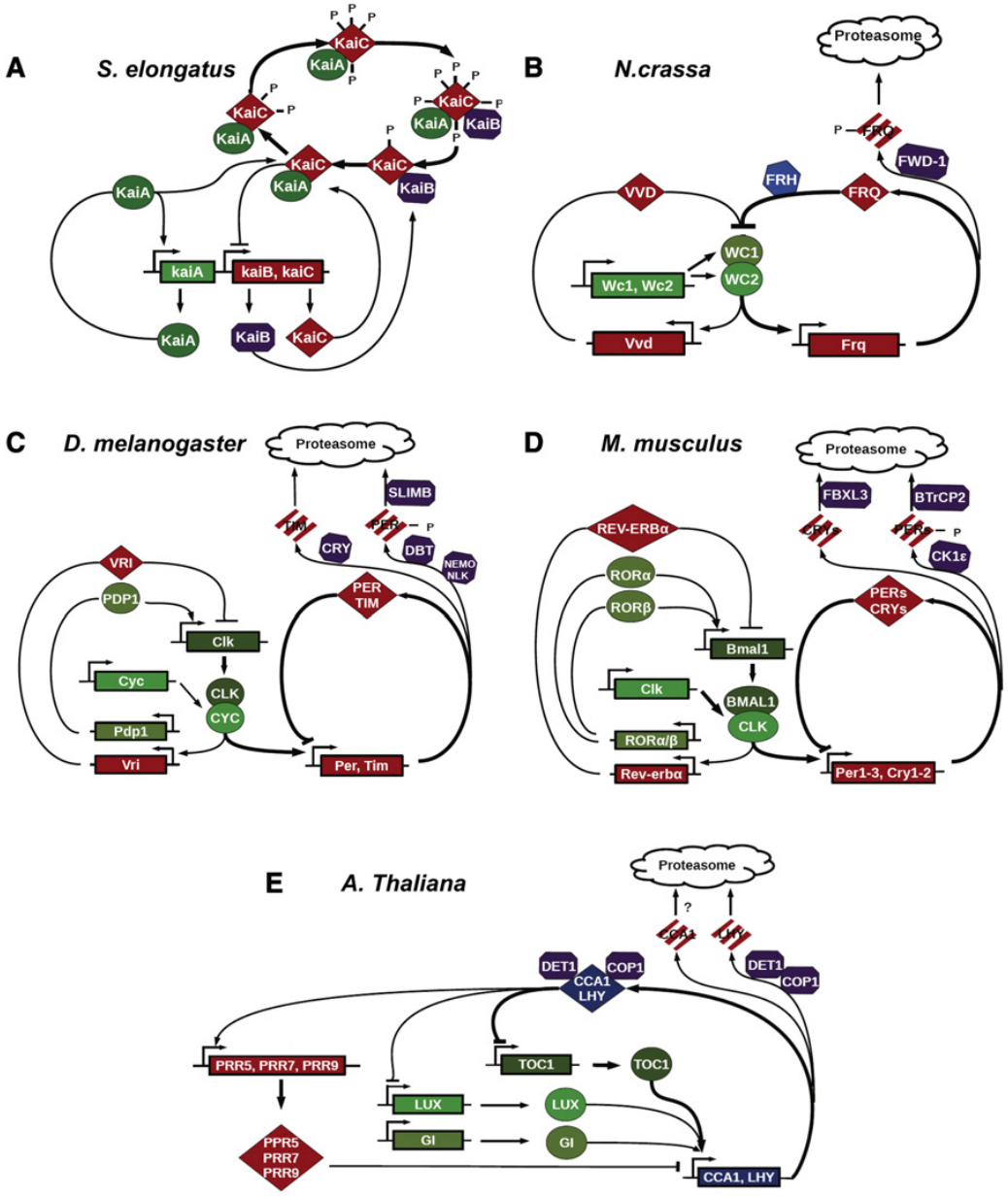

Figure 1.1. Schematic outline of canonical circadian core and interlocked feedback loops.

Clocks of *Synechococcus elongatus* (A), *N. crassa* (B), *D. melanogaster* (C), *Mus musculus* (D), and *A. thaliana* (E). In each case, a principal loop (bold lines) is supported by interlocked parallel loops (lighter lines) sharing common components, either activators (green) or repressors (red). In cyanobacteria (A), the principal loop is a posttranslational feedback loop (PTFL), based on cycles of KaiC phosphorylation. In the four eukaryotes, the primary known loop is a transcription-translation feedback loop; this complex architecture show accessorial transcriptional regulators modulating core-clock components, except in the *N. crassa* where none have been yet identified (Brown et al. 2012)

In Neurospora, different studies have revealed that several chromatin regulators act upon the promoters of core clock genes, modulating their normal circadian function (BELDEN *et al.* 2011; CHA *et al.* 2013; HUANG *et al.* 2013; RADUWAN *et al.* 2013). While WCC appears to play a key role in mastering *frq* expression, other transcriptional regulators allow -directly o indirectly- its fine-tuning by interaction with the *frq* promoter, as IEC-1 (GAI *et al.* 2017), or modulating WCC levels allowing metabolic compensation in response to sugar availability, as observed for CSP-1 and RCO-1 (SANCAR *et al.* 2012; OLIVARES-YANEZ *et al.* 2016). These regulators are capable of affecting core-clock component directly or through interaction with other TFs, showing an effect on the circadian function (SANCAR *et al.* 2011; SANCAR *et al.* 2012; OLIVARES-YANEZ *et al.* 2016; GAI *et al.* 2017) yet, no additional information is known regarding other TFs impacting clock function in this organism. Based on this evidence, in Neurospora and other clocks (ZHANG and KAY 2010; ZHENG and SEHGAL 2012), it is evident the roles of ancillary TFs as critical in proper circadian regulation.

N. crassa, along with *D. melanogaster*, has been two key model organisms that opened the doors to the era of molecular clocks (DUNLAP 1993; DUNLAP 2008; BRODY 2011). Indeed, work on both organisms was central in the dissection of the molecular basis of circadian rhythms, paving the road for the elucidation of circadian mechanisms in mammals (LOWREY and TAKAHASHI 2011; BROWN *et al.* 2012; PARTCH *et al.* 2014). The primary strategy used in early studies was based on classical genetic screens, scoring circadian phenotypes and mapping genes affected in mutants exhibiting altered clock properties. The ease to conduct genetic analyses in Neurospora (BEADLE and TATUM

1941) represented a clear advantage for forward genetics studies, whether it involved mapping naturally occurring mutations or mutants obtained by radiation or chemicals (FELDMAN and HOYLE 1973; FELDMAN and ATKINSON 1978). Importantly, the circadian phenotype of daily spore production in Neurospora (banding) was key in the use of this organism as a circadian model. Indeed, the use of the "*band*" (*bd*) strain, which corresponded to a mutant strain exhibiting reduced growth rate and enhanced circadian conidiation in race tubes assays, was quickly adopted as the WT background for most circadian studies (SARGENT *et al.* 1966; MONTENEGRO-MONTERO *et al.* 2015). Only in recent years *bd* was finally identified as a spontaneous mutation in the *ras-1* gene (*ras-1*^{bd}), exhibiting enhanced circadian output, particularly regarding rhythmic regulation of conidiation genes (SARGENT and WOODWARD 1969; BELDEN *et al.* 2007; BAKER *et al.* 2012).

However, race tube-based screens can provide confounding results, since this assay depends on the banding phenotype, and mutations that affect conidiation *per se* (and not necessarily the clock) can yield "mutants of interest". Nevertheless, in recent years it has become possible to conduct bioluminescence-based screens, utilizing luciferase as a proxy to follow circadian gene expression analyzing, unambiguously, the status of the core-clock (GOOCH *et al.* 2008; LARRONDO *et al.* 2012; MONTENEGRO-MONTERO *et al.* 2015). Such luciferase assays have helped analyze in 96-well format, multiple strains. Interestingly, recent studies have shown that in particular mutants related to carbon metabolism, the constant growth on fresh medium (as seen in race tubes) can yield circadian alterations that are not necessarily observed when performing luciferase assays on static 96-well cultures (EMERSON *et al.* 2015). Importantly, luciferase screens

can reveal normal clock function in strains that otherwise may appear arrhythmic, or devoid of circadian banding in race tubes assays (SHI *et al.* 2007; MONTENEGRO-MONTERO *et al.* 2015). A clear example of such situation is the analysis of *fwd1* mutants. FWD1 is a protein that regulates FRQ degradation and that was first described as an essential element of the Neurospora clock (HE *et al.* 2003); nevertheless, recent studies utilizing luciferase reporters revealed that neither FWD1 nor FRQ degradation *per se* are necessary for the proper functioning of the clock (LARRONDO *et al.* 2015), changing the paradigm regarding clock negative element half-life and period determination in the majority of the clock models (KRAMER 2015; ODE *et al.* 2017). While the lack of *fwd-1* leads to absence of conidial bands and altered FRQ levels (when analyzed from liquid culture media), growth of Neurospora on static cultures (96-well plates or agar-petri plates) allow to evidence the presence of unambiguous rhythms in FRQ expression as detected by luciferase quantification.

Taking these data into account, and taking advantage of the availability of an ample Knockout collection in Neurospora (COLOT *et al.* 2006), we sought to embark upon a comprehensive search of novel regulators of the circadian clock. Particularly, we focused on transcription factors (TFs), aiming at identifying the ones that could modulate clock components expression, and therefore clock parameters. Thus, we adopted a reverse genetic approach, utilizing the *Knockout* (KO) collection available from *Neurospora crassa* as well as luminescent reporters to analyze the circadian phenotypes in different genetic backgrounds. In this chapter, we report several TF whose absence cause clock mutant phenotypes. Particularly, we focus on the CBF1-like transcription factor

identified as Kpr-16, capable of affecting circadian period and clock response to shifts to high-temperature. Such results allow proposing a role for Kpr-16 as a regulator of the Neurospora clock, particularly in the integration of environmental temperature changes. Together, these results provide a conceptual framework to further understand the composition of the circadian regulatory network.

MATERIALS AND METHODS

Strains and Crosses

For this reverse genetic screen, we analyzed the sexual progeny obtained from crosses between circadian luciferase reporter strains and TFs KOs available from the Fungal Genetic Stock Center (FGSC, Kansas City, MO). The KOs, currently available in the lab, were constructed as part of the Neurospora Functional Genomic Project, where individual loci were replaced with a drug-resistance gene cassette for hygromycin B phosphotransferase (*hph*) (COLLOPY *et al.* 2010). We generated a list of putative TFs genes based on the presence of the DNA binding domain sequence described for the *N. crassa* genome (WEIRAUCH *et al.* 2014). Such list included other genes previously related to transcriptional function (BORKOVICH *et al.* 2004; TIAN *et al.* 2011), obtaining a final list of 272 putative TFs loci, which has been recently compiled (CARRILLO *et al.* 2017).

The reporter utilized in the primary screening was a firefly luciferase gene under the control of a minimal *frq* clock promoter, $frq_{c\text{-}box}$-*luc,* integrated into the *his-3* locus in LG I (*his-3::*$frq_{c\text{-}box}$-*luc*)(GOOCH *et al.* 2008). Alternatively, another reporter consisting on a destabilized firefly luciferase (containing a PEST domain), under the control of the same minimal $frq_{c\text{-}box}$ promoter was utilized, this time targeted to the *csr-1* locus (*csr-1::*$frq_{c\text{-}box}$-luc^{PEST})(OLIVARES-YANEZ *et al.* 2016). Both loci are apart by ~3 millions of bp., improving the number of successful crosses to that linkage group, preventing the sexual non-

segregation of reporter and hygromycin cassette (BARDIYA and SHIU 2007; GOOCH *et al.* 2008; HONDA and SELKER 2009; GOOCH *et al.* 2014). To confirm that the observed effects were due to the eliminated gene, and not to some other factor produced in the cross, and to also test the extent of potential circadian alterations we evaluated the expression of circadian output reporters. The two different clock reporters used were: *con-10*^{luc} and *medA*^{luc} (*con-10-luc*^{bar} and *med-A-luc*^{bar} respectively) (LAUTER and YANOFSKY 1993; OLIVARES-YANEZ *et al.* 2016). Both reporters correspond to fusions of luciferase to the respective gene ORFs at the corresponding endogenous loci. Therefore, they are translational reporters and technically not transcriptional reporters, as *frq*$_{c\text{-}box}$*-luc*. Nevertheless, they faithfully recapitulate overall circadian alterations (OLIVARES-YANEZ 2015), and therefore help further characterizing the mutants of interest.

Out of a list of 272 putative TFs encoding genes in the Neurospora genome (MONTENEGRO-MONTERO 2014), 45 KO strains were not available in the Neurospora FGSC KO collection (http://www.fgsc.net/) at the time we started the screening. This left us a with a total of 227 plausible crosses to analyze in this reverse genetic screen: one for each TF. From those crosses, KOs for 56 putative TFs did not yield successful offspring, as evidenced by absence of protoperithecia or unfruitful spore germination in 10 of the crosses. Thus, progeny derived from 171 crosses, including the WCC KOs *Δwc-1* (NCU02356) and *Δwc-2* (NCU00902), were obtained based on selection for hygromycin resistance and luciferase activity, and posteriorly analyzed for circadian behavior.

Growth Conditions

Culture conditions for vegetative growth and asexual reproduction utilized Vogel minimal medium (VM) (VOGEL 1956), whereas conditions for sexual development used synthetic Crossing medium (SCM) (WESTERGAARD and MITCHELL 1947). Sorbose-containing medium (FIGS) was used for colony isolation on plates and ascospore germination assays (DAVIS and DE SERRES 1970). Picked ascospores were then grown on slants containing VM media supplemented with hygromycin (200 mg/ml; Calbiochem, San Diego, CA) and luciferin (GoldBio) (10 μM), in order to select for progenies carrying Knockout cassettes and reporter activity respectively.

To conduct the circadian screen (see below), spores from the selected progeny were inoculated in a 96 well plate containing LNN-CCD media (OLIVARES-YANEZ 2015), containing 25 μM of Luciferin (GoldBio). Cultures were grown for 24 hours in constant light (LL) at 25˚C and then were analyzed under free-running conditions, consisting of constant darkness (DD) at 25˚C, for 4 to 5 days in Percival incubators equipped with CCD PIXIS 1024B cameras (Princeton Instruments). For the experiments contemplating analyses of temperature input, we varied the DD free-running temperature: 20˚C (DD20C), 25˚C (DD25C), 30˚C (DD30C), and also one temperature outside the range of compensation: 35˚C (DD35C).

Circadian Luciferase Screen

The 171 crosses were analyzed for circadian phenotypes in two high-throughput screens. 96-well plates inoculated with strains of interest were grown in constant light (see above), and then luciferase expression was analyzed for 4 to 5 days in complete darkness under a CCD camera. The resulting images were analyzed with a customized software (LARRONDO *et al.* 2012; STEVENS-LAGOS 2012). The acquired datasets varied in some cases containing, 2 or 3 pictures per hour, with exposure times of 10 or 5 minutes respectively. Nevertheless, these differences do not affect the analyses as information can be compared throughout the datasets. Importantly, control wild type (WT) strains were included in each 96-well plate and in each experimental run. The obtained luciferase traces obtained were analyzed as rough as well as normalized data.

For the circadian analysis of the recorded time series, the data was uploaded in a free-available online platform BioDare (***Bio***logical ***Da***ta ***Re***pository) (MOORE *et al.* 2014; ZIELINSKI *et al.* 2014); this platform provides information on *Period*, *Amplitude*, *Phase* and *RAE* (***R***elative ***A***mplitude ***E***rror), using different algorithms (ZIELINSKI *et al.* 2014). The data were processed in the following manner: first, detrending to remove stationary effects over our time series, as such trends can cause distortions in the data, masking circadian information. Secondly, we discarded the first and last 12 hours of the data to minimize noise effects associated with the transition from light to darkness and the mathematical process of detrending over the data, respectively. Finally, we normalized and aligned every data point with respect to the average of all points in their time series

to enhance their visualization and calculate period using a fast Fourier transform-nonlinear least-squares analysis (FFT-NLLS) (PLAUTZ *et al.* 1997; ZIELINSKI *et al.* 2014). All data sets produced in this screen are stored in the BioDare platform, as an open access repository, with the spirit of encouraging further analysis within the circadian and *N. crassa* community. The information will become available once the work is published, linking it to the publication. Importantly, the data sets are enlisted in the platform, describing reporters, TF KOs, and dates of the experiments.

All the BioDare parameters retrieved, except for *RAE*, were re-calculated (see below) to compare such parameter for each strain with the internal control of the *wild type* strains in the same 96-well plate and experiment, and to minimize the potential noise when comparing different plates in different experiments. The data were analyzed in the following form:

Period as **Period Change**: a positive or negative value measured in hours.

$$Period_{KO} - Period_{wt}$$

Phase as **Phase Shift**: a value obtained comparing the circadian time (CT) of Phase measure by the first peak.

$$(Phase_{KO} / CT_{KO}) - (Phase_{wt} / CT_{wt})$$

$$CT = (Period/24\ hours)$$

Amplitude as **Fold of Expression**: a ratio between the amplitude calculated by the average peaks of luminescence.

$$\text{Amplitude}_{KO}/\text{Amplitude}_{wt}$$

As a proof of principle, we analyzed the behavior of the luciferase reporter in KOs of core clock components: *wc-1* (NCU02356) and *wc-2* (NCU00902). As expected, the oscillations on these Knockouts disappeared, and the LUC levels were extremely low and completely arrhythmic (Figure 1.2 and 1.3).

To define which TF KOs displayed important circadian alterations, we focused on strains outside three or more standard deviation from normally distributed mean of WT controls (ZHANG *et al.* 2009) (Shapiro-Wilk test, $p<0.05$).

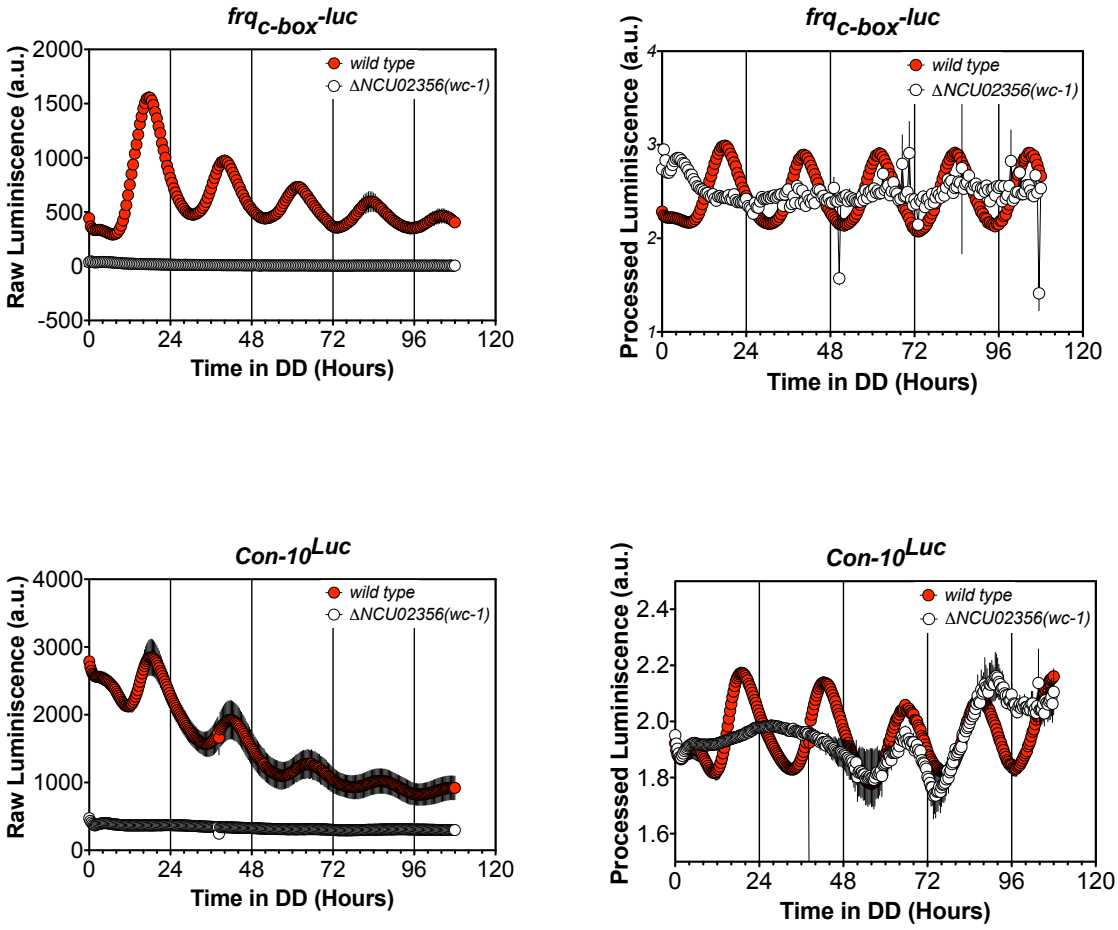

Figure 1.2. Circadian reporters are arrhythmic in *Δwc-1* strain.
Both reporters, *frq*$_{c\text{-}box}$*-luc* and *con-10*^{Luc}, confirm the absence of a functional clock in a *Δwc-1* strain.

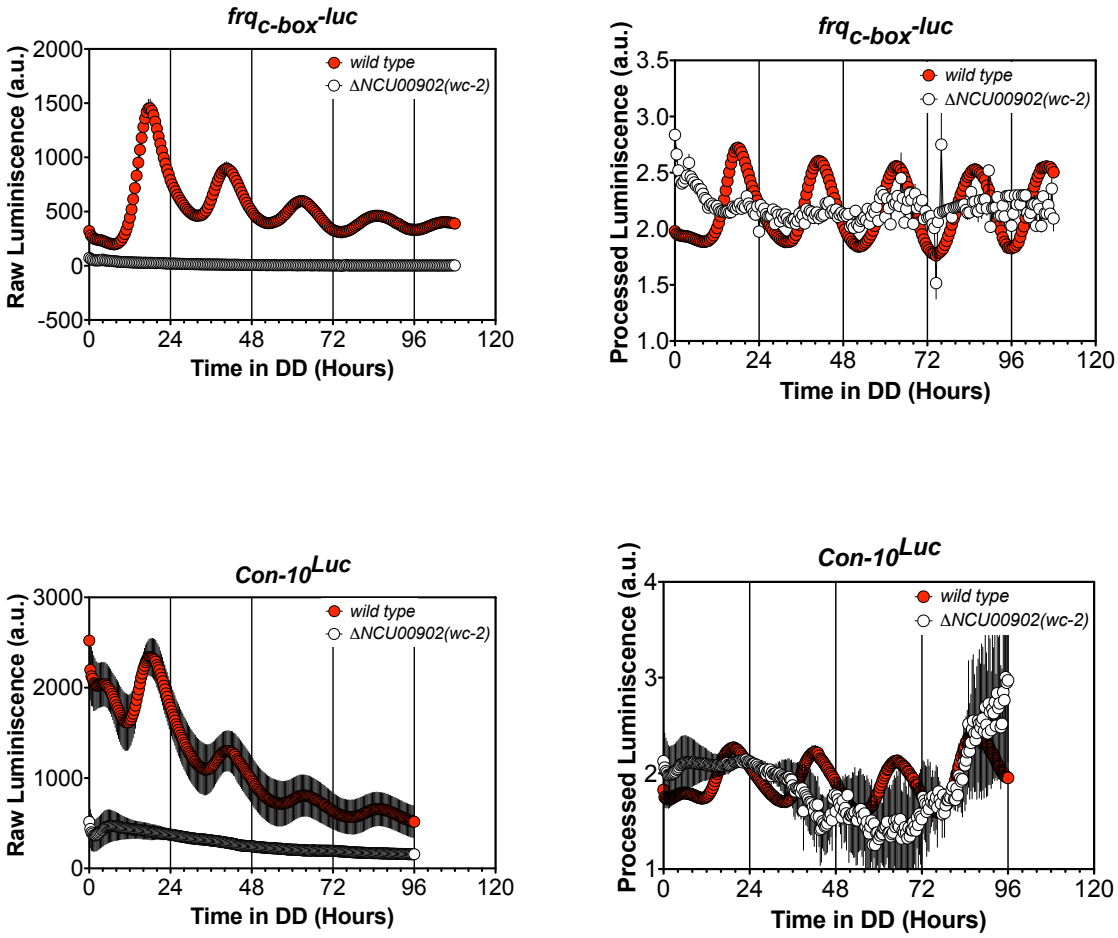

Figure 1.3. Circadian reporters are arrhythmic in a *Δwc-2* strain.
frq$_{c\text{-}box}$*-luc* and *con-10*^{Luc} reporters confirm the lack of a functional clock in the absence of WC-2.

RESULTS

Primary Circadian Screening

To identify novel transcriptional regulators impacting the Neurospora circadian clock we adopted a reverse genetic strategy, focusing on the transcription factors (TFs) identified in the genome of this organism (BORKOVICH *et al.* 2004; TIAN *et al.* 2011; WEIRAUCH *et al.* 2014). The screen consisted in analyzing the behavior of circadian luciferase reporters in the absence of individual TFs. As indicated in the methods section, this was achieved by crossing TF KOs, available from the Neurospora KO collection, with the indicated circadian luciferase reporters. The resulting progeny were monitored for LUC expression, focusing the analyses on four parameters: **Period Change**, **Phase Shift**, **Fold of expression** (see Methods) and the relative amplitude error (**RAE**) for the oscillatory component in the time series (MOORE *et al.* 2014; ZIELINSKI *et al.* 2014). To select the stronger circadian phenotypes in our crosses, we defined upper and lower thresholds at three standard deviations of the WT mean values for each parameter. With this approach, we covered 99.7% of the WT group (assuming a normal distribution, Shapiro-Wilk test $p<0.05$), selecting our TFs Knockout candidates from the most plausible population (ZHANG *et al.* 2009), assuming a $p<0.003$ for further statistical test analysis.

The quality of the reporter's oscillations was measured according to the **RAE** index. The latter considers values from 0 to 1, where 0 is assigned to perfect oscillatory components on the data set while 1 indicates a null oscillatory component (MICHAEL and MCCLUNG 2002; SHI *et al.* 2007). The WT population had a **RAE** of 0.2536, similar to the values obtained for most of the TFs KO strains (Figure 1.4).

Only one TF KO (NCU02666) fell outside the WT RAE, suggesting an arrhythmic phenotype, although this RAE alteration was not replicated in a secondary screening using a different clock reporter (*con-10*^{luc}) (Figure 1.5). Moreover, visual analysis of the Δ*NCU02666* primary data showed robust oscillations in the first two days, but then the traces started to show noise (Figure 1.5, upper right panel). Observing the raw data, it is possible to see low levels of *frq*$_{c\text{-}box}$*-luc* expression in this strain, low levels that probably make the oscillations noisy, increasing its **RAE** index. Comparing the **RAE** index from *ΔNCU02666* strain (0.79) with the **RAE** from the arrhythmic strains: *Δwc-1* (0.99) and *Δwc-2* (0.95) (grey line and black line in Figure 1.4, respectively) shows that the **RAE** for the KO of NCU02666 is different to that observed in the core-clock mutant arrhythmic strains. Such results highlight that the luciferase traces exhibiting altered RAE need to be visually examined in order to understand the nature of such alteration. Moreover, the access to additional clock reporters helps to confirm or disregard the relevance of certain alterations.

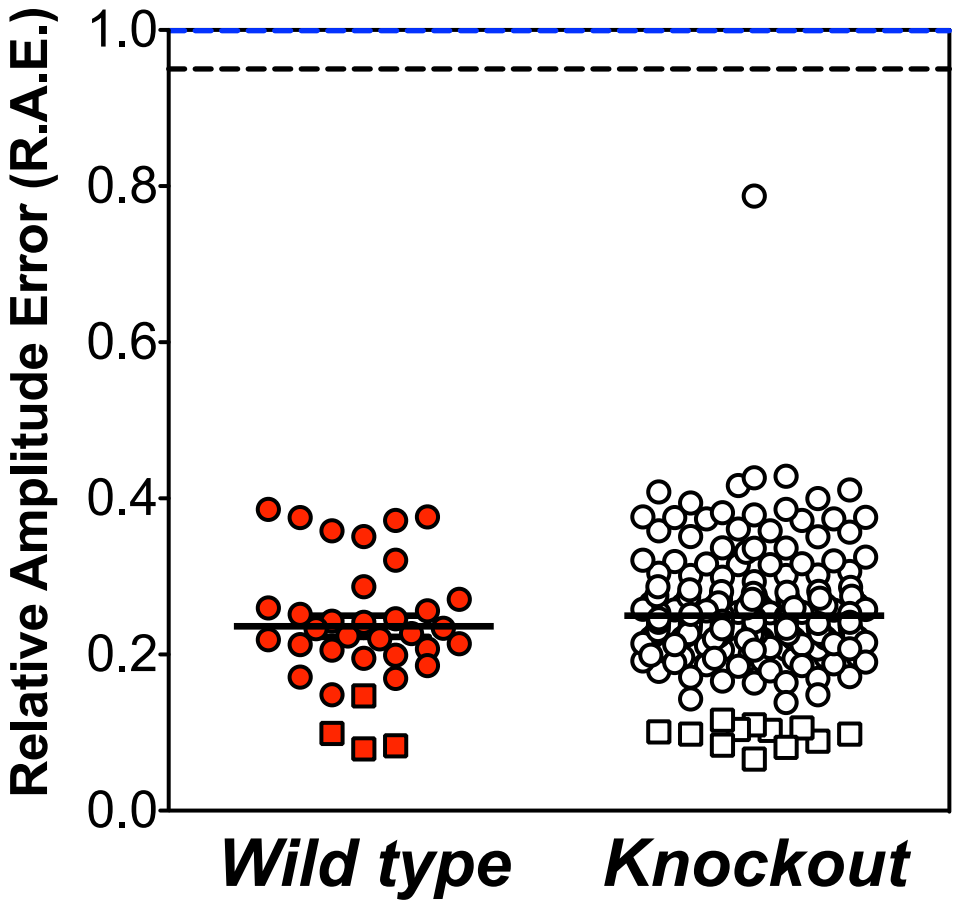

Figure 1.4. Comparison of WT and KOs overall quality of circadian oscillations. Progeny coming from the 171 crosses analyzed in this study were compared to the RAE index established on the BioDare platform (ZIELINSKI *et al.* 2014). We compared the Relative Amplitude Error mean of progeny coming from all 171 KO strains (white) and WT (red) from every 96-wells plate analyzed; the bar is the mean for each data set. The dots are data from *frq*$_{c\text{-}box}$*-luc* reporter, whereas the squares are data from *frq*$_{c\text{-}box}$*-luc*^{PEST} reporter. The blue line (---) and the black line (---) is the RAE value for *Δwc-1* and *Δwc-2*, respectively.

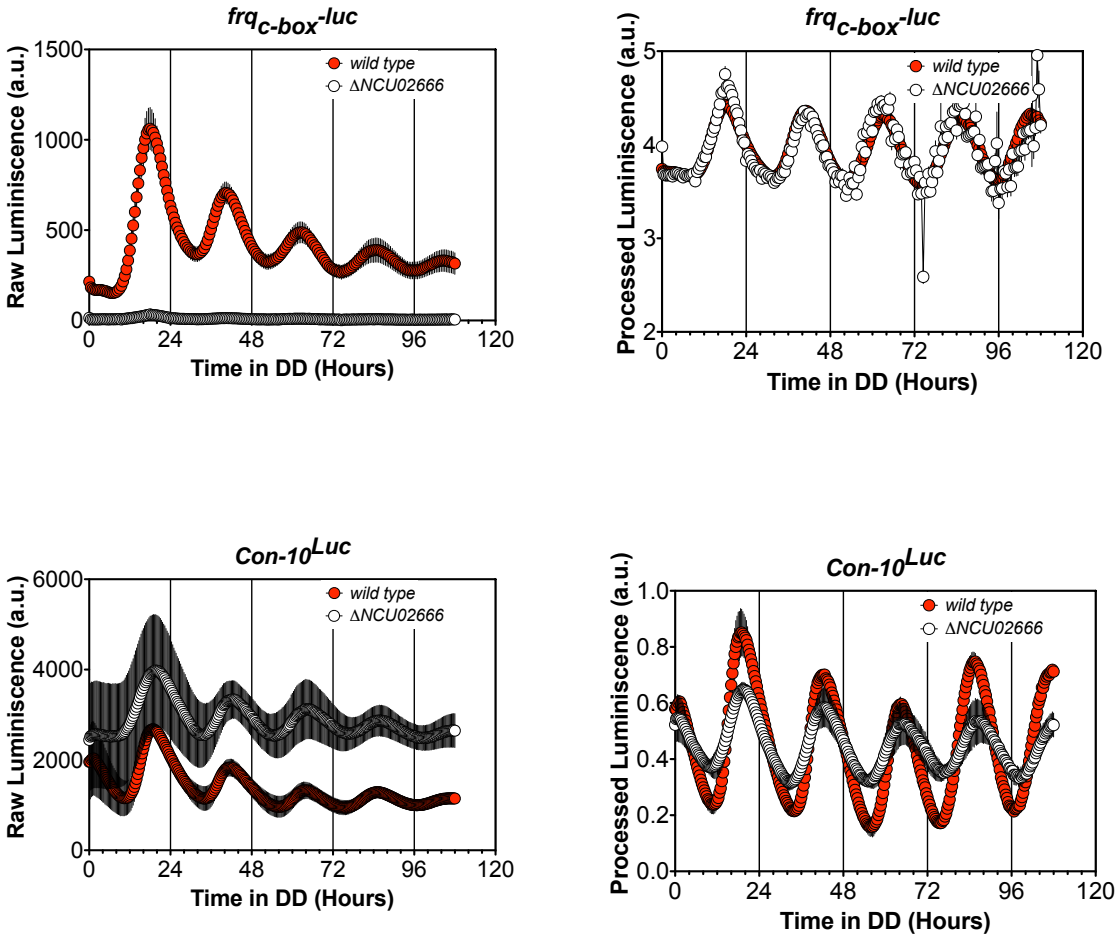

Figure 1.5. Characterization of clock reporters in a *ΔNCU02666* strain.
The *frq*$_{c\text{-}box}$*-luc* reporter in *ΔNCU02666* exhibited extremely low levels of LUC activity (upper right panel). When such traces are normalized it is possible to confirm the presence of rhythmic traces particularly during the first two days, as these oscillations become noisy thereafter, contributing to high a RAE index for this strain. When the *con-10*^{luc} reporter is analyzed in the absence of *ncu02666* it is possible to observe robust circadian rhythms in this strain.

For the other circadian parameters, **period change**, **phase shift** and **fold of expression**, we observed several strains with strong phenotypes differing from the WT population. In the case of **period changes**, most of the TFs KOs identified as interesting exhibited shorter periods (Figure 1.6) and only two TF KOs (NCU08999 and NCU01243) displayed longer periods (Figure 1.7). These results contrast with the results observed in similar screens conducted in other circadian models, where most of the identified genes affecting the circadian function yielded longer periods (MATSUMOTO *et al.* 2007; HIROTA *et al.* 2008; ZHANG *et al.* 2009; AGRAWAL and HARDIN 2016).

In order to assess **phase shift**, we considered the first circadian peaks at their respective circadian time (CT), to eliminate the **period change** component from the difference (see Methods) between WT and a KO of interest. For a better visualization of this effect we plotted **phase shift** vs **period change** (Figure 1.8). The WT population, from the different camera runs, showed a phase dispersion of ~45 minutes (±0.75 h of SD). Similarly, the phase distribution of the analyzed KOs showed little alterations, except for four strains that exhibited phase delay. One of them corresponded to *ΔNCU08999*, which showed a strong phase delay, beyond the phase value expected for its lengthened **period.** The other three strain were *ΔNCU03352*, *ΔNCU04628*, *ΔNCU00223* which exhibited a phase delay, despite of not having any period defect (Figure 1.8).

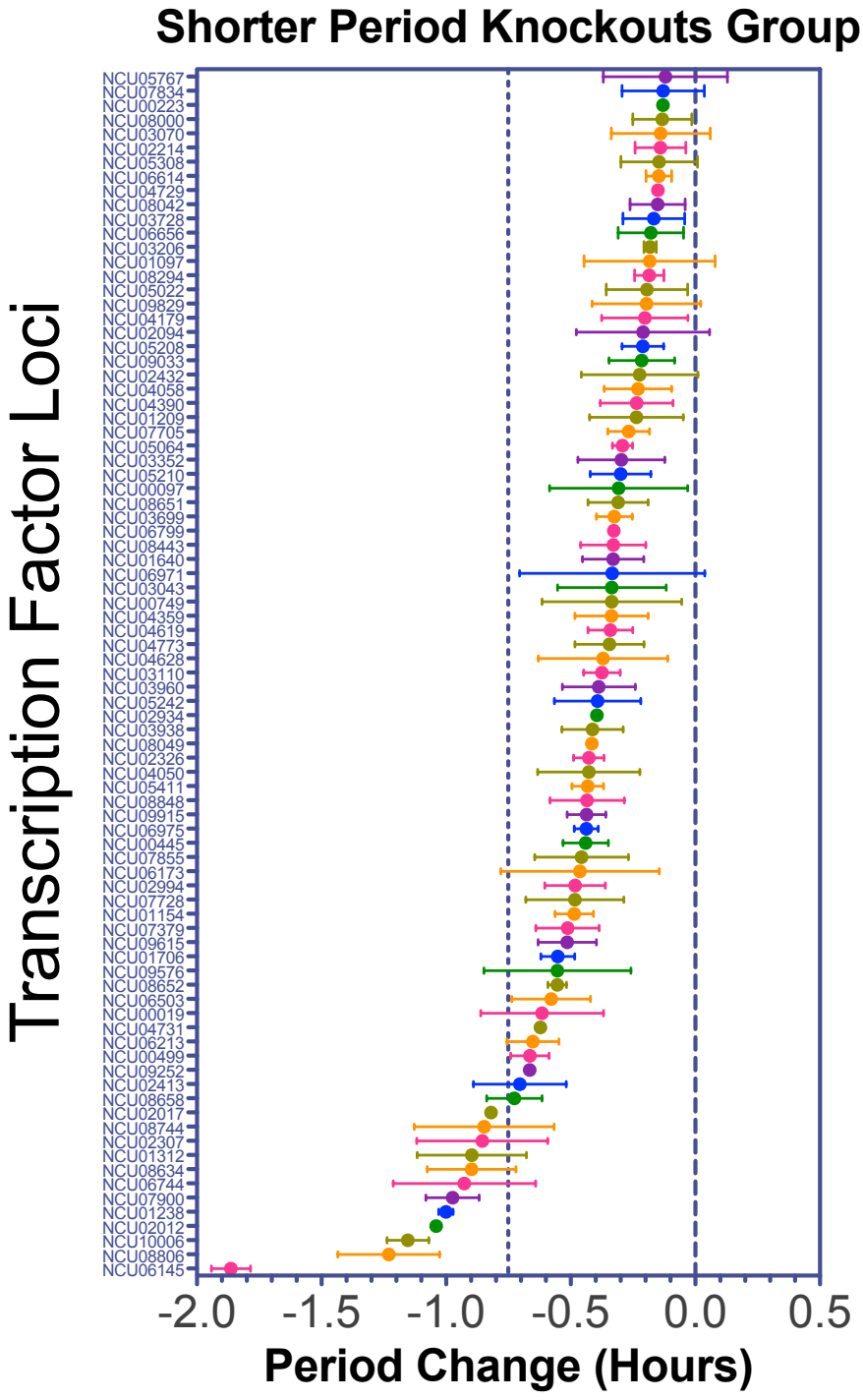

Figure 1.6. KOs exhibiting shorter circadian period.
The plot depicts 85 KOs crosses resulting in negative **period change** values; each dot reflects the mean and the error bars the standard error for each TF KO analyzed with at least three replicates. From the small dashed lines to the right reflect the behavior of the 99.7% of the WT population.

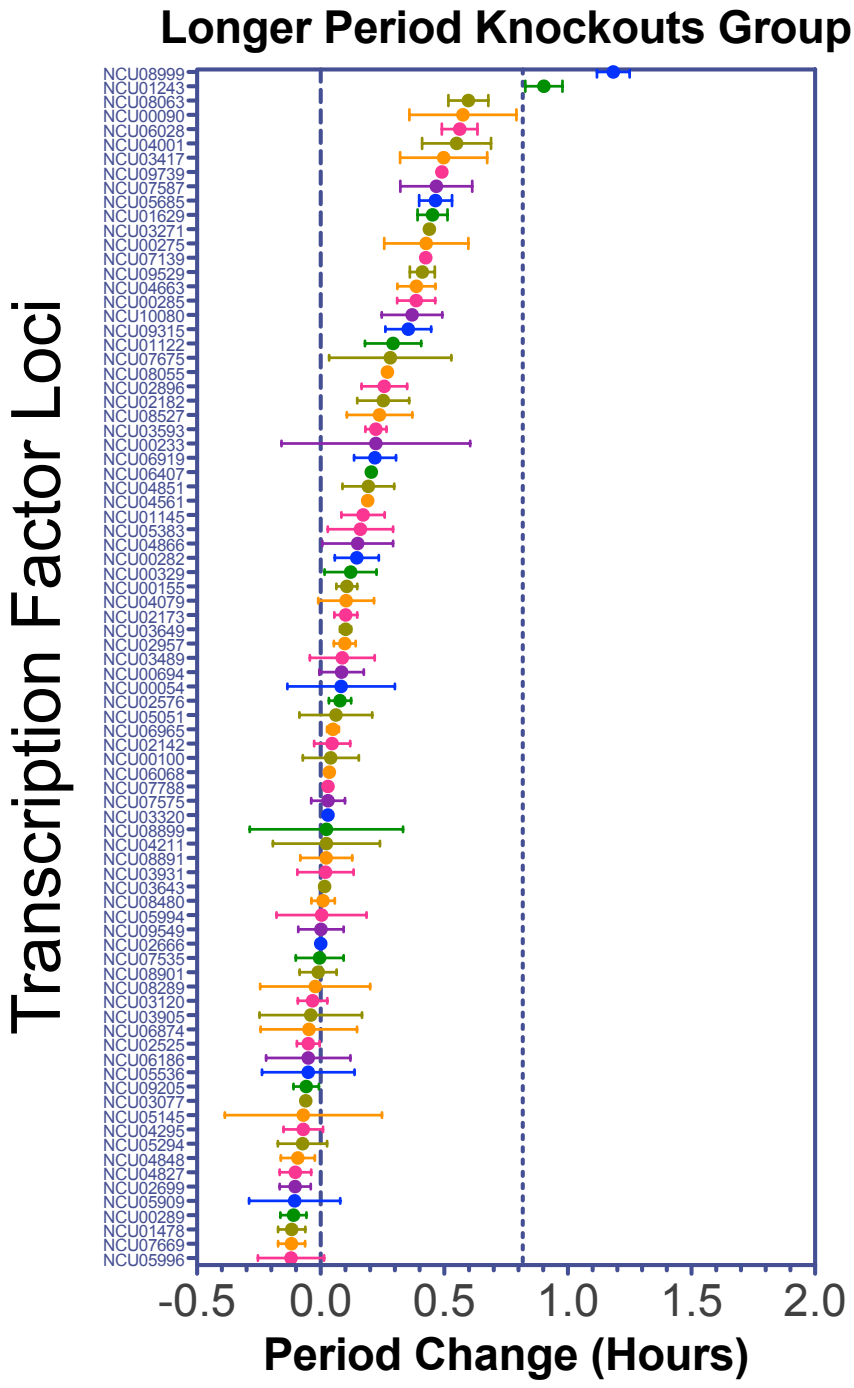

Figure 1.7. KOs exhibiting longer circadian period.
The plot summarizes the data of 84 TF KOs, focusing on the ones that present positive **period changes**. Each dot reflects the mean and the error bars is the standard error for each KO for TF analyzed with at least three replicates. From the small dashed lines to the left reflect the behavior of the 99.7% of the WT population.

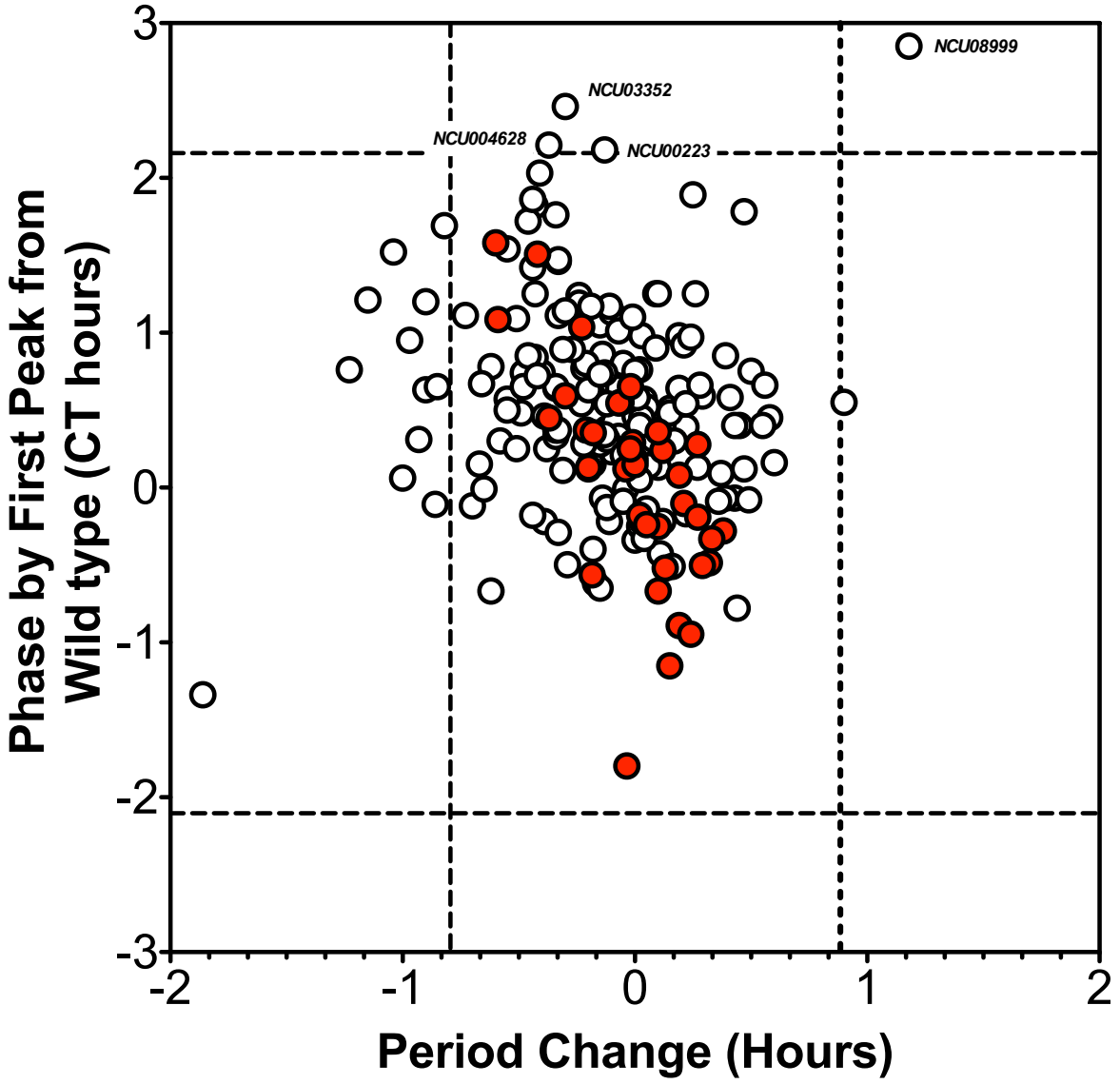

Figure 1.8. Circadian phase shift analysis of Neurospora TF KOs.
Out of 169 analyzed TF KOs (excluding *Δwc-1* and *Δwc-2*) phase shifts versus period change values were analyzed and compared for the KOs (white dots) and WT (red dots) from each plate. The dashed lines reflect the 99.7% of the WT population for each parameter.

Defining mutants with **fold of expression** defects may prove more complicated, as some TF KOs can display decreased growth, which may also translate to overall lower luminescence leading to false positives when screening for this parameter (ZHANG *et al.* 2009). **Fold of expression** is tightly associated to **RAE** index as we indicated in Figure 1.4, that's why we plot these two variables for each strain to better appreciate the defects. Regardless, we were capable of defining five strains with increased amplitude and two strains with decreased amplitude (Figure 1.9). Contrary to our initial expectations, just one strain *ΔNCU02666*, exhibited low expression and high **RAE** index, although other strains, such as *ΔNCU06173* had expression levels that were one third of WT. On the other hand, the strains that almost double the WT expression levels tend to have lower **RAE** indexes, probably due to the reduction of noise oscillations.

The results of our primary screening are summarized in Table 1.1, indicating the number of replicates and the four parameters calculated for each of the analyzed TF KOs. From this list we selected 20 KOs exhibiting substantial alterations in their circadian parameters. To be sure that the circadian change was caused by the genetic perturbation, provoked by the removal of the specific locus of interest, we also analyzed WT sibling for each selected KO. In addition, we analyzed the corresponding KOs utilizing different clock reporters (output reporters, see Methods) as part of a secondary circadian screen (see below). This additional evaluation helps reducing the error rate associated with technical noise derived from the analysis of different plates and/or camera runs, or for unlinked spontaneous mutations present in the KO or emerging during the sexual crosses (WATTERS and STADLER 1995).

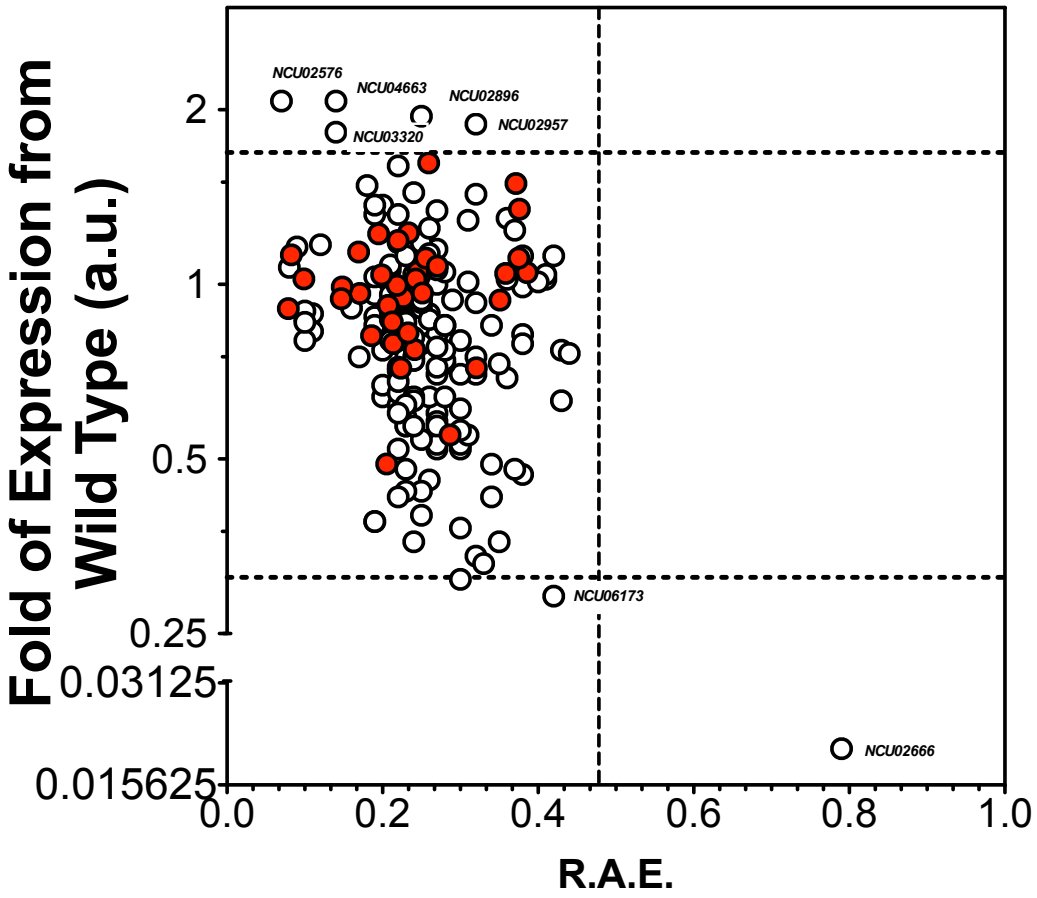

Figure 1.9. Several Neurospora TF KOs exhibit circadian fold of expression defects. 169 TF KOs (excluding *Δwc-1* and *Δwc-2*) analyzed in this study were plotted for RAE and fold of expression values. KOs (white dots) were compared to WT strains (red dots) present in each plate. The dashed lines reflect the 99.7% of the WT population for each parameter.

Table 1.1. Circadian Parameters measured in the primary screen of 171 TFs analyzed for their role in the Neurospora Clock

Locus	n	Period Change	Phase Shift	Fold of Expression	R.A.E.
NCU00019	4	-0.62	0.78	0.64	0.23
NCU00054	3	0.08	0.25	1.34	0.27
NCU00090	5	0.58	0.45	1.02	0.26
NCU00097	4	-0.31	0.11	1.02	0.25
NCU00100	7	0.04	0.57	0.88	0.24
NCU00155	8	0.11	-0.43	1.01	0.31
NCU00223	1	-0.13	2.18	1.48	0.18
NCU00233	3	0.22	0.39	0.57	0.23
NCU00275	4	0.43	-0.07	0.87	0.21
NCU00282	7	0.15	0.51	0.87	0.23
NCU00285	3	0.39	0.85	0.71	0.22
NCU00289	4	-0.11	-0.22	0.80	0.24
NCU00329	8	0.12	-0.22	1.44	0.24
NCU00445	4	-0.44	1.42	0.97	0.23
NCU00499	4	-0.66	0.67	1.29	0.31
NCU00694	4	0.09	1.25	0.52	0.22
NCU00749	4	-0.34	0.64	1.18	0.22
NCU00902	***3***	***NR***	***NR***	***1.20***	***0.95***
NCU01097	3	-0.18	0.59	0.76	0.24
NCU01122	3	0.29	0.59	0.90	0.21
NCU01145	7	0.17	0.30	1.00	0.27
NCU01154	7	-0.49	0.48	0.31	0.30
NCU01209	3	-0.24	1.24	0.61	0.30
NCU01238	8	-1.00	0.06	0.52	0.30
NCU01243	3	0.90	0.55	0.75	0.23
NCU01312	3	-0.90	1.20	0.62	0.23
NCU01478	5	-0.12	0.38	1.30	0.36
NCU01629	6	0.45	0.40	0.77	0.20
NCU01640	3	-0.33	1.11	0.64	0.20
NCU01706	5	-0.55	0.57	0.89	0.26
NCU02012	1	-1.04	1.52	0.60	0.27
NCU02017	1	-0.82	1.69	0.47	0.38
NCU02094	3	-0.21	0.31	1.15	0.27
NCU02142	6	0.05	-0.14	1.16	0.09
NCU02173	6	0.10	1.25	0.62	0.22
NCU02182	3	0.25	1.89	0.99	0.20
NCU02214	5	-0.14	0.86	1.02	0.41
NCU02307	3	-0.86	-0.11	0.87	0.23
NCU02326	4	-0.43	0.78	0.70	0.32
NCU02356	***3***	***NR***	***NR***	***0.98***	***0.99***

NCU02413	9	-0.70	-0.12	0.53	0.30
NCU02432	3	-0.22	0.77	0.98	0.24
NCU02525	5	-0.05	0.23	0.87	0.10
NCU02576	6	0.08	0.31	2.07	0.07
NCU02666	3	0.00	-0.34	0.02	0.79
NCU02699	7	-0.10	0.23	0.89	0.11
NCU02896	4	0.26	1.25	1.89	0.32
NCU02934	1	-0.40	0.74	0.39	0.19
NCU02957	4	0.10	0.13	1.95	0.25
NCU02994	4	-0.48	0.74	0.63	0.43
NCU03043	4	-0.34	1.76	1.01	0.20
NCU03070	7	-0.14	-0.07	0.83	0.11
NCU03077	1	-0.06	0.50	0.70	0.30
NCU03110	6	-0.38	0.25	0.90	0.10
NCU03120	4	-0.03	0.21	0.80	0.10
NCU03206	14	-0.18	0.15	0.86	0.10
NCU03271	1	0.44	-0.78	0.67	0.20
NCU03320	1	0.03	0.98	1.83	0.14
NCU03352	3	-0.30	2.46	0.36	0.35
NCU03417	4	0.50	0.75	0.46	0.26
NCU03489	4	0.09	0.90	1.24	0.37
NCU03593	4	0.22	-0.17	1.05	0.28
NCU03643	1	0.02	-0.25	0.34	0.32
NCU03649	4	0.10	0.47	0.75	0.24
NCU03699	4	-0.33	1.46	0.80	0.21
NCU03728	3	-0.17	-0.62	0.97	0.22
NCU03905	4	-0.04	-0.01	0.82	0.38
NCU03931	4	0.02	0.76	0.96	0.19
NCU03938	7	-0.41	2.03	0.21	0.36
NCU03960	4	-0.39	-0.22	0.79	0.25
NCU04001	5	0.55	0.40	0.83	0.23
NCU04050	4	-0.43	1.82	1.06	0.21
NCU04058	3	-0.23	0.54	1.37	0.20
NCU04079	6	0.10	0.37	0.84	0.27
NCU04179	4	-0.20	0.63	1.18	0.25
NCU04211	3	0.02	0.53	0.88	0.19
NCU04295	4	-0.07	0.64	1.12	0.42
NCU04359	4	-0.34	0.65	1.07	0.25
NCU04390	4	-0.24	1.19	0.85	0.34
NCU04561	1	0.19	0.98	0.65	0.22
NCU04619	5	-0.34	0.33	0.55	0.31
NCU04628	3	-0.37	2.21	1.04	0.24
NCU04663	4	0.39	-0.07	2.07	0.14
NCU04729	1	-0.15	1.05	0.98	0.23
NCU04731	3	-0.62	-0.67	1.32	0.22

NCU04773	9	-0.34	0.37	0.85	0.25
NCU04827	5	-0.10	1.13	0.75	0.17
NCU04848	4	-0.09	0.42	1.03	0.19
NCU04851	6	0.19	0.64	0.64	0.26
NCU04866	6	0.15	0.48	0.72	0.22
NCU05022	5	-0.19	0.16	0.78	0.24
NCU05051	6	0.06	0.14	0.79	0.38
NCU05064	4	-0.29	-0.50	1.32	0.19
NCU05145	4	-0.07	0.33	0.82	0.27
NCU05208	8	-0.21	0.80	0.64	0.24
NCU05210	3	-0.30	1.14	0.44	0.25
NCU05242	7	-0.39	0.46	0.52	0.27
NCU05294	6	-0.07	1.01	0.48	0.23
NCU05308	4	-0.15	0.28	1.04	0.41
NCU05383	6	0.16	-0.51	0.59	0.23
NCU05411	5	-0.43	0.84	0.99	0.38
NCU05536	6	-0.05	0.80	0.93	0.32
NCU05685	6	0.47	0.12	1.08	0.21
NCU05767	3	-0.12	0.31	0.74	0.24
NCU05909	4	-0.11	1.17	0.59	0.23
NCU05994	4	0.00	0.46	0.91	0.25
NCU05996	7	-0.12	-0.13	1.37	0.19
NCU06028	4	0.56	0.66	0.87	0.26
NCU06068	1	0.04	-0.33	0.57	0.23
NCU06145	3	-1.86	-1.34	1.43	0.32
NCU06173	4	-0.46	0.85	0.29	0.42
NCU06186	6	-0.05	-0.09	0.68	0.22
NCU06213	6	-0.65	-0.01	0.63	0.24
NCU06407	1	0.21	0.92	0.44	0.23
NCU06503	7	-0.58	0.30	0.40	0.25
NCU06614	11	-0.15	-0.65	0.73	0.28
NCU06656	9	-0.18	-0.40	0.43	0.34
NCU06744	3	-0.93	0.31	0.53	0.27
NCU06799	1	-0.33	1.47	0.33	0.33
NCU06874	4	-0.05	0.21	0.94	0.29
NCU06919	6	0.22	0.54	0.62	0.23
NCU06965	7	0.05	0.52	0.86	0.23
NCU06971	3	-0.33	-0.29	0.60	0.22
NCU06975	4	-0.44	-0.18	1.25	0.26
NCU07139	1	0.43	0.40	0.36	0.24
NCU07379	4	-0.51	0.25	0.96	0.21
NCU07535	5	0.00	0.18	0.75	0.32
NCU07575	5	0.03	0.45	1.02	0.36
NCU07587	5	0.47	1.78	0.49	0.34
NCU07669	4	-0.12	0.54	0.58	0.27

NCU07675	5	0.28	0.66	0.38	0.30
NCU07705	4	-0.27	0.89	1.13	0.26
NCU07728	3	-0.48	0.65	0.74	0.24
NCU07788	1	0.03	-0.22	0.43	0.22
NCU07834	7	-0.13	0.74	0.54	0.25
NCU07855	3	-0.46	1.72	0.87	0.21
NCU07900	6	-0.97	0.95	0.69	0.36
NCU08000	12	-0.13	0.34	0.80	0.30
NCU08042	8	-0.15	0.73	0.74	0.28
NCU08049	1	-0.42	0.72	1.06	0.27
NCU08055	1	0.27	0.13	0.79	0.25
NCU08063	5	0.60	0.16	0.85	0.28
NCU08289	6	-0.02	0.57	0.77	0.43
NCU08294	4	-0.19	1.17	0.85	0.28
NCU08443	7	-0.33	0.37	0.85	0.28
NCU08480	4	0.01	0.58	0.91	0.16
NCU08527	6	0.24	0.97	0.70	0.27
NCU08634	4	-0.90	0.63	0.89	0.21
NCU08651	3	-0.31	0.89	0.80	0.22
NCU08652	5	-0.55	0.50	0.64	0.28
NCU08658	4	-0.73	1.11	1.01	0.40
NCU08744	4	-0.85	0.65	0.73	0.35
NCU08806	6	-1.23	0.76	0.76	0.44
NCU08848	5	-0.43	1.25	0.56	0.30
NCU08891	7	0.02	0.40	1.12	0.38
NCU08899	3	0.02	0.05	0.72	0.27
NCU08901	3	-0.01	1.10	0.93	0.25
NCU08999	7	1.18	2.85	0.77	0.28
NCU09033	4	-0.22	0.28	0.81	0.24
NCU09205	5	-0.06	0.55	1.07	0.08
NCU09252	1	-0.67	0.15	0.85	0.19
NCU09315	6	0.36	-0.09	1.17	0.12
NCU09529	7	0.41	0.58	1.12	0.23
NCU09549	7	0.00	0.75	0.57	0.24
NCU09576	3	-0.55	1.54	1.60	0.22
NCU09615	5	-0.51	1.09	0.87	0.21
NCU09739	1	0.49	-0.08	0.39	0.19
NCU09829	5	-0.20	0.12	0.78	0.27
NCU09915	3	-0.44	1.86	0.48	0.37
NCU10006	3	-1.15	1.21	0.57	0.27
NCU10080	4	0.37	0.09	0.94	0.25

The final list of TF KOs studied in the reverse genetic screen. In this table, we showed the info for each locus under study.

Secondary Circadian Screen

To confirm the clock defects observed in our minimal clock reporter were indeed due to circadian alterations, and not to unidentified technical errors, for example low expression, as previously described for the NCU02666 KO, we evaluated the behavior of clock output reporters in defined TF KOs. For this, we focused on the output genes *con-10* (NCU07325), a gene expressed in late stages of conidial differentiation (ROBERTS *et al.* 1988), with a circadian expression (LAUTER and YANOFSKY 1993); and *medA* (*acon-3*; NCU07617), a gene required for early conidiophore development and female fertility (CHUNG *et al.* 2011), with an anti-phasic circadian expression to *frq* expression (unpublished data).

Regarding period alterations, 12 short periods and 2 long periods KO had been originally identified and were subjected to a secondary screen. KOs for *NCU08744*, *NCU08634*, *NCU01312*, *NCU02012*, *NCU08806* and *NCU06744* were dismissed, as the secondary screen did not confirm considerable **period change** defects (Figure 1.10). For the KOs *NCU01243*, *NCU02307* and *NCU06744*, it was not possible to analyze the output reporters in these strains, leaving their confirmation pending. However, several KOs showed variation in the magnitude of the defects previously observed, reducing the number of strong candidates to 6 TFs in which their individual absence leads to **period changes** (Figure 1.11 and Figure 1.12).

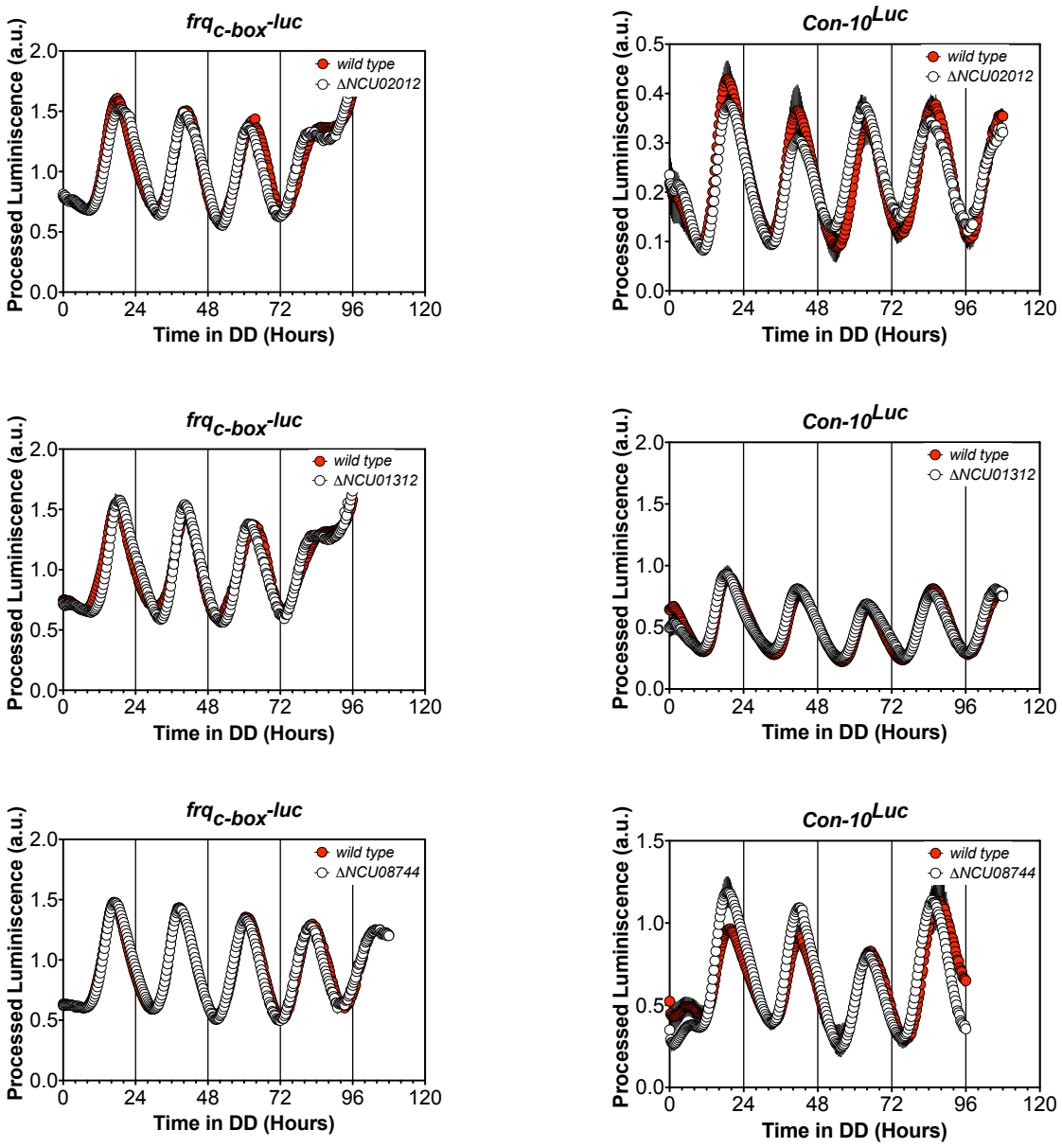

Figure 1.10. KOs for which a secondary screen did not confirm period alterations. Three examples of KO identified in the primary screen are shown, depicting luciferase expression coming from a *frq*$_{c\text{-}box}$*-luc* reporter in the KOs and WT sibling strains (left column). The right column shows the expression of a *con-10*^{luc} reporter. As it can be seen for both reporters, no appreciable period changes are evident.

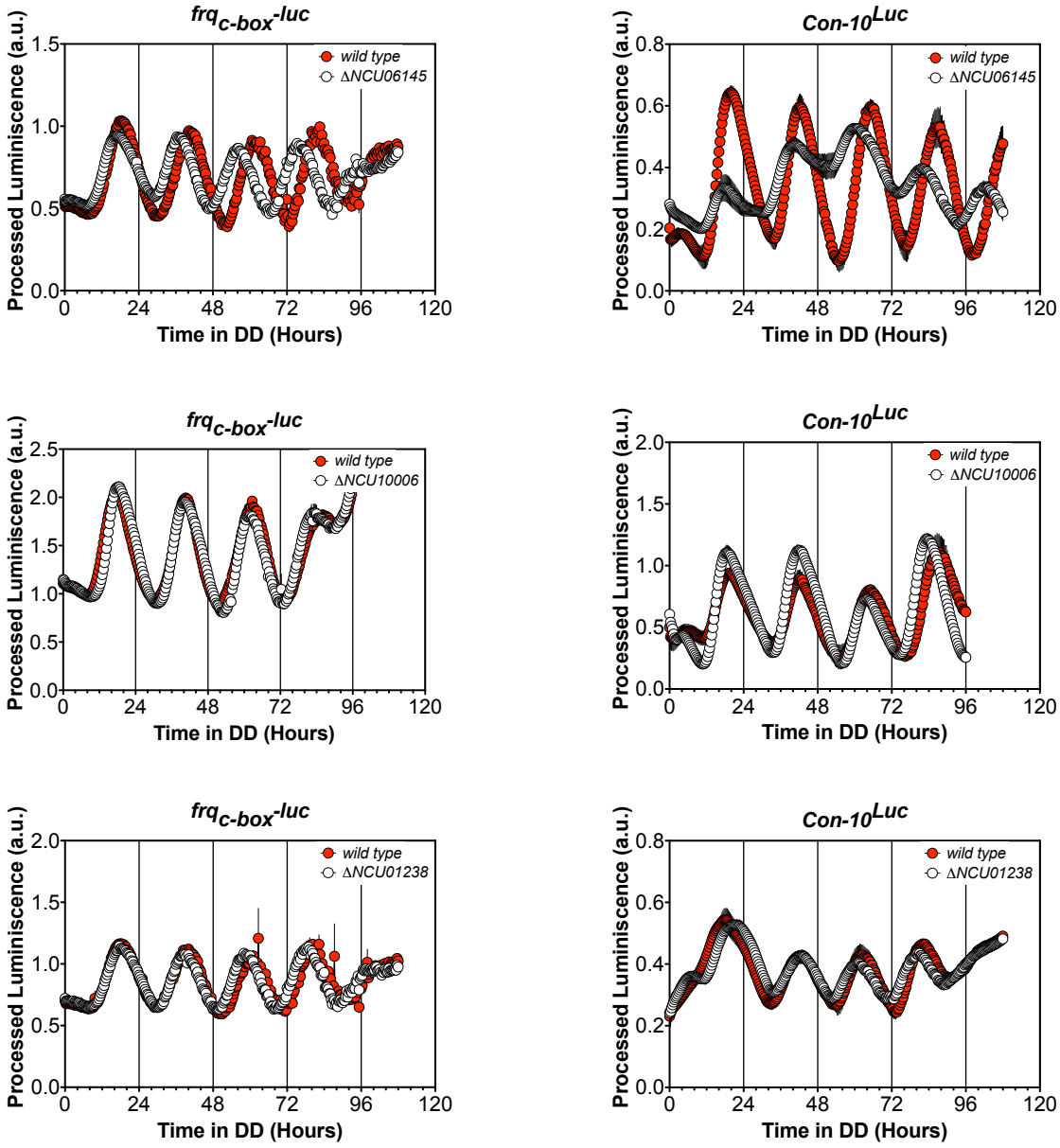

Figure 1.11. Putative Neurospora TFs whose absence leads to a change in circadian period.

The left column depicts luciferase expression coming from *frq*$_{c\text{-}box}$*-luc* reporters in selected TF KOs and their WT sibling strains. The right column shows the expression of a *con-10*^{luc} reporter. For both reporters it is possible to confirm changes in period for the indicated TF KOs.

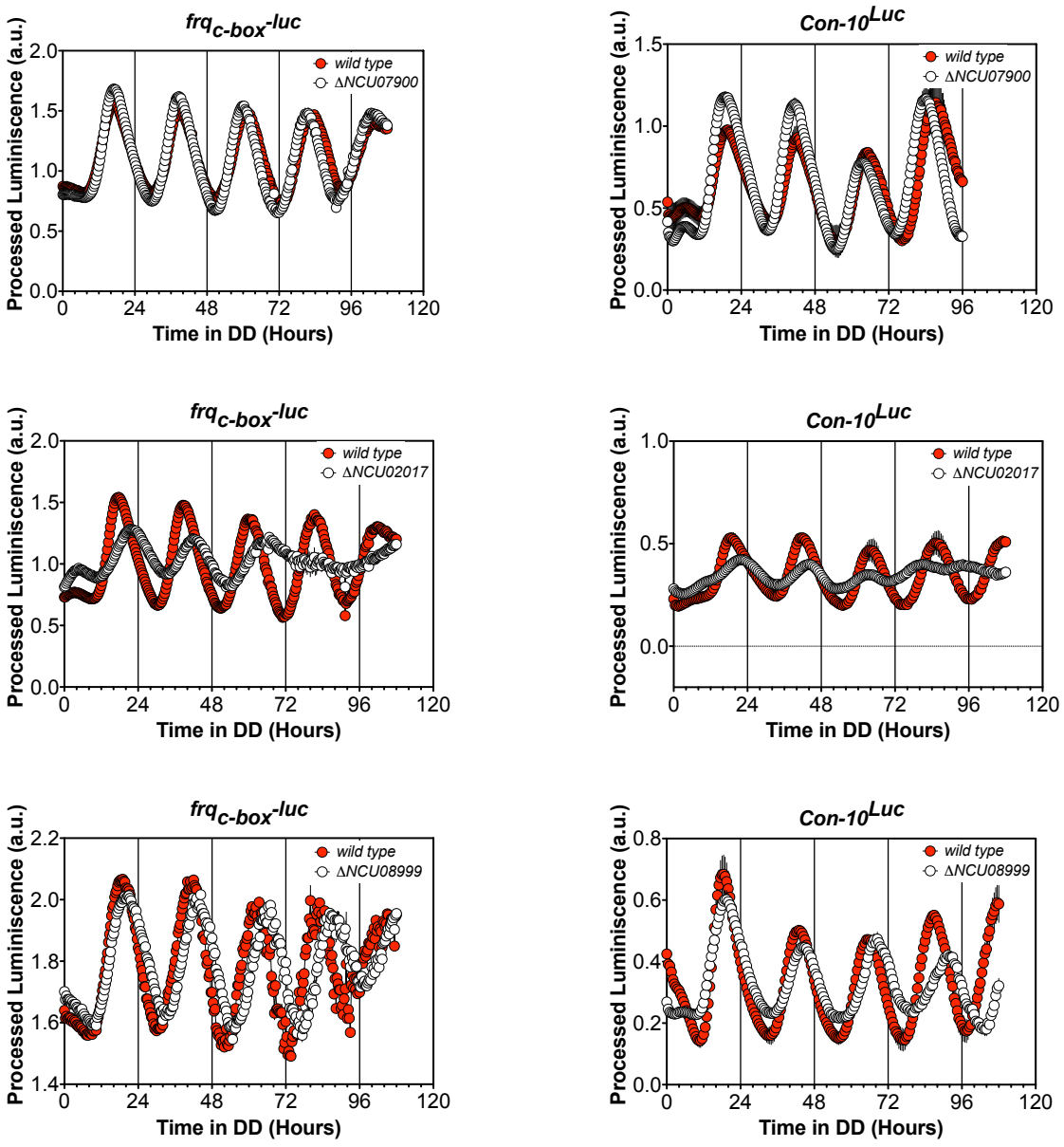

Figure 1.12. Putative Neurospora TFs whose absence leads to a change in circadian period.

The left column depicts luciferase expression produced by *frq*$_{c\text{-}box}$*-luc* reporters in selected TF KOs and their WT sibling strains. The right column shows the expression of a *con-10*^{Luc} reporter. For both reporters it is possible to confirm changes in period for the indicated TF KOs.

In the case of **phase shift**, the four strains with a delayed first peak failed to show the same defect in the secondary circadian screen (Figure 1.13), and therefore we decided to dismiss both strains from further circadian analyses. In particular, in the case of Δ*NCU08999*, we noticed that the phase shift was not observed when comparing it with that of its sibling (see below, Figure 1.15).

As for **fold of expression**, it was interesting to confirm that most of the strains analyzed did not exhibit the same degree of signal, as seen on the expression of the minimal reporter, compared to what was observed with the translational reporters. Based on this, *NCU04663* and *NCU03320* appear to be the only TFs affecting overall levels of clock expression amplitude in Neurospora (Figure 1.14).

Finally, we summarize the results of our Secondary Circadian Screen from the first 22 possible TFs identified in our Primary Circadian Screen in the Table 1.2. We decided to concentrate, based on the criteria outlined from the analyses of both screens, in 8 TFs as novel regulators impacting properties of the Neurospora circadian clock.

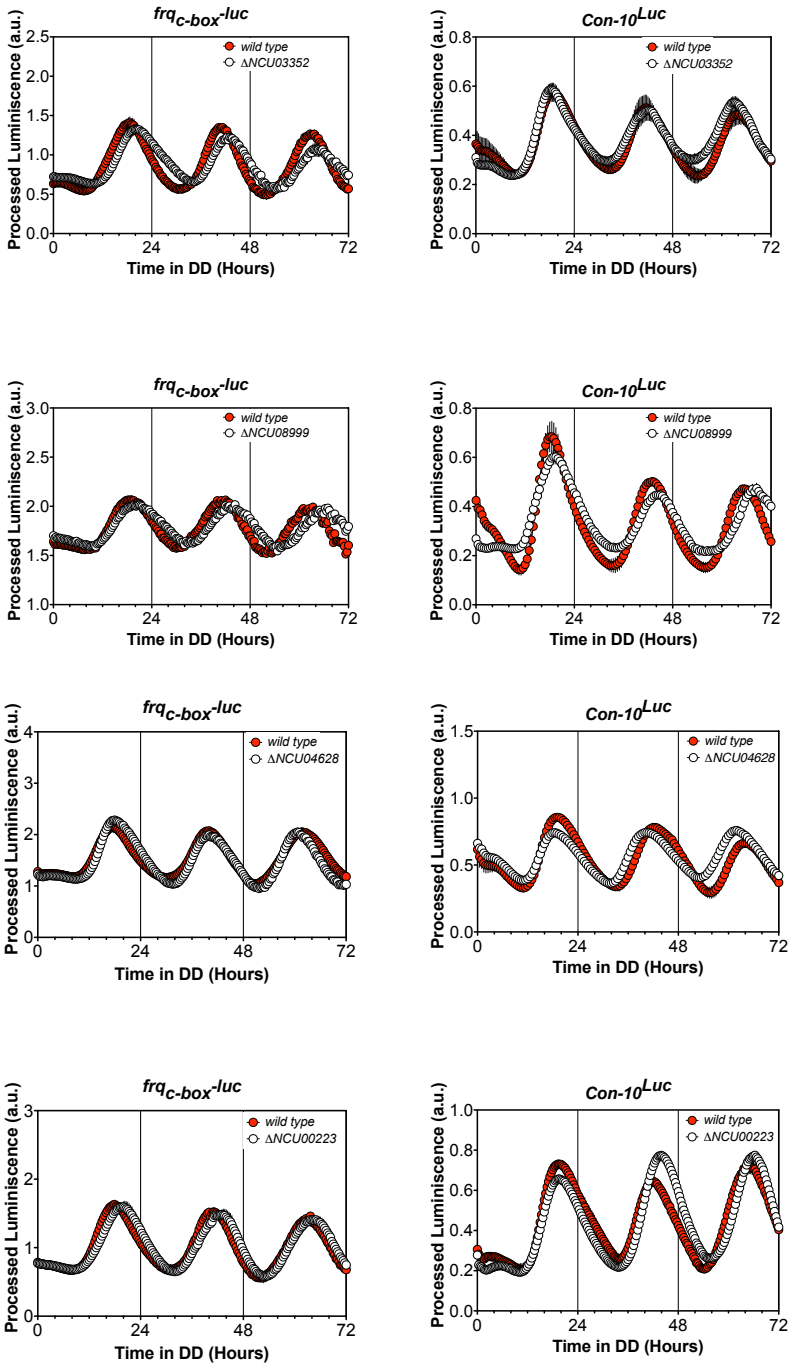

Figure 1.13. Secondary Screen of selected TF KOs exhibiting Phase Shift defects. The four strains selected in the primarily screen did not show the same defects using the circadian output reporter *con-10*^{luc}, and two of the TFs of interest were discarded.

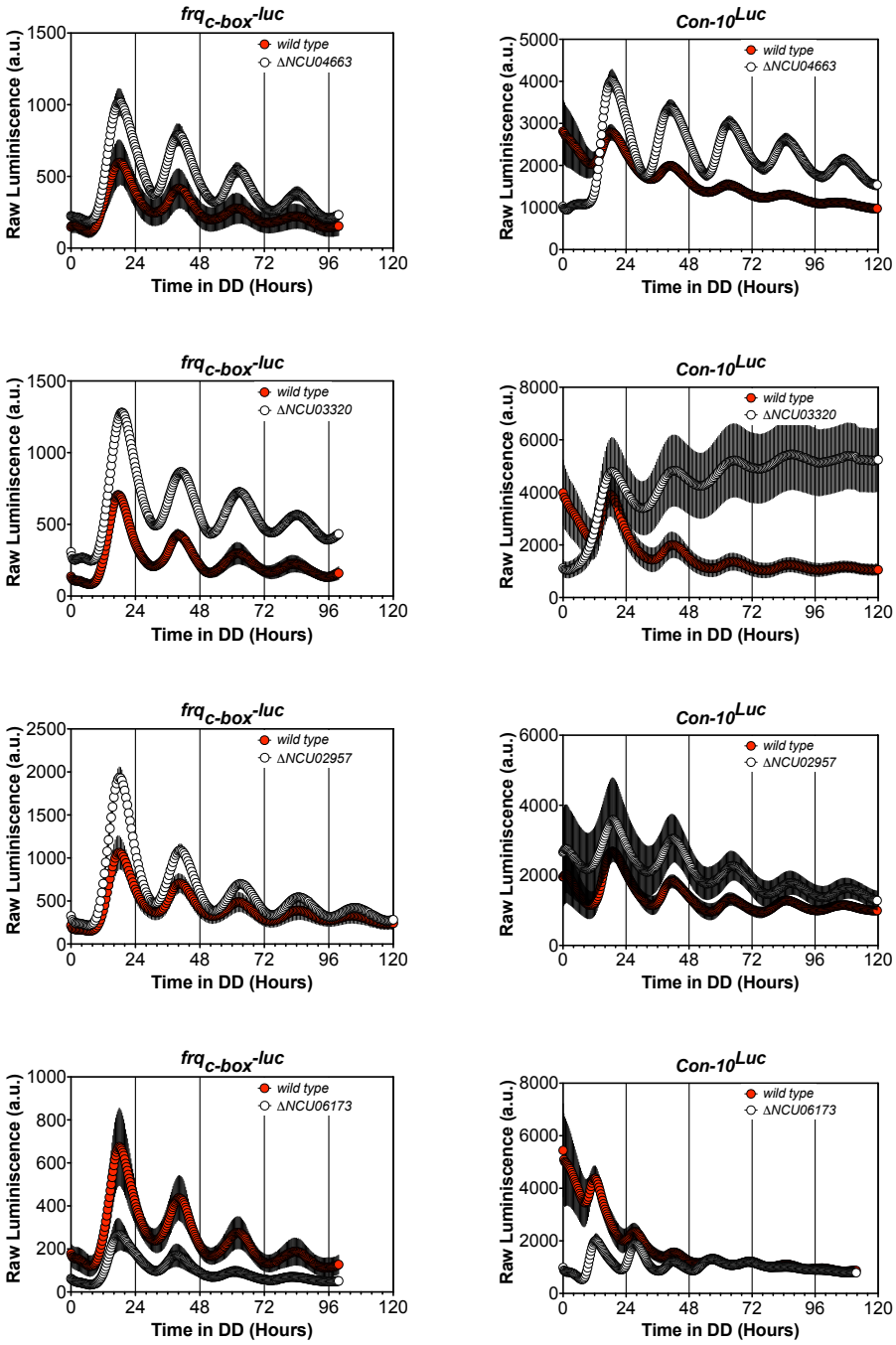

Figure 1.14. Secondary Screening for Fold of Expression parameter for KO strains. Just two from the 6 strains primarily selected show similar defects using the circadian output reporter *con-10*^{luc}. (first two rows). The other two KO strains with *con-10*^{luc} do not show the same phenotypes in fold of expression (last two rows).

Table 1.2. Summary of the Secondary Circadian Screen.

Period Change (hours)

Locus	*frq$_{c\text{-}box}$-luc*	*con-10^{Luc}*
NCU01238	-0.78 ± 0.05	-1.02 ± 0.06
NCU01243	0.65 ± 0.07	N.A.
NCU01312	-0.66 ± 0.15	0.14 ± 0.04
NCU02012	-0.83 ± 0.08	-0.26 ± 0.13
NCU02017	-1.76 ± 0.68	N.A.
NCU02307	-1.07 ± 0.18	N.A.
NCU06145	-1.51 ± 0.07	-1.52 ± 0.14
NCU06744	-0.51 ± 0.25	N.A.
NCU07900	-0.41 ± 0.08	-1.08 ± 0.09
NCU08634	-0.54 ± 0.21	-0.46 ± 0.03
NCU08744	0.02 ± 0.12	-0.60 ± 0.04
NCU08806	-0.06 ± 0.13	0.15 ± 0.13
NCU08999	0.97 ± 0.13	1.80 ± 0.24
NCU10006	-0.75 ± 0.08	-1.18 ± 0.17

Fold of Expression (A.U.)

Locus	*frq$_{c\text{-}box}$-luc*	*con-10^{Luc}*
NCU02576	2.14 ± 0.14	N.A.
NCU02896	1.89 ± 0.33	N.A.
NCU02957	1.92 ± 0.12	0.98 ± 0.12
NCU03320	1.83 ± 0.24	1.51 ± 0.29
NCU04663	2.07 ± 0.20	3.87 ± 0.22
NCU06173	0.36 ± 0.10	0.97 ± 0.11

Phase Shift (hours)

	frq$_{c\text{-}box}$-luc	*con-10^{Luc}*
NCU00223	2.46 ± 0.33	-0.07 ± 0.16
NCU03352	3.19 ± 0.38	-0.55 ± 0.44
NCU04628	3.22 ± 0.28	-0.99 ± 0.26
NCU08999	0.46 ± 0.16	-1.02 ± 0.21

The 22 KO strains selected from the primary screen were tested using *frq$_{c\text{-}box}$-luc* and *con-10^{Luc}* for Period Change, Fold of expression and phase shift. (N.A. are not available crosses)

Thus, the analyses revealed that different putative TFs appear to impact the Neurospora clock. Finally, we identify 8 TFs which, when deleted, cause strong and reproducible circadian defects, which fall in broad range of function or processes, as it is indicated below:

- *NCU01238*: PHD transcription factor capable of interacting with the co-repressor RCO-1, the latter being a transcriptional regulator known to impact clock regulation (OLIVARES-YANEZ *et al.* 2016),
- *NCU02017*: all development altered-2, *ada-2* transcription factor
- *NCU03320*: all development altered-4 *(ada-4)*, a Fungal Zn-Cys transcription factor involved in all stage of development (COLOT *et al.* 2006).
- *NCU04663*: a hypothetical protein with a Zn-Cys domain, which is a DBD only present in fungal transcription factors.
- *NCU06145*: "really interesting new gene-6" *(ring-6)*, a C2H2 transcription factor for which Knockout strains, period was the shortest period found in this screening.
- *NCU07900*: “slow growth rate-26” (*sgr-26*), a conserved hypothetical protein (CARRILLO *et al.* 2017).
- *NCU08999*: kinetochore protein-16 *(kpr-16)*, this locus encodes a bHLH transcription factor.
- *NCU10006*: “slow growth rate-30” (*sgr-26*), a Zinc finger C2H2 transcription factor (CARRILLO *et al.* 2017).

The kpr-16 gene affects the clock responses to temperature steps.

One of the putative TFs identified by this reverse genetic screen was NCU08999, since in its absence the circadian period was lengthened in ~1.3 h, while not changing other parameters. Indeed, although visual inspection of the data may suggest that there is a slight phase defect, when correcting them by circadian time calculation, it is possible to confirm that phase its not affected. As mentioned earlier, NCU08999 encodes a Kpr-16 (kinetochore protein-*16*), a hypothetical protein with a bHLH DNA binding domain (GALAGAN *et al.* 2003). This putative gene shares sequence identity with *S. cerevisiae* CBF1/YJR060W, Centromere Binding Factor 1 (BRAM and KORNBERG 1987; CAI and DAVIS 1990). In yeast, the Knockout for this gene exhibits several traits, among them an increased innate thermotolerance (JAROLIM *et al.* 2013), the natural ability of a cell to survive after the exposure to an acute heat shock without pretreatment. This evidence hinted a plausible connection between this TF of interest and additional aspects to look at, such as the input pathway of Temperature to the circadian clock.

To test the relevance of this putative TF in the processing of temperature input signals to the clock (Figure 1.15), we examined the response of this KO to temperature perturbations. Two specific features were measured: temperature resetting (TR), the ability of the clock to respond to temperature changes; and temperature compensation (TC), the ability of the clock to maintain its period in a physiological range of temperatures. To do this, we grew the strains under constant temperature and light conditions (25˚C, and LL), to reset the clock during 24 hours, and then we transferred the

strains to free running conditions of constant darkness and various constant temperature (see methods). The **period change** and **phase shift** parameters were then calculated as described in methods.

We observed that temperature compensation (TC) was not affected in the absence of *kpr-16*, as similar profiles were observed in WT and the KO strains. Likewise, different temperature steps did not reveal obvious differences between both genotypes, except for a temperature steps from LL25C to DD35C (Figure 1.15, bottom left and right respectively), which revealed unexpected differences between WT and the Δ*kpr-16*. A normal clock, as previously described (LIU *et al.* 1998), exhibits an advanced phase shift when exposed to the temperature step, which is explained by the low to high temperature step. On the contrary, the *kpr-16* KO mutant appeared to not be capable of perceiving the temperature change, since it kept the same phase as at the other temperatures. These results provide a possible connection between Kpr-16 and circadian input pathways feeding to the *Neurospora* circadian clock.

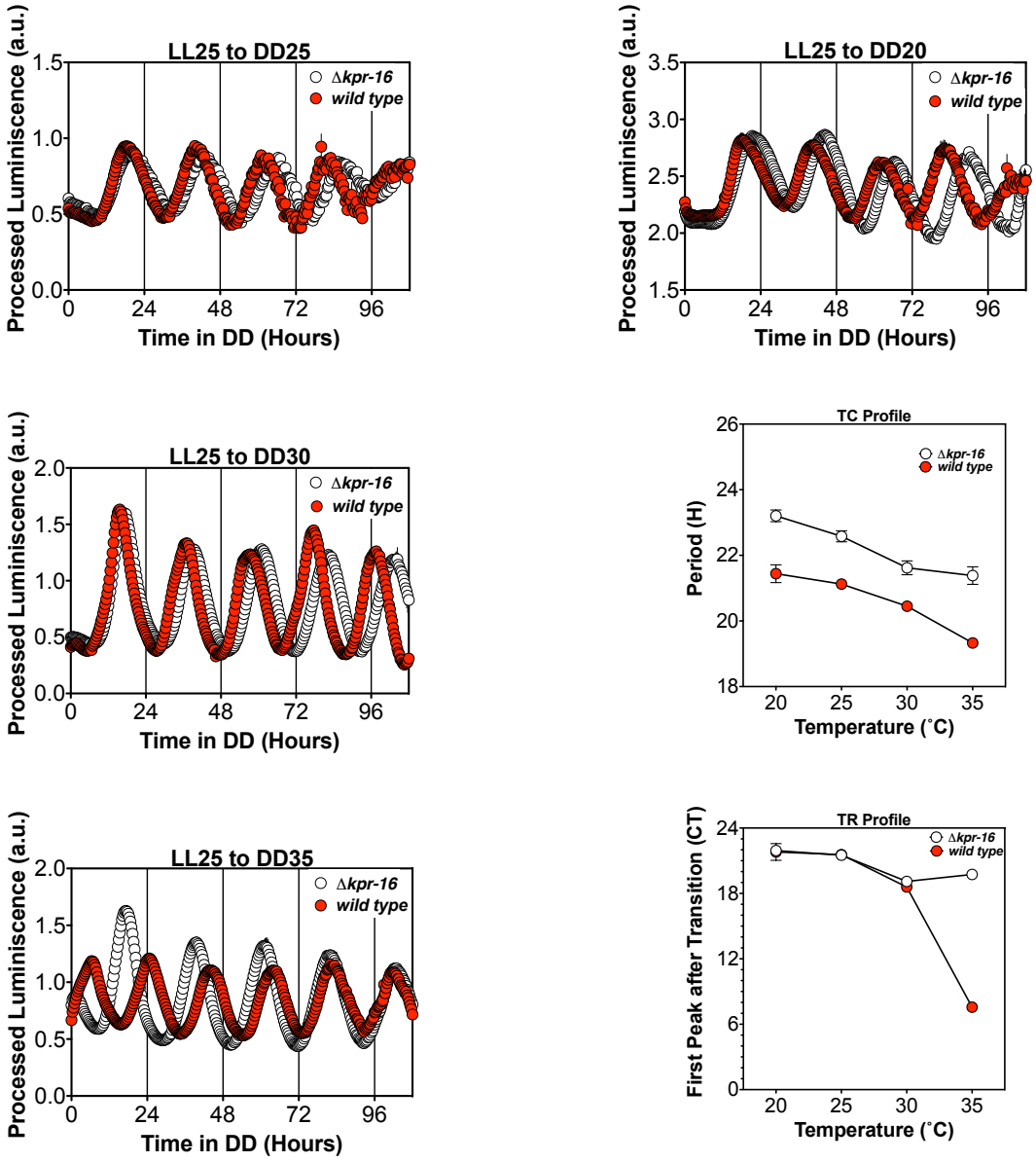

Figure 1.15. Circadian temperature compensation and response to temperature steps in the absence of *kpr-16*.

The effect of different temperature shifts was analyzed in terms of the circadian profiles of the *N. crassa* clock, utilizing a *frq*$_{c\text{-}box}$*-luc* reporter. Cultures were grown at LL25 and then monitored in constant darkness (DD) at the following temperatures: 20˚C, 25˚C, 30˚C, and 35˚C, indicated as DD20, DD25, DD30, and DD35, respectively. The Temperature Compensation (TC) profile and response to temperature steps (temperature resetting, TR) profiles at the indicated temperatures were calculated using the BioDare platform. as detailed in Methods. Error bars correspond to the s.e. for at least four biological replicates.

DISCUSSION

To identify novel transcriptional factors regulating the circadian clock in *N. crassa*, we carried out a comprehensive screening for circadian alterations in a large list of strains lacking specific TFs, utilizing robust luciferase circadian reporters. Thus, we studied 169 KOs (excluding the KOs for the WCC), identifying at least 20 putative TFs whose absence affected, in different degrees, the Neurospora clock in terms of period, phase or amplitude. Remarkably, one of them (NCU08999), encoding for a Kpr-16 ortholog, displayed and unexpected alteration in response to defined temperature steps.

Diverse biological and technical issues limited the comprehensiveness of this screening. We started our study with a list of 272 genes encoding for putative TFs in the Neurospora genome (MONTENEGRO-MONTERO 2014; WEIRAUCH *et al.* 2014; CARRILLO *et al.* 2017). Nevertheless, KOs for only for 227 of these genes were available in the *N. crassa* KO collection, as some of these genes are essential and therefore obtaining such mutants as homokaryons is not possible (COLOT *et al.* 2006; GIAEVER and NISLOW 2014). For the available 227 KOs, sexual crosses were conducted with a clock-luciferase reporter-containing strain in order to analyze the effect of deleting specific TFs on circadian parameters. Of 227 TFs selected, 71 crosses were unsuccessful in producing hyg^R, luc^+ offspring, which in some cases can be explained by genetic linkage problems: 38 KO locus are in the chromosome 1. Therefore, for these crosses that failed (based on a

luciferase reporter inserted on *his-3* locus) (HONDA and SELKER 2009), we conducted new crosses, but this time utilizing the same type of reporter but inserted at the *csr-1* locus, (BARDIYA and SHIU 2007), which allowed obtaining progeny for 15 additional TF Knockouts, leaving 56 unsuccessful crosses of which, 23 can be explained by genetic linkage. Thus, this screen finally examined a total of 171 TFs *Knockouts*, corresponding to ~75% of the putative Neurospora transcriptional regulators.

Out of these TF KO strains (not considering the ones for *wc-1* and *wc-2*), only 20 showed significant differences in the primary screen, based on our established criteria of three standard deviation from the wild type population mean (ZHANG *et al.* 2009). These 20 candidates were subjected to a secondary screen, comparing also data between such KOs and the WT siblings of each cross, as well as using additional clock reporters (circadian output reporters, see above). These secondary analyses reduced the list of candidates to a final number of 8 TFs of interest, which would correspond to ~3% of the putative TFs in *Neurospora* having an impact in its circadian clock. Importantly, other clock screens based on similar reverse genetics approaches have shown similar results. For example, in a broad gene screen using human cells the success rate was near to 1% of a total of 22,468 genes (MAIER *et al.* 2009; ZHANG *et al.* 2009), but depending on the size of the screen and the kind of genes searched the success rates could go up; in Drosophila, for instance, the rate rose to ~3.8% in a screening from 133 circadian expressed genes (MATSUMOTO *et al.* 2007), and even ~22% in a phosphatase centered screening (86 total genes) (AGRAWAL and HARDIN 2016). To our knowledge, there are no published studies

focusing exclusively on the circadian impact of TFs, although TFs with a clock related function have been already identified in unbiased screens (MATSUMOTO *et al.* 2007).

Our study constitutes one of the most extensive ones concentrating on the Neurospora clock utilizing luciferase as a proxy for circadian molecular regulation. Such approach is a major advance compared to previous Neurospora circadian forward genetic screens based on race tubes (FELDMAN and HOYLE 1973; FELDMAN and ATKINSON 1978). Indeed, the latter can lead to false positives due to mutations of interest, as any alteration that impacts conidiation *per se* would show overt arrhythmicity, although core circadian function may remain intact. Indeed, KOs of TFs associated with asexual growth (COLOT *et al.* 2006), could yield confusing or inconclusive results on race tube assays, as conidiation banding would be obscured (BAKER *et al.* 2012).

Unexpectedly, the use of diverse circadian reporters allowed us to appreciate differences in the degree of circadian alteration of several TFs KOs strains, depending on the luciferase reporter used. These differences could come from a direct effect of the candidate TF in both pathways: the expression of the output reporter (*medA* and *con-10*), along with the effect on the Neurospora clock, versus a major effect only in the latter and compensatory mechanisms in the former. A clear example of a KO impacting both core clock function and the output pathways in *Δada*-2 (*NCU02017)*, where the expression of *con-10*^{Luc} appears severely affected.

The low number of TFs of interest identified in this screen could be explained by two possibilities: the *Transcription Factor* explanation or the *Circadian System* explanation. The *Transcription Factor* explanation is the most parsimonious answer and proposes that the circadian oscillator is not regulated by a significant number of transcription factors, unlike what occurs in other regulatory systems. On the other hand, we have the *Circadian System* explanation, which argues for the pervasive robustness of circadian systems, taking the clock phenotype as a robust structure that is resilient to individual genetic perturbations. A corollary of the latter premise is that some genetic perturbations may require particular environmental stimuli to reveal an important role in the circadian system (KITANO 2002; FELIX and BARKOULAS 2015). Thus, a given regulator may not be identified as important in metabolic compensation or temperature compensation unless defined sugar levels or temperatures are tested (SANCAR *et al.* 2012; OLIVARES-YANEZ *et al.* 2016). Indeed, regarding this idea there are additional pieces of evidence to discuss. CSP-1, a TF implicated in the metabolic compensation of the clock (SANCAR *et al.* 2011; SANCAR *et al.* 2012), has been shown to modulate circadian period only under high glucose conditions, since under low glucose levels, no circadian alterations are appreciated (as is also seen in this thesis for *Δcsp-1*). On the other hand, the TF IEC-1, was recently reported as a key element required for the Neurospora clockworks (GAI *et al.* 2017). Nevertheless, although such report indicates that there is an arrhythmic phenotype when this gene is absent, our screen did not reveal circadian phenotypes for this KO (Figure 1.16). Such difference could be due to the fact that arrhythmic IEC-1 plays an important role only under defined culture conditions, and it may be that our conditions were different.. They used similar experimental conditions to

ours, except that media (FGS media) utilized for luciferase monitoring consisted of fructose (0.05%), glucose (0.05%) and sorbose (2%)(GAI *et al.* 2017), while our media used 0.03% of glucose and no other carbon source; we replicate our experiments in their same conditions, but the differences observed in their work were still not reproduced. It will be necessary to repeat this experiment using their strain under their and our conditions, to compare and evaluate this differential behavior.

Thus, such data in addition to the singular defect appreciated in the Δ*kpr-16* to a given temperature shifts, support a *Circadian System* explanation to conceptualize the role of different TFs in the Neurospora clock. Further experiments based on external variations using this now available "Circadian+TFs" KO repository are necessary to define novel signals to the clock; also, double TFs *Knockouts*, could help assessing the genetic robustness of the Neurospora clock and further explore the *Circadian System* explanation.

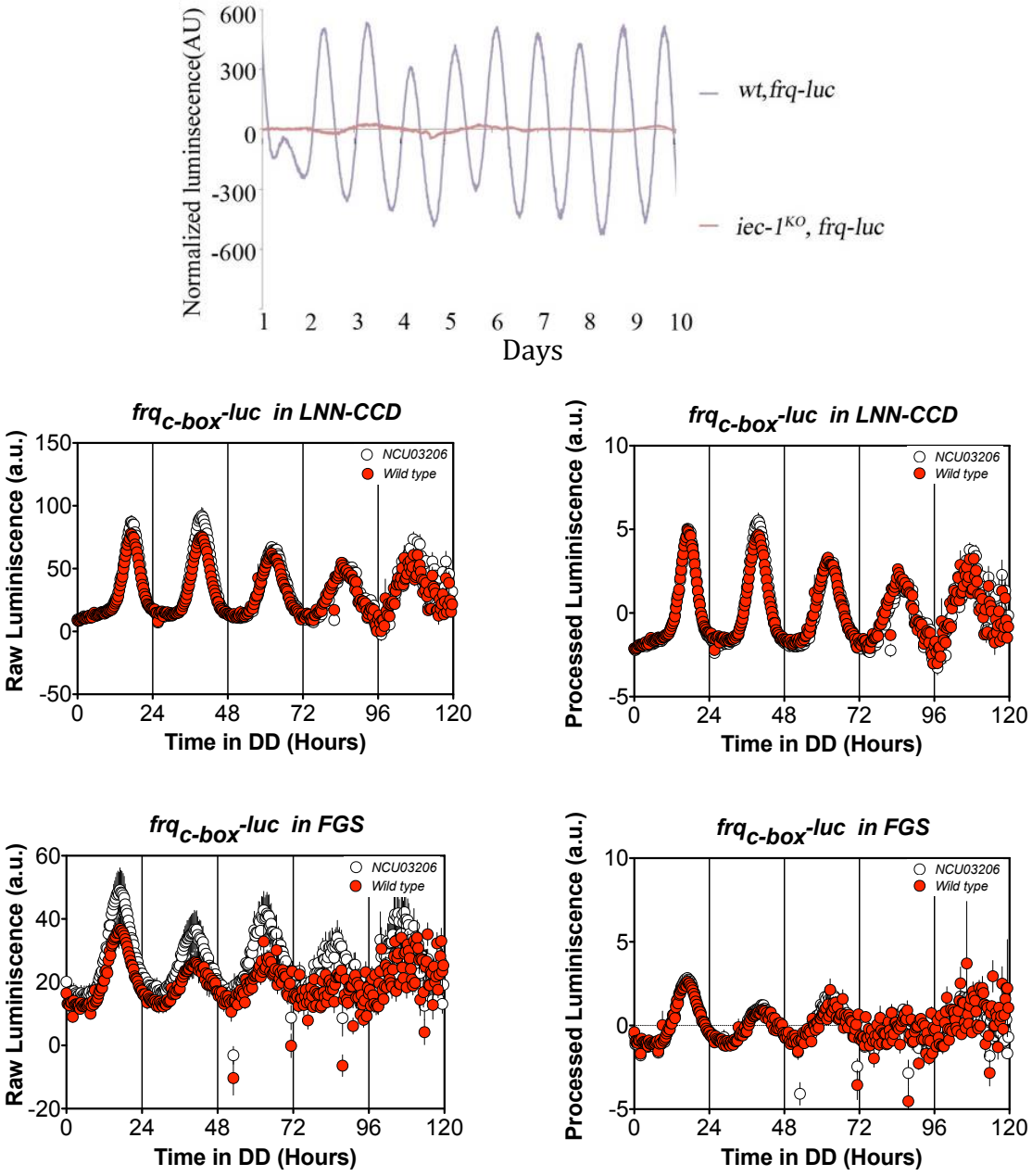

Figure 1.16. Comparison of inconsistent *Δice-1* datasets.
The upper panel was extracted and modified from (GAI *et al.* 2017), depicting luciferase traces coming from a full length frequency promoter reporter (*frq-luc*). Therein, it is possible to appreciate (red line) that *Δiec-1* appears arrhythmic. Such conclusion contradicts our own data (middle panel), obtained during the circadian screen, that indicated that *Δiec-1* displays no difference, whatsoever, with WT. Finally we replicate the experiment Gai et al. 2017 with our strain using FGS media, the data present a high signal noise in the last days, but does not shown differences between *Δiec-1* and a WT strain.

In order to define potential environmental perturbations that could be modulating the clock, causing large alterations in the absence of TF of interest, the final list of 8 TFs could be subdivided, but there is little information regarding these 8 putative TFs. In addition, they don't appear to be linked to any well-studied biological pathways, opening up the possibility of finding novel signals or inputs that could feed into the Neurospora clock. Among them, we found TFs affecting the development and growth of Neurospora (COLOT *et al.* 2006) probably altering numerous transcription pathways. The two TF KOs showing stronger circadian phenotypes have not been previously studied in *Neurospora*; *ring-6*, when KO exhibits the shortest period, encodes an unknown transcription factor not associated to any defined pathway, while *kpr-16*, which encodes a kinetochore protein-16 ortholog, is the only KO that led to a significant lengthening of period. Interestingly, this KO is not associated to any well-studied pathway in Neurospora. We tried to cross both long and short mutant, to observe the resulting phenotype, but the cross was not viable (data not shown).

With the information obtained from the *S. cerevisiae* database it was possible to see potential links with thermotolerance and viability to high temperatures (JAROLIM *et al.* 2013). Thus, we found that thermal resistance could be a phenotype of interest in *Δkpr-16*, which led us to challenge the strain under different temperature paradigms; detecting a specific phase responsive signal of the clock in this genetic background. This finding allows us to propose that Kpr-16 in signal transduction of temperature to the circadian input pathways, an area still unexplored (LIU *et al.* 1998; DUNLAP and LOROS 2017). The period defect in a KO strain for Kpr-16 was recently confirmed for other

group (CAO *et al.* 2018), but they not shown the effect associated to temperature change. In that work, they shown how Kpr-16 promotes the recruitment of the WCC in the *frq* promoter interacting with the complex. As a proposal model, the recruitment of the WCC for Kpr-16 is possibly being regulated by temperature signals.

The results obtained for Kpr-16 establish a proof of principle on how using the "Circadian+TFs" KO repository to unveil novel signals capable of affecting the Neurospora clock. The next step involves dissecting the mechanism behind this process and deconstructing the circadian transcriptional network in this fungus, which in return will allow extrapolating such lessons to other circadian systems. Further validations of the phenotypes observed for each TF KO are necessary to confirm their effects on the Neurospora circadian system, together with defining the signals associated with them. Thus, this could provide additional tools to further establish a complete framework for the transcriptional regulation of the *Neurospora crassa* circadian clock.

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Chapter 2

Identification of *ck1a*^{ctm6}, a novel mutation affecting clock period in *Neurospora crassa*

Keywords: SNPs, Sequencing, Casein Kinase, Circadian Clocks

ABSTRACT

One of the main strategies that have helped characterize the molecular basis of circadian rhythms has been the identification of mutants exhibiting a clock phenotype of interest, followed by the mapping and identification of the underlying gene(s). Thus, this *forward genetics* approach for the characterization of naturally occurring or lab-generated mutants with clock phenotypes, coupled with the flourishing of deep-sequencing technologies, has led to the identification of novel and unmapped clock mutations in various model systems. In this chapter, we report the serendipitous discovery of a new Neurospora genetic variant that strongly affects circadian period, a mutation in the *casein kinase 1a* locus (NCU00685). This mutant, a missense single point mutation resulting in the change of a histidine to an aspartic acid at position 165, encodes for a novel *ck1a* allele and leads to a period defect of over 6 h. Notably, other key circadian parameters appear to be unaltered in the mutant. Phosphorylation of clock elements is a key regulatory mechanism controlling clock function and alterations in phosphorylation dynamics can drastically impact period length. While core clock components differ across phyla, their posttranslational modifications still appear to be largely controlled by the same conserved kinases and phosphatases, where CK1a plays a pivotal role in all eukaryotic models studied to date. Our results provide new details for the understanding of posttranslational regulatory mechanisms influencing circadian period determination.

INTRODUCTION

The molecular bases of circadian rhythms have been widely studied in several model organisms during the past 30 years, which has allowed obtaining a rather clear description of the molecular components underlying biological clocks (DUNLAP 1999; YOUNG and KAY 2001; BRODY 2011). One of the main strategies that has helped identifying novel genes encoding for circadian clock components has been the selection of random mutants, followed by the mapping and identification of the genes of interest. Thus, forward genetics of naturally occurring, or lab-generated mutants with clock phenotypes, have paved the road towards the molecular identification of clock genes (TAKAHASHI 2004; SIEPKA and TAKAHASHI 2005; NOLAN and PARSONS 2009). Nevertheless, despite all these major advances, a complete understanding of each gene product in the context of rhythm generation and maintenance still remains incomplete (ODE and UEDA 2017)

Neurospora crassa is a fungus that exhibits overt circadian phenotypes, a defining characteristic that has allowed its development as a successful circadian model and as a platform for genetic screens (DUNLAP 1999; DUNLAP 2008; BAKER *et al.* 2012; HURLEY *et al.* 2016). In past years, with the flourishing of deep-sequencing technologies, the identification of yet unmapped clock mutations has become an easier task, which has led to new circadian discoveries (SMITH *et al.* 2008; EMERSON *et al.* 2015)

Phosphorylation of clock elements is now widely accepted as a general key mechanism controlling clock function and period accuracy (MEHRA *et al.* 2009; MONTENEGRO-MONTERO *et al.* 2015). Remarkably, despite the fact that clock components differ across phyla (i.e FRQ and PER), their posttranslational modifications (PTM) still appear to be largely controlled by the same kinases and phosphatases (HEINTZEN and LIU 2007; MEHRA *et al.* 2009; REISCHL and KRAMER 2011). Although sequence homology is not conserved among clock negative elements (PER, FRQ etc.), *in silico* analyses of Neurospora FRQ and both insect and mammalian PERs, predicts that they all contain intrinsically disordered regions, a structural characteristic that, in combination to extensive phosphorylation could help understand general mechanisms underlying circadian rhythmicity even in the absence of sequence conservation (HURLEY *et al.* 2013; HEVIA *et al.* 2016).

Different kinases, such as casein kinase 1 and 2, PKA, or PRD-4 (checkpoint kinase-2), have been shown to phosphorylate FRQ, impacting Neurospora circadian function (MEHRA *et al.* 2009; GUO and LIU 2010; BAKER *et al.* 2011; DIERNFELLNER and SCHAFMEIER 2011). Likewise, several phosphatases, such as protein phosphatase-1 (PP1) and -2 (PP2a), or PP4 (BAKER *et al.* 2011), have also been shown to modulate circadian function.

In Neurospora two casein kinases 1 orthologs have been identified: CK1a and CK1b. FRQ has been shown to strongly interacts with CK1a through two specific domains (FCD1 and FCD2) (QUERFURTH *et al.* 2011), while CK1a can phosphorylate dozens of FRQ sites, distributed widely through different FRQ domains, according to *in vitro* (TANG *et al.* 2009), and *in vivo* studies (BAKER *et al.* 2011). In addition, several lines of evidence indicate that CK1a physically interacts with FRQ, modulating its phosphorylation state, impacting period and FRQ turnover (HE *et al.* 2006b), (QUERFURTH *et al.* 2007).

In this chapter, we report the unexpected and serendipitous characterization of a new Neurospora mutation affecting the *ck1a* (NCU00685) locus, which strongly modifies circadian period. This mutant, consisting of a single point mutation, encodes a new *ck1a* allele, as mentioned earlier, a well-known kinase with a pivotal role in all eukaryotic models studied to date (REISCHL and KRAMER 2011; ODE and UEDA 2017). Our results provide new details into the understanding of posttranslational mechanisms that regulate circadian period-determination.

MATERIALS AND METHODS

Strains and Crosses

The *ctm-6* mutation was unexpectedly found in strain FGSC11366 (FGSC, Kansas City, MO), a KO strain for gene NCU06173, which was available as part of the Neurospora Functional Genomic Project (COLOT *et al.* 2006). The latter initiative aimed to replace loci of all individual Neurospora genes with a drug-resistance cassette containing a hygromycin B phosphotransferase (*hph*) gene (COLLOPY *et al.* 2010). While analyzing a sexual cross between strain FGSC11366 and *a frq$_{c\text{-}box}$-luc* (*his-3::frq$_{c\text{-}box}$-luc*, *ras-1^{bd}*) strain it became evident that the circadian defect seen in this cross was not linked to the *hph* cassette. Thus, the mutation was segregated away from the *hph* marker, and the resulting strain (depicting the circadian defect of interest) was backcrossed with new KOs, as that provided a way to use different chromosomal positions of the corresponding *hph* markers as a measurement of segregation, which allowed mapping the mutation to a certain region within a linkage group (chromosome). In addition, to better characterize the circadian defects associated with this mutation, we crossed strain FGSC11366 to different clock reporters available in our laboratory. Besides utilizing the minimal clock promoter of Neurospora fused to luciferase *frq$_{c\text{-}box}$-luc*, (GOOCH *et al.* 2008; GOOCH *et al.* 2014) we also crossed it to a FRQ-LUC translational fusion containing strain (LARRONDO *et al.* 2012), or two different circadian output reporters: CON-10^{LUC} (OLIVARES-YANEZ *et*

al. 2016) and MedA^{LUC} (*con-10^{luc}-bar*, *medA^{luc}-bar* respectively) (LAUTER and YANOFSKY 1993).

The circadian screen was based on the LUC analysis of the progeny in search for circadian defects, utilizing luciferase reporters of interest. Additionally strains containing a *his-3::frq$_{c\text{-}box}$-luc*, *ctm-6* genotype were further crossed with strains bearing *his-3::frq$_{c\text{-}box}$-luc, frq^{V5H6}-bar,* as the latter allele provided a means to follow FRQ easily due to the presence of a V5 tag (BAKER *et al.* 2009; LARRONDO *et al.* 2015).

In order to map the *ctm-6* mutation in the Neurospora genome, we crossed a strain containing the *ctm-6* mutation in a genetic background that also carried a *frq$_{c\text{-}box}$-luc,* reporter (see above) together with different *Knockout strains* for each Neurospora Linkage Group (LG). The following set of KOs (Linkage Group number indicated in brackets) were chosen (PERKINS 1959; HAMMOND *et al.* 2012): *NCU00833*(I), *NCU09973*(I), *NCU01706*(II), *NCU00100*(III), *NCU06975*(IV), *NCU03649*(V), *NCU04058*(VI). All those loci were known not to cause clock phenotypes, as identified in our previous global screening for transcription factors (Chapter 1). Recombination rate for Linkage Group VII was obtained using the offspring from the *frq^{luc}* reporter. Recombination rates calculations were made based on the percentage of *ctm-6* phenotypes out of the total number of the Hygromycin resistant (HygR) offspring analyzed produced by the cross (PERKINS 1959; JIN *et al.* 2007; HAMMOND *et al.* 2012).

In order to infer genetic interactions, we took the same *ctm-6* parental reporter strain used for the genetic mapping, and crossed it with different *frq* alleles: frq^{1}, frq^{7}, $frq^{\Delta C\text{-}term}$ and frq^{S900A} (FELDMAN and HOYLE 1973; GARDNER and FELDMAN 1980; ARONSON *et al.* 1994a; BAKER *et al.* 2009). Each of these alleles confers a period defect, which can be easily detected by including a $frq_{c\text{-}box}$-*luc* reporter in the genetic background. The analysis of the circadian phenotypes in the resultant offspring was made through luciferase analyses recorded using a CCD-Camera.

Growth Conditions

Culture conditions for vegetative growth and asexual development included the use of Vogel minimal medium (VM) (Vogel 1956), whereas sexual development was assessed using synthetic crossing medium (SCM) (WESTERGAARD and MITCHELL 1947), as already described (MONTENEGRO-MONTERO 2014; OLIVARES-YANEZ 2015). Sorbose-containing medium (FIGS) was utilized for the isolation of germinating ascospores (DAVIS and DE SERRES 1970). For crosses selection, we used VM supplemented with hygromycin for KO (200 mg/ml; Calbiochem, San Diego, CA) and Luciferin (at a final concentration of 25μM) for reporter activity. Race tube media contained 1X VM, 0.1% glucose, 0.17% arginine, 50 ng/mL biotin, and 1.5% agar (BELDEN *et al.* 2007).

For circadian luciferase analyses, we utilized a 96-well plate containing LNN-CCD media, supplemented with 0.03% of glucose, adding Luciferin (GoldBio) to a final concentration of 25μM. Cultures were grown 24 hours in constant light at 25˚C and then were analyzed in constant darkness at 25˚C for 4 to 5 days in Percival incubators equipped with a PIXIS 1024 CCD camera (LARRONDO *et al.* 2012; LARRONDO *et al.* 2015).

Whole Genome Sequencing

Genomic DNA for screening and whole-genome sequencing was extracted with the following protocol: cultures were grown for two days in 3 ml of Vogel's Solution (VOGEL 1964) supplemented with 2% sucrose; then mycelia was dried and frozen in liquid nitrogen. Frozen mycelia were ground utilizing a Tissuelyser and 800 μL of Nuclei Lysis Solution (Promega) were added, and then vortexed intensively for 2 min. Samples were then incubated for 1 hour at 65˚C, vortexed again for another 2 mins, 3 μL of RNAse A (4mg/mL; Promega) added, and then incubated at 37˚C for 1 additional hour to eliminate excess RNA. Samples were then transferred to Ice for 15 min, and 450 μL of Protein Precipitation Solution (Promega) were added. The mixture was vortexed for 2 min, to then centrifuge 5 min at max. speed. The supernatant was transferred to a new tube and centrifuged 5 min at max. speed. To precipitate the nucleic acids, 700 uL of cold isopropanol were added to the supernatant and immediately centrifuged for 10 min at maximum speed. The pellet was washed twice with 500 μL of cold 70% ethanol and centrifuged 5 min at max speed. The pellet was dried at 55˚C for 15 min, and 50 μL of water or TE Buffer was added, then incubated at 55˚C for 15 min. The DNA samples were

quantified using a Nanodrop 2000, and the integrity of the genomic DNA was confirmed by 1% agarose gel electrophoresis.

Good-quality DNA was purified, taking 4 µg of each sample and mixed generating two pools: a WT one, conformed by DNA from 14 individuals with WT clock behavior, and a pool of mutant progeny (as inferred by the *ctm-6* clock phenotype) conformed by DNA from 16 individuals. The pooled samples were sent to Macrogen Inc Seoul, Korea for whole-genome re-sequencing. The libraries were prepared with the TruSeq DNA PCR-free kit system and then sequenced using Paired-end Illumina HiSeq2500 technology. Each sample gave 1900 millions bases, with a total of 18 millions sequencing reads. The Neurospora genome is about 40MB, which means an average coverage of 48X, this provides more that enough sequencing depth to identify point mutations in the genome, based on previous work requiring a 20X minimal coverage (VAZQUEZ-GARCIA *et al.* 2017).

The reads of each sequenced pool were aligned separately against the reference genome of *N. crassa* using Bowtie2 in High sensibility (LANGMEAD and SALZBERG 2012). This allowed us to align 17 million of reads on the reference genome. Reads were visualized using Geneious version 8.1.8 (Biomatters, USA). Through the genetic mapping and the use of the fungiDB platform (http://fungidb.org/fungidb/) (STAJICH *et al.* 2012), we identified 20 candidate genes related to circadian function in the Linkage Group I of *N. crassa*; we then extracted the consensus sequence obtained from the reads aligned against the reference genome from both pools, the WT pool and the mutant pool. The consensus sequences coming from each pool were aligned using the Clustal Omega

software (SIEVERS *et al.* 2011). This multiple alignment was made for each of the 20 candidate genes independently, including the sequence from the reference genome of the *N. crassa* OR74-A strain (GALAGAN *et al.* 2003).

The final non-synonymous SNP found (see Results) was evaluated through a SIFT (Sporting Intolerant From Tolerant) analysis, which predict how an amino acid change affects the function of a protein, based on the homology of sequence from different species and considering the physical properties of the amino acids (NG and HENIKOFF 2001). The SIFT analysis was made using its on line platform (http://sift.bii.a-star.edu.sg/), using the UniProt-SwissProt 2009-2010 data base with an average conservation of sequence equal to 3 (KUMAR *et al.* 2009). The results of this analysis showed that the nucleotide 493 change, leading to H165D in the *ck1a* gene (see Results) had a low frequency, making it tolerable, and that the protein should retain partially its function.

RFLP Screen

To check for the presence or absence of the *ck1a*^{ctm6} mutant allele in different progeny under analysis we designed an RFLP diagnostic analysis. For that, we extracted gDNA for each strain (see above) and amplified a 241 bp region of the NCU00685 locus with the following primers: Forward, 5'-tcgagtacatccatgccaagt-3'; and Reverse, 5'-atgtcgtcacggcgagactg-3'. The amplicons were digested with the restriction enzyme *HinfI*, and finally checked by agarose gel electrophoresis of the fragments. The digestion of the

wild-type amplicons produced two fragments of 107 bp and 134 bp. Since the *ctm-6* mutation alters the restriction enzyme recognizing site, the amplicon containing the mutation is not digested.

Circadian Luciferase Screen

All of the offspring from the different crosses, as well as complemented strains were analyzed by quantifying luciferase levels, and by evaluating the circadian parameters through a system that CCD camera. Data coming from the CCD camera runs were processed with WinView/32, and the data were extracted from each 96-well plate utilizing a customized ImageJ toolset from our lab (OLIVARES-YANEZ 2015). For circadian analysis, time series datasets were uploaded to the BioDare online platform (MOORE *et al.* 2014). The processed data were detrended, removing amplitude and baseline trends for better visualization, and the period estimation was calculated through two different methods, FFT-NLLS and Spectrum Resampling, discarding the first and last 12 hours of the data (ZIELINSKI *et al.* 2014). The circadian parameters were calculated as explained previously (Chapter 1)

RESULTS

The mutation ctm-6 modifies the period of the Neurospora circadian clock.

By serendipity, in a genetic screen analyzing the *Neurospora crassa Knockout* Stock strains (Chapter 1) (COLOT *et al.* 2006; COLLOPY *et al.* 2010), we found a mutation causing an increase in circadian period, mutation that was unlinked to the hygromycin cassette present in that cross (strain FGSC11366). The latter strain was intended to be isogenic to a WT strain, except that it contains a drug resistance marker replacing the NCU06173 ORF (GALAGAN *et al.* 2003; BORKOVICH *et al.* 2004). NCU06173 encodes for the transcription factor SGR-21 (*s*low *g*rowth *r*ate 21) (CARRILLO *et al.* 2017). Thus, analysis of the progeny coming from the cross between the luciferase clock reporter and the NCU06173 KO strain revealed that only some of the luc^{+}, $HygR^{+}$ progeny (and not all of them) had a circadian period phenotype. Likewise, analysis of the LUC+, hygromycin sensitive progeny showed that not all of them expressed a WT circadian period. Therefore, the most likely interpretation of the genetic data was that, in addition to the corresponding KO cassette, strain FGSC11366 happened to contain an unexpected mutation that provided a circadian phenotype. This mutation strongly affected period, lengthening it by 6 h (5.99±0.09, s.e.), as measured with a minimal circadian transcriptional reporter (Figure 2.1). This clock defect was also confirmed with additional circadian output luciferase reporters: $CON\text{-}10^{Luc}$ and $MedA^{Luc}$; and by *race*

tube assay (Figure 2.2). From now on, we refer to this mutation as *ctm-6*, for ***c***lock ***t***iming ***m***odifier by ***6*** hours.

The growth rate of the *ctm-6* mutant strains was compared to that of its WT siblings in a race tube assay. For this, it was important to analyze strains having, in addition to the *ctm-6* mutation, the *ras-1^{bd}* allele as part of their genetic backgrounds. This is since the latter allele enhances circadian phenotypic output and helps seeing conidial "bands", allowing easy visualization of overt conidiation rhythms in the race tube assay (SARGENT and WOODWARD 1969; BELDEN *et al.* 2007). Strains containing the *ctm-6* allele exhibited only a slightly decreased in growth rate: 3.7±0.1cm/per day for *ctm-6* strain versus 4.1±0.1cm/per day for WT strain. Period, calculated from the race tubes (by measuring the positions of the conidial bands), confirmed the alteration already observed using the LUC reporters (Figure 2.2) (BAKER et al. 2012), although with a slightly shorter period (26.5 h for *ctm-6* strain, and 21.9 h for WT) compared to the analysis of the LUC traces. Such difference could be associated peculiarities of clock behavior (and the role of the allele under study), as we are comparing assays involving tissue continuously grown in fresh media (race tube) versus static cultures (96-well plates). Indeed, there have been a couple of examples where the detection (or strength) of the circadian phenotype varies depending on the growth conditions of the assay (EMERSON *et al.* 2015; OLIVARES-YANEZ *et al.* 2016).

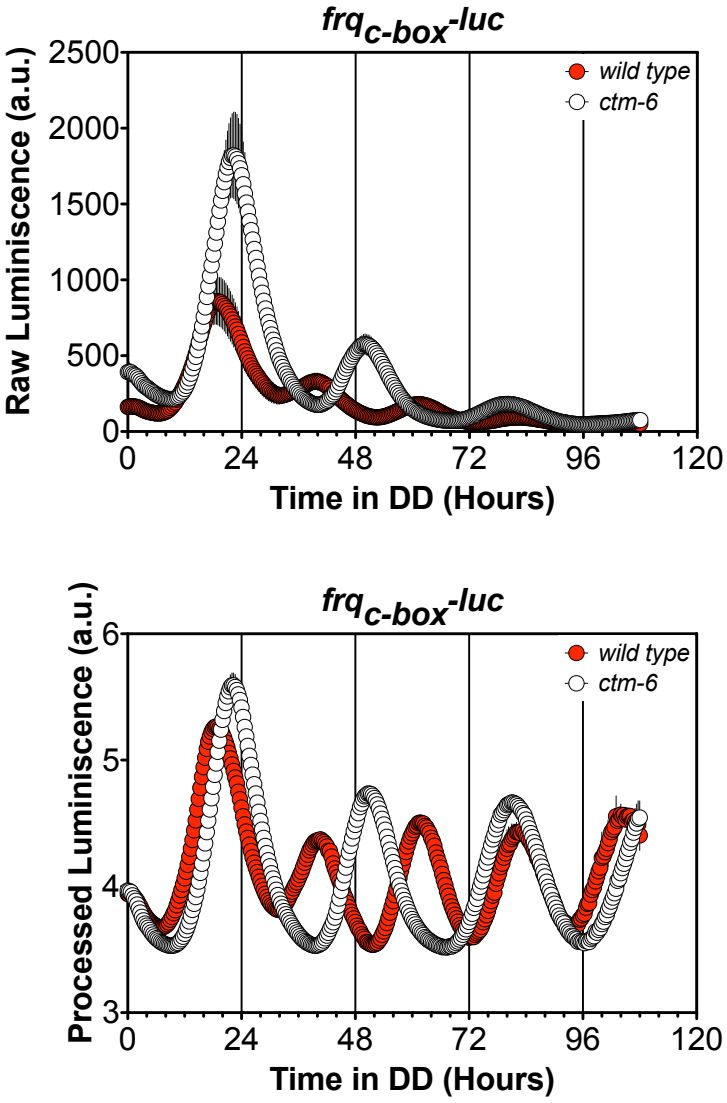

Figure 2.1. The *ctm-6* mutation affects period of the Neurospora clock.
The increase in period can be observed utilizing the clock minimal reporter *frq*$_{c\text{-}box}$*-luc*, by comparing WT and mutant strains. The upper panel depicts raw luminescence data, whereas the lower panel shows processed bioluminescence (see Methods). The data shown the greatly lengthened period of ~27 hours in the mutant strain.

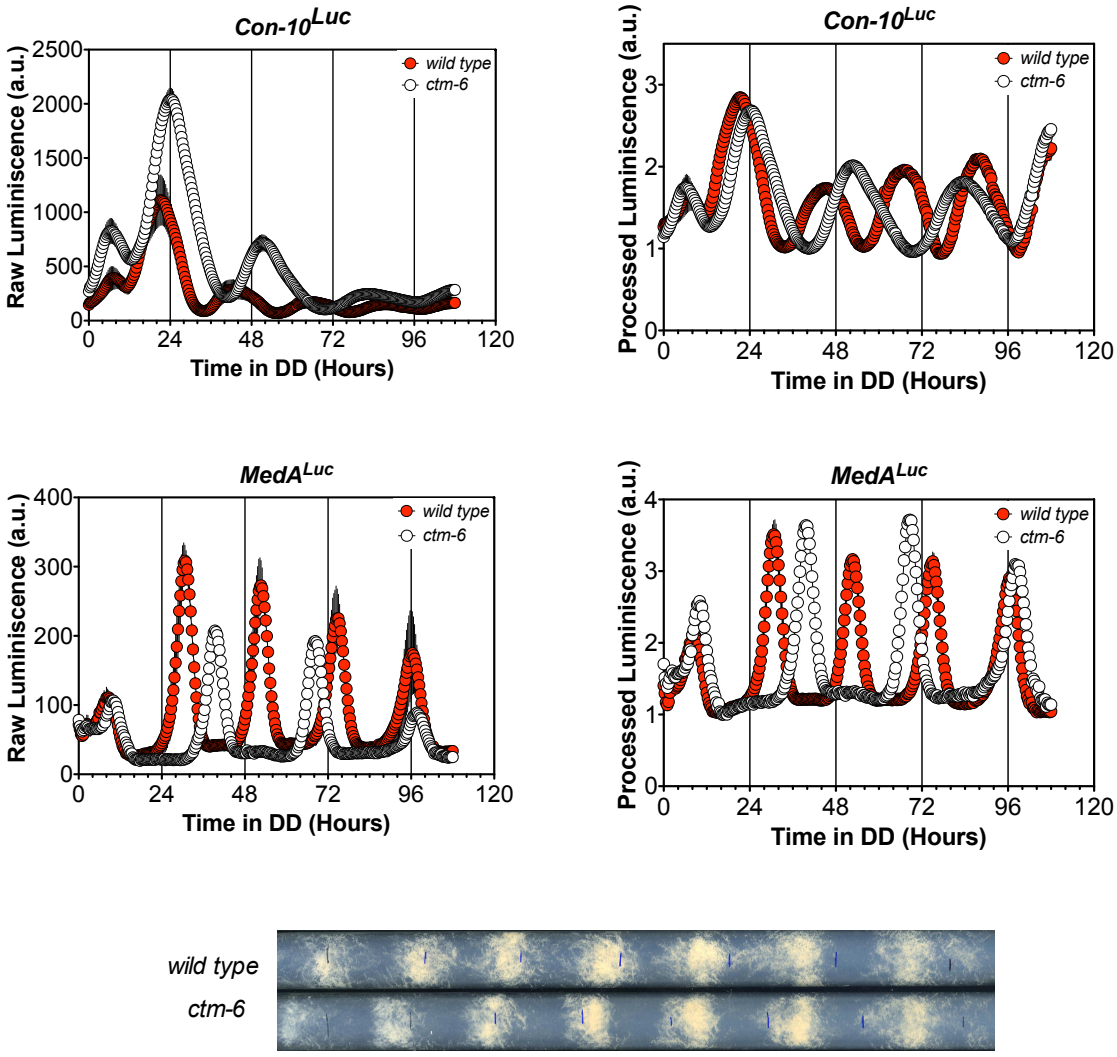

Figure 2.2. The *ctm-6* period phenotype is also visible when examining circadian output.

Period lengthening caused by the *ctm-6* mutation was also visualized utilizing two LUC translational output reporters, CON-10^{LUC} (upper panels) and MedA^{LUC} (middle panels). The left panels shows raw luminescence data, whereas the right panels correspond to processed data (see Methods). The lower panel shows to representative race tubes of a WT and *ctm-6* strain.

Notably, when the *ctm-6* mutation was analyzed in the presence of a FRQ^{LUC}, or a FRQ^{V5} allele, a synthetic effect in period was observed. Thus, in *ctm-6*, frq^{luc} strains, period was near 36 hours (36.2±0.1 h)(Figure 2.3, upper panels). This effect is synthetic, as it is not an independent property of the *ctm-6* or frq^{luc} alleles on their own. Nevertheless, it is important to highlight that, as previously reported (LARRONDO *et al.* 2012) period of frq^{luc} strains is already slightly longer (~1.5 h) compared to the one conferred by a WT *frq* allele. This slight alteration is probably a defect caused by a minor conformational alteration of the recombinant protein, because FRQ is fused to LUC at its C-terminus, which could alter some of its conformational dynamics. Remarkably, combining the *ctm-6* allele with frq^{luc} leads to an enhanced circadian defect, while a similar synthetic effect, but of a smaller magnitude, was appreciable when combining in the same strain frq^{v5} with *ctm-6* (BAKER *et al.* 2009), yielding a period close to 30 hours (29.6±0.12 h) (Figure 2.3, lower panels). Thus, the data appear to indicate that *ctm-6* sensitizes the clockworks to small alterations that may affect/modify FRQ structure.

Other circadian parameters, such as phase or amplitude, were not drastically affected in *ctm-6*. This mutation caused a subtle phase shift with a phase delay of ~1 hour (see calculations in Chapter 1)(ZIELINSKI *et al.* 2014), a defect that was also observed when analyzing this mutant with luciferase reporters. These changes can be compared between the reporters along with their associated periods in Figure 2.4. In terms of overall luciferase expression the reporters in *ctm-6* revealed a subtle increased in overall levels as observed in the raw-data, a situation that was not all that obvious when peak/valley relationship were considered after detrending (ZIELINSKI *et al.* 2014).

Finally, the R.A.E. indexes were similar between the strains with or without the *ctm-6* mutation (Figure 2.5).

The ctm-6 mutation is a non-synonymous point mutation in the casein kinase 1 gene.

In order to determine the molecular identity of the *ctm-6* mutation, we sough to pinpoint the mutated locus, taking parallel and complementary strategies based on classical genetic mapping and whole-genome sequencing. Thus, to define the location of the *ctm-6* mutation, we mapped it to a defined linkage group and chromosome arm (GALAGAN *et al.* 2003). For this, we crossed the *ctm-6* mutant with different KO strains, in order to analyze the segregation of the KO cassettes (containing *hph*, hygromycin resistance) relative to the *ctm-6* locus. The selected KO strains, which span the different chromosomes, were previously used in Chapter 1, and contain the same luciferase reporter (*his-3::frq*$_{c\text{-}box}$*-luc*) present in the *ctm-6* strain to be crossed (COLOT *et al.* 2006; COLLOPY *et al.* 2010; LARRONDO *et al.* 2012). Importantly, as identified in the previous chapter, the selected KO genes don't cause any circadian alteration. Thus, by characterizing the progeny of the different crosses, in terms of altered period and hygromycin sensitivity/resistance, we calculated the recombination rate for each cross. A recombination rate close to 50%, would indicate no-linkage between the *hph* cassette and the *ctm-6* mutation.

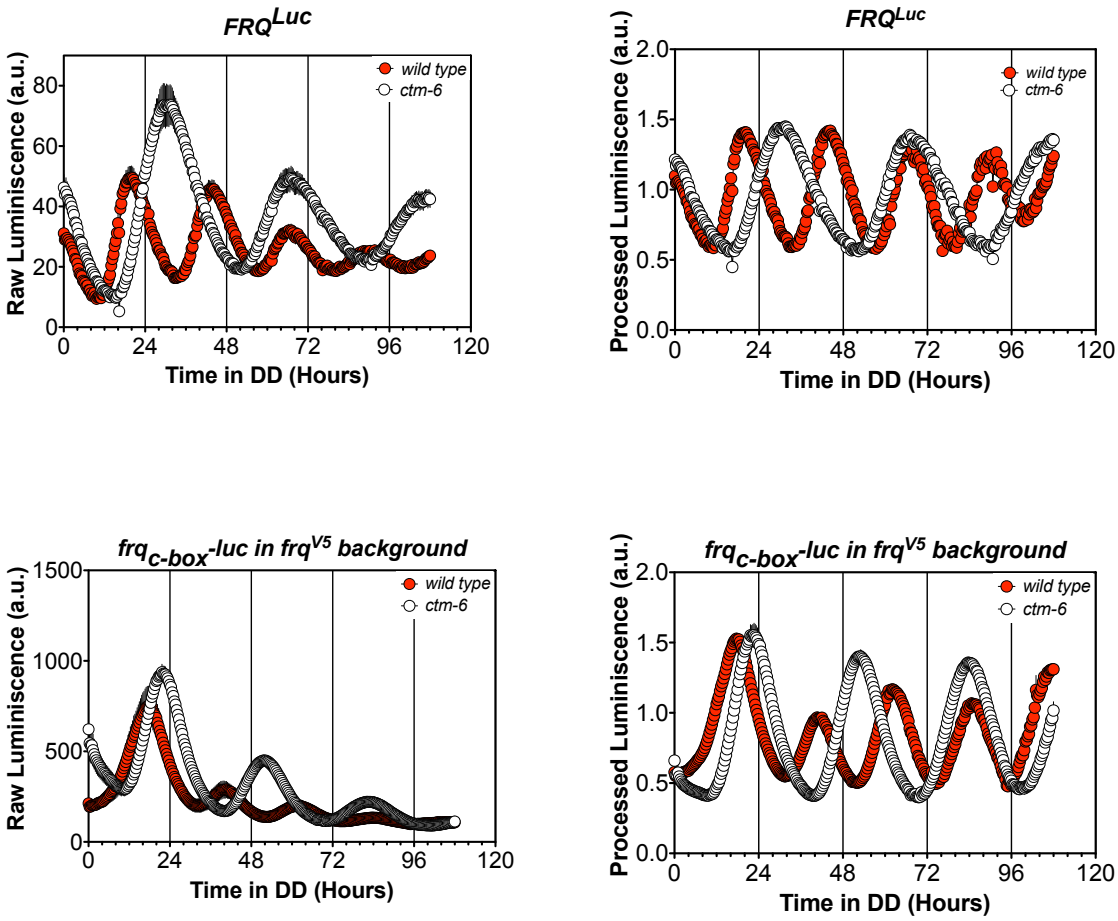

Figure 2.3. *ctm-6* causes a synthetic effect when combined to particular *frq* recombinant alleles.

A strong period increment is observed when *ctm-6* is analyzed in a background containing a FRQ-LUC translational fusion reporter: FRQ^{LUC} (upper panels). Likewise, when *ctm-6* is analyzed in a background containing a frq^{v5} allele, the $frq_{c\text{-}box}$-*luc* traces reveal changes in period that exceed those observed in a *frq* WT background. The left panels contain raw luminescence data whereas the right panels correspond to the processed luminescence data (see Methods)

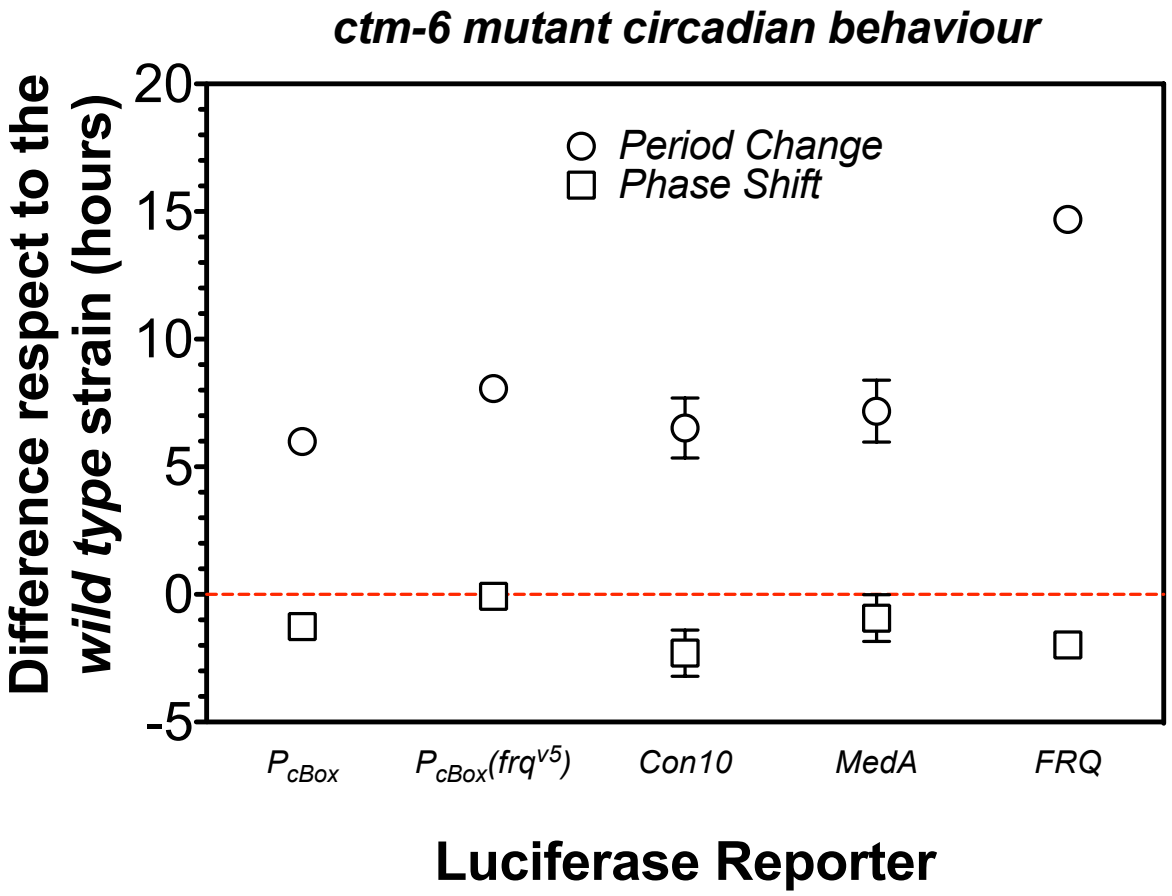

Figure 2.4. Summary of period and phase changes caused by the *ctm-6* mutation, in different genetic backgrounds.
Values, derived from luciferase results analyses, are summarized comparing *Period Change* (**O**) and the *Phase Shift* (□) for each reporter and in different genetic backgrounds (WT, frq^{v5} or frq^{luc}) .

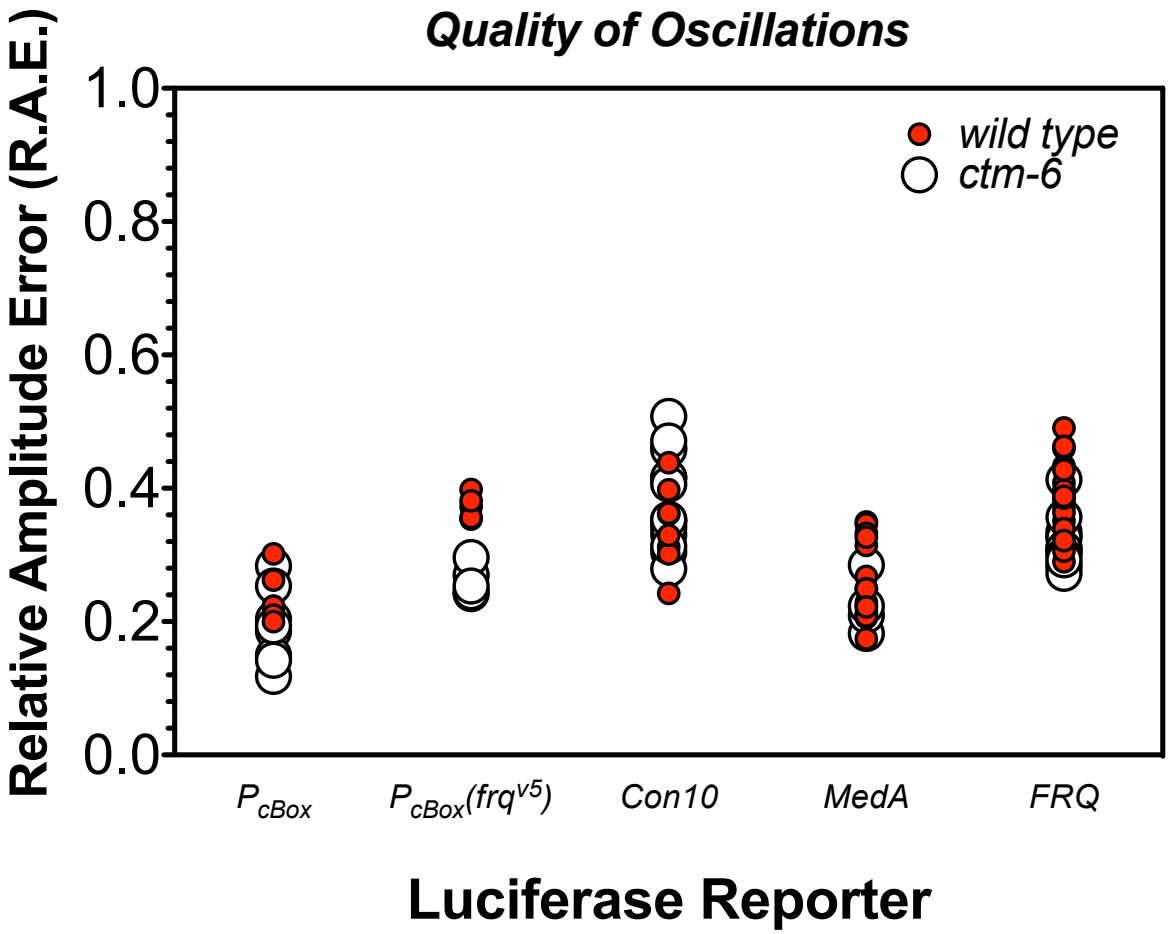

Figure 2.5. Quality of oscillations of *wild type* and *ctm-6* strains, in different genetic backgrounds.
Values, derived from luciferase results are summarized comparing *Relative Amplitude Error* (RAE) for each strain in different genetic backgrounds (WT, frq^{v5} or frq^{luc}). The *wild type* strains are shown as red dots and the *ctm-6* strain as white dots.

A recombination rate close to 0% between *ctm-6* phenotype (long period) and a *KO* cassette (hygromycin resistance) indicated that the *ctm-6* mutation and *hph* cassette are near each other, in other words, linked (PERKINS 1959; JIN *et al.* 2007; HAMMOND *et al.* 2012). Crosses between KOs located in different positions of Linkage Groups II through VII, yielded offspring *(BAKER et al. 2012)* where segregation of the *ctm-6* mutation and the resistance to hygromycin was near 50%. However, when crossing a KO located in Linkage Group I with the *ctm-6* containing strain, the analysis of the progeny revealed a recombination percentage under 20%, a result that was further confirmed with a second cross with another KO also located in the same Linkage Group, which showed a recombination percentage of 10%. Interestingly, Linkage Group I is the most extensively mapped chromosome in the *Neurospora crassa* genome (GALAGAN *et al.* 2003; BORKOVICH *et al.* 2004) and contains several genes associated with circadian function (LAKIN-THOMAS and BRODY 2004; HEINTZEN and LIU 2007; BAKER *et al.* 2012).

In parallel we selected strains containing the *ctm-6* mutation in order to extract DNA and perform whole genome sequencing. For this we backcrossed the mutant with a frq^{luc} strain, and selected progeny exhibiting the expected period for frq^{luc} (~23 h), and also isolated progeny with long period due to the synthetic effect of *ctm-6* plus a frq^{luc} (Figure 2.3). Thus, based on clear phenotypes we were able to sort out the two groups (WT and mutant), proceeding to extract DNA from 14 and 16 progeny each, respectively. DNA samples were pooled in equal amounts within each group and sent for sequencing to an external provider (see Methods). Since we had pooled the DNAs, we buffered any random mutation that could be present in some of the progeny, and which would not be

linked to the phenotype of interest, as that would be equally present in all DNA pools. Among the mutations that were present only in the *ctm-6*-derived pool, we concentrated on the SNPs (single nucleotide polymorphism) with a 100% of read frequency (Table 2.1), that were located in in the Linkage Group I, area that the genetic data had identified as likely to contain the mutation. We identified a single mutation in the locus for NCU00685, which encodes for the *casein kinase 1a* gene (*ck1a*).

This particular SNP is a transversion from a Cytosine (C) to a Guanidine (G) in the nucleotide 493 (taking as +1 de start codon of the ORF), causing a non-synonymous change from a basic Histidine (H) to an Aspartic Acid (D) at position 165 of the predicted protein. *ck1a* encodes the Neurospora homologue of casein kinase I, a highly conserved kinase, widespread across the tree of life, and known to play a key role in most of the eukaryotic circadian clocks characterized to date (EIDE and VIRSHUP 2001; REISCHL and KRAMER 2011). Members of this family exhibit a highly evolutionary conserved catalytically domain in the amino-terminal region (~300 amino acids), while possessing a variable and not so conserved C-terminal tail, which has been associated to specific regulation in each species (KNIPPSCHILD *et al.* 2005; QUERFURTH *et al.* 2007). The *ctm-6* mutation is localized in the highly conserved N-region, but nevertheless, corresponds to an evolutionarily rare conserved amino acid (Figure 2.6, upper panel).

Table 2.1. SNPs found through the whole genome sequencing for the *ctm-6* pooled samples.

Linkage Group	Total SNPs with 100% of variant frequency		Coding genes affected by non-synonymous point mutation	
	wild type	***ctm-6***	***wild type***	***ctm-6***
I	223	308	N/F	*ck-1a*
II	101	108	N/F	*bud-6*
III	2165	2248	*aab-1/stk-42/stk-41/vad-3/xyk-1*	*aab-1/stk-42/stk-41/vad-3/xyk-1*
IV	57	60	*ras-1*	*ras-1*
V	67	61	N/F	N/F
VI	34	31	N/F	N/F
VII	235	238	*ars-1*	*ars-1*

We identify several SNPs with a 100% of variant frequency through the whole genome sequencing for each Linkage Group (chromosome) in *N. crassa*; however, a few were identified in coding genes, affecting them by non-synonymous change, as we see *ctm-6* mutants pooled samples shown *ck1a* and *bud-6* as possible target of the *ctm-6* mutation, but *ck1a* is later confirmed as the carrier for the ctm-6 mutation by genetic mapping. N/F, not found.

For a better understanding of the relevance of this mutation for CK1a function, we used the available crystal structure of the human Casein kinase delta (Ck1δ) as a 3D model rendering (LONGENECKER *et al.* 1996). And mapped on to the structure of Ck1δ the corresponding amino acid that is mutated in CK1a^{ctm-6} (ARNOLD *et al.* 2006; BIASINI *et al.* 2014). Ck1δ exhibits a 76.74% of identity to Neurospora CK1a, and their catalytic domains are well identified (XU *et al.* 1995; KIEFER *et al.* 2009; BIENERT *et al.* 2017; MARCHLER-BAUER *et al.* 2017). The amino-terminal region of the protein contains the active site, including the ATP-binding site (MASHHOON *et al.* 2000; LONG *et al.* 2012), which is tightly associated to the peptide substrate-binding site (BROWN *et al.* 1999; FAVELYUKIS *et al.* 2001), and the activation loop (A-loop). In most of the described kinases, including the casein kinases, the A-loop undergoes a conformational change or a disorder-to-order transition upon phosphorylation of Ser, Thr or Tyr residues within the loop. This phosphorylation event allows ATP and substrates to access the active site, allowing catalysis to occur (RUSSO *et al.* 1996; HUBBARD 1997; HUANG *et al.* 2003; WU *et al.* 2003). With the predicted comparative structural model for CK1a, we noted that the mutated amino acid (165) is not localized near the identified catalytic domains close to the active site. Instead, it appears to be located inside the A-loop structure (Figure 2.6, lower panels), which could still have an effect over the activity of this enzyme.

As a way to further confirm the connection between the SNP mutation present in the NCU00685 locus and the circadian phenotype under study, we developed an RFLP, a PCR-digestion assay (see Methods), to confirm the presence of the mutation in all the strains exhibiting the *ctm-6* circadian phenotype. Thus, we unambiguously confirmed the

presence of the *ck1a* SNP in all the circadianly strains characterized as *ctm-6*, which include all the *ctm-6* mutant strains in the different genetic backgrounds, being a total of 116 confirmed strains. Therefore, we can reliably associate this mutation with the *ck1a*^{ctm6} allele.

Different Kinase inhibitors selectively affect the ck1a^{ctm6} *mutant selectively.*

One plausible explanation for the molecular alteration present in *ck1a*^{ctm6} could be a dysfunctional catalytic activity of the mutant CK1a protein (REISCHL and KRAMER 2011; ODE and UEDA 2017). This change in activity would provoke changes in the phosphorylation of clock proteins throughout the day, as already seen for Ck1 mutants in several other organisms, including Neurospora (PREUSS *et al.* 2004; HE *et al.* 2006a; MENG *et al.* 2008; REISCHL and KRAMER 2011). In order to evaluate this potential effect, we took a pharmacological approach, by using general and specific kinase inhibitors (COMOLLI *et al.* 1994; LIU *et al.* 2000; KON *et al.* 2015). Thus, we utilized 6-dimethylaminopurine (6-DMAP), a broad-spectrum kinase activity inhibitor already shown to block clock protein phosphorylation, and causing a lengthening of the clock (COMOLLI *et al.* 1994; LIU *et al.* 2000). In addition, we used two others specific Ck1 inhibitors: IC261 (QUERFURTH *et al.* 2007; KON *et al.* 2015) and PF-670462 (BADURA *et al.* 2007; WALTON *et al.* 2009).

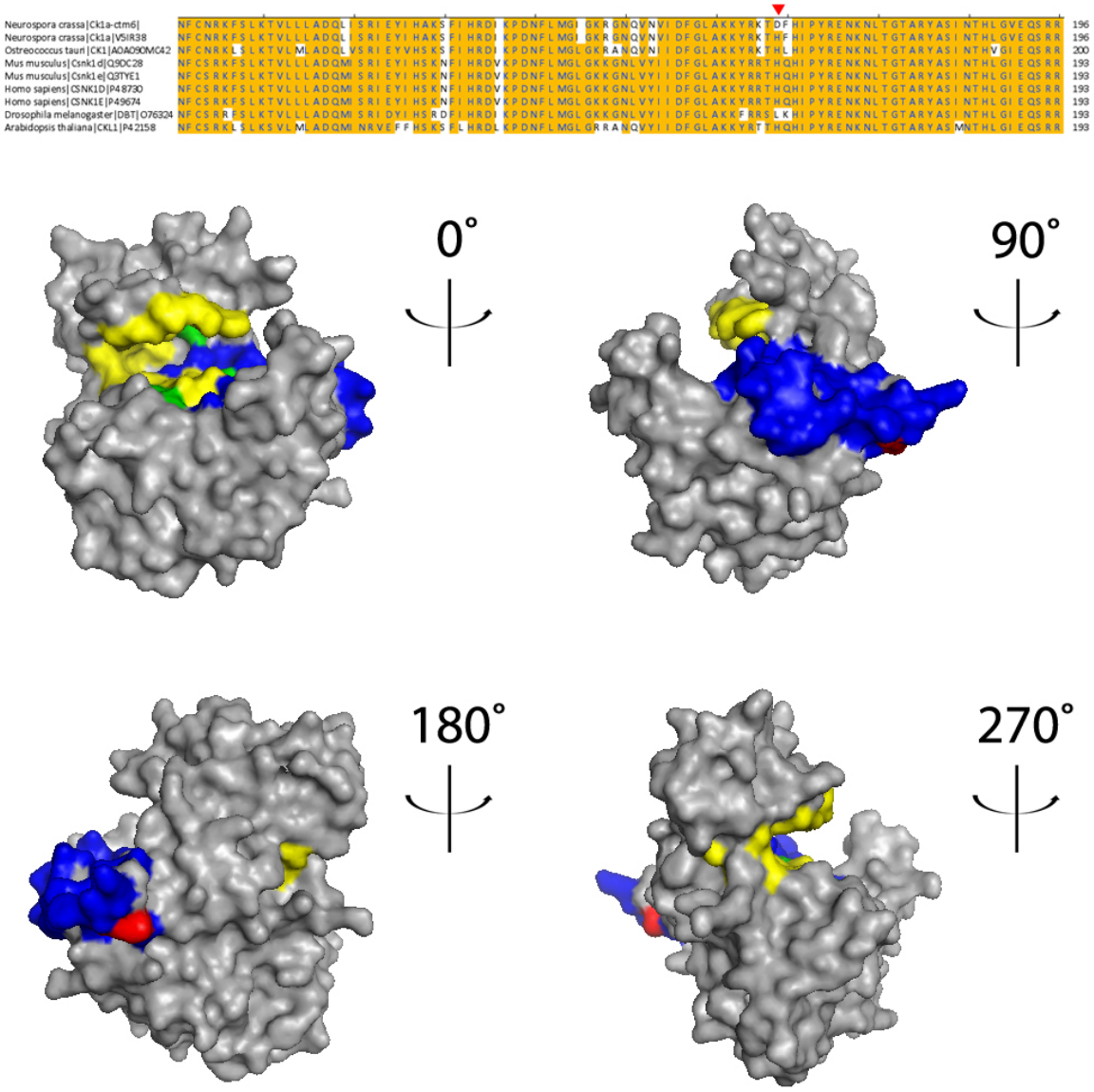

Figure 2.6. Sequence conservation and the structural model for Neurospora CK1a^{ctm6}.

The multiple alignments (upper panel), highlight the region where the *ctm-6* point mutation is located. Comparing the equivalent regions with CK1s from others circadian models, it becomes evident that there exists a high conservation, including at the position of the mutated residue, signaled by a triangle. A structural model of Neurospora CK1a, obtained by using as a render the crystallized structure of Ck1δ from human, is shown in the lower panels**.** Four structural views are provided, where the structure has been rotated around the Y-axis at 0˚, 90˚, 180˚, and 270˚ degrees. Yellow was used to decorate the active site, including the nucleotide-binding site in green. The A-loop is depicted in blue and the residue 165, where the *ctm-6* mutation occurs, is highlighted in red.

These compounds have been shown to provoke dose-dependent increments in period length in several clock models (LIU *et al.* 2000; EIDE *et al.* 2005; MENG *et al.* 2010; VAN OOIJEN *et al.* 2013; LARRONDO *et al.* 2015). We reasoned that if the *ck1a*^{ctm6} is a hypomorphic mutation that decreases CK1a kinase activity, an additional decrease of kinase activity by these drugs should be partially masked in a *ck1a*^{ctm6} mutant strain. Thus, we hypothesized that there wouldn't be an additional increase in period (caused by a reduction of FRQ phosphorylation) in the presence of the drugs if *ck1a*^{ctm6} would already exhibited decreased kinase activity.

When analyzed in a WT background, the broad range inhibitor 6-DMAP lead to period increment, in a dose-dependent manner (Figure 2.7), as already described. Notably, it also led to period lengthening when tested in a *ck1a*^{ctm6} background, indicating that chemical kinase inhibition can further lengthen period (to a comparable degree) even in the presence of this mutant *ck1a* allele and that, therefore, our original assumption regarding the hypomorphic nature of this mutation may be incorrect. In order to better focus our question on CK1a, we also utilized specific casein kinase 1 family inhibitors at different concentrations, producing saturation curves. The specific and robust drug PF-670462 binds tightly to the ATP-binding site of Ck1 (LONG *et al.* 2012), causing period lengthening in different circadian systems (MENG *et al.* 2010; VAN OOIJEN *et al.* 2013). Importantly, and as seen when assessing 6-DMAP, the effect of PF-670462 on the Neurospora clock was similar in both WT and *ck1a*^{ctm6} backgrounds (Figure 2.8), which also help dismiss the hypothesis that *ck1a*^{ctm6} is a hypomorphic mutation

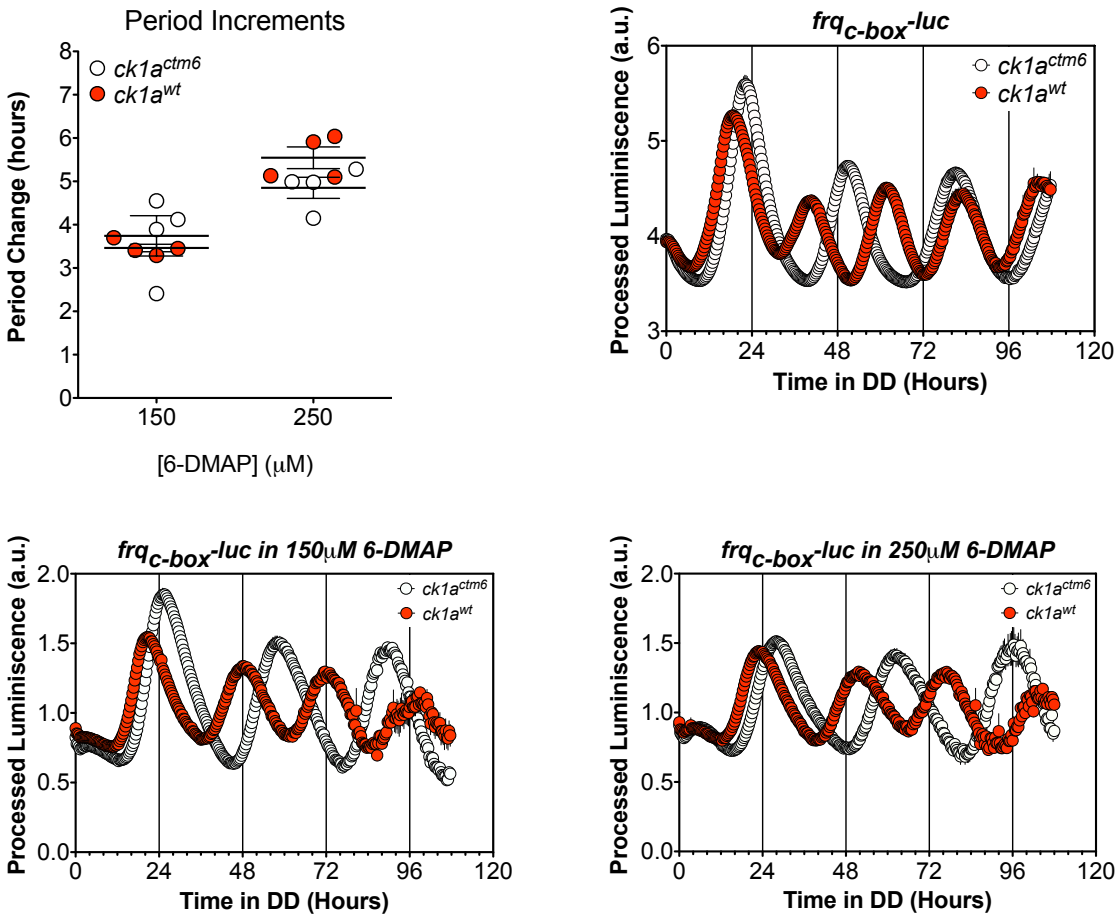

Figure 2.7. The *ck1a*^{ctm6} mutant allele responds to the general inhibitor 6-DMAP increasing the period same as the WT strain.
The effect of 6-DMAP, at two concentrations (150 μ M and 250μM) was tested in strains of the indicated genetic backgrounds.

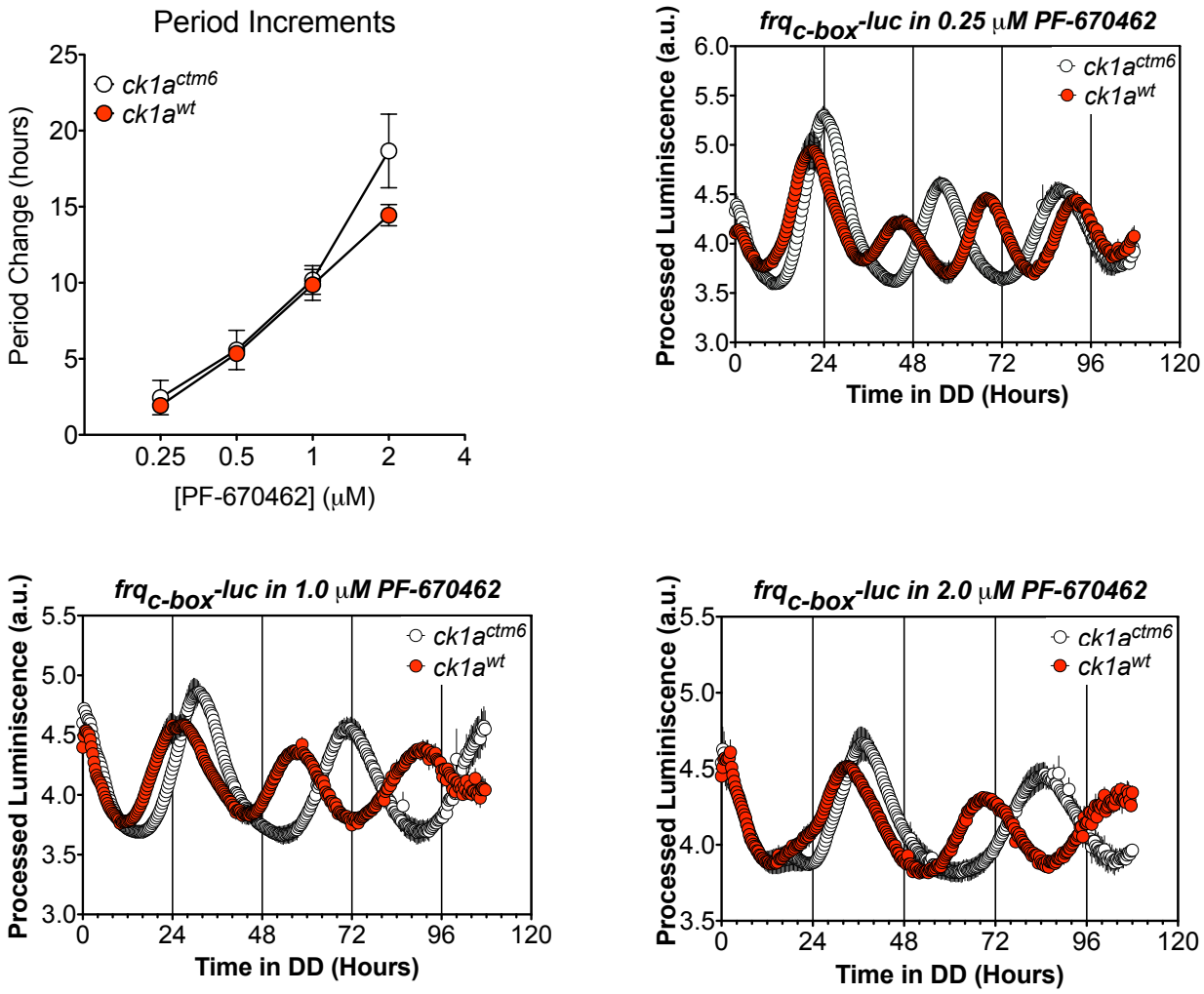

Figure 2.7. The *ck1a*^{ctm6} and *ck1a*^{wt} alleles respond in a dose-dependent manner to the specific CK1 inhibitor PF-670462.
Saturation curves generated with the specific CK1 inhibitor PF-670462 are shown in the upper left panel. The other panels depict the rhythmic patterns observed for the minimal *frq*$_{c\text{-}box}$*-luc* reporter in *ck1a*^{wt} (red dots) and *ck1a*^{ctm6} (white dots) strains, at the indicated concentrations of the inhibitor.

However, the inhibitor IC261 showed differential effects on period length between *ck1a*^{ctm6} and control strains (Figure 2.8). Paradoxically, we observed that IC261 did not provoke major period lengthening in a WT strain, even when high concentrations of the drug were utilized. On the other hand, the period alterations for *ck1a*^{ctm6} were unexpected: at low IC261 concentrations the drug lengthened period in ~2 hours compared to the untreated control. As higher concentrations of IC261 were tested the effect on period decreased, to the point where it was no different than the untreated *ck1a*^{ctm6} control. IC261 has been described as a far less potent inhibitor of Ck1 than PF-670462 (RODRIGUEZ *et al.* 2012; VAN OOIJEN *et al.* 2013). However, when tested in the context of its clocks effects, it has been shown to cause a dose-dependent increase in period in others circadian systems (EIDE *et al.* 2005; VANSELOW *et al.* 2006; REISCHL *et al.* 2007; VAN OOIJEN *et al.* 2013). This drug has also been shown to cause to cell cycle arrest (BEHREND *et al.* 2000; CHEONG *et al.* 2011). Thus, the results observed for IC261 and probably this differential behavior will be associated to these secondary effects of the drug, and the influence of the *ctm-6* mutation in the structure of the protein kinase activation loop provoke the different behavior between strains.

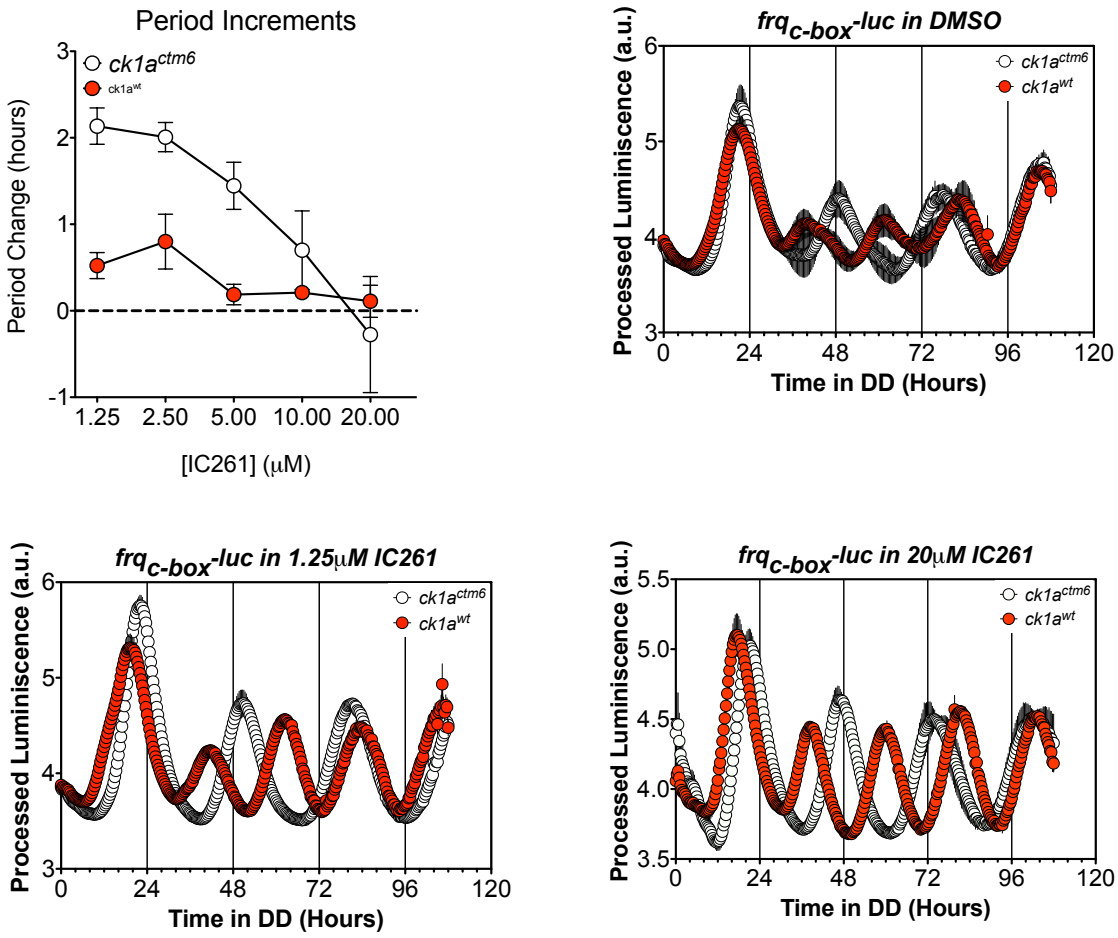

Figure 2.8. The *ck1a*^{ctm6} and *ck1a*^{wt} alleles respond differentially to the specific inhibitor IC261.

The saturation curves generated with the specific CK1 inhibitor IC261 are shown in the upper left panel. The other panels show the rhythmic patterns observed for the minimal *frq*$_{c\text{-}box}$*-luc* reporter in *ck1a*^{wt} (red dots) and *ck1a*^{ctm6} (white dots) strains, at the indicated concentrations of kinase inhibitor.

The ck1a^{ctm6} allele shows varying genetic interactions with different frq alleles

As we previously noted, a synergistic effect on circadian period was observed when the *ck1a^{ctm6}* mutation and particular *frq* alleles (*frq^{V5}* or *frq^{luc}*) were combined in the same genetic background (Figure 2.3). Nevertheless, both alleles are artificial, as they result from the addition of protein tags (V5-tag or luciferase respectively) to FRQ (BAKER *et al.* 2009; LARRONDO *et al.* 2015). Therefore, we sought to understand what would be the effect of combining *ck1a^{ctm6}* with natural occurring *frq* alleles (FELDMAN and HOYLE 1973) or *frq* alleles containing mutations that affect known *in vivo* phosphorylation sites (BAKER *et al.* 2009). It is well known that post-translational modifications of clock proteins, mainly phosphorylations, are critical events in circadian period determination (TOMITA *et al.* 2005; LARRONDO *et al.* 2015; ROMANOWSKI and YANOVSKY 2015). Moreover, Casein kinase 1 has been long before studied as key component of the phosphorylation machinery that progressively modifies the negative elements of the clock in diverse clock models (REISCHL and KRAMER 2011; ODE and UEDA 2017). In an attempt to better understand the relationship and the mechanisms involved in the *ck1a^{ctm6}* phenotype on circadian period, we analyzed the genetic interactions of *ck1^{ctm6}* with different *frq* alleles known to produce a wide range of period defects. The selected *frq* alleles correspond to point mutations or a C-term deletion, with clear and defined circadian period phenotypes (BAKER *et al.* 2009; LARRONDO *et al.* 2015). Thus, it is possible to determine the genetic interactions between both loci and further classified them.

The haploid nature of Neurospora allows eliminating dominance effects from the equation, and to efficiently characterize the type of genetic interaction between loci of interest (LAKIN-THOMAS and BRODY 1985; MORGAN and FELDMAN 2001; MORGAN *et al.* 2001). If the effect of each locus is independent, an additive or multiplicative effect is expected, whereby each mutation can contribute to the phenotype in a fixed amount or a fixed percentage, respectively (MORGAN *et al.* 2001). The alternative to this is the interdependence of each locus, whereby the relationships could be epistasis, intergenic suppression, or synergy. In the first case, epistasis, the effect of one mutation masks the effect of the other; in intergenic suppression, the interaction of both mutations produces a WT phenotype; and synergy, where the expected phenotype cannot be predicted (MORGAN and FELDMAN 2001; MORGAN *et al.* 2001).

Two of the *frq* alleles selected for analysis correspond to classical SNPs mutants not related to phosphorylation sites, but that lead to strong period alterations: frq^{7}, a well-known mutant allele has Aspartic Acid (D) instead of an Glycine (G) at position 459 amino acid (G459D) which increases period by ~7 hours. The other allele is frq^{1}, that leads to a period decrease of ~6 hours, and corresponds to a change of a Serine (S) to Glycine (G) in the position 482 (G482S) (FELDMAN and HOYLE 1973; GARDNER and FELDMAN 1980; ARONSON *et al.* 1994a). Interestingly, both alleles showed a synergistic relationship with $ck1^{ctm6}$, instead of additive (+) or multiplicative (×) effected (Figure 2.9).

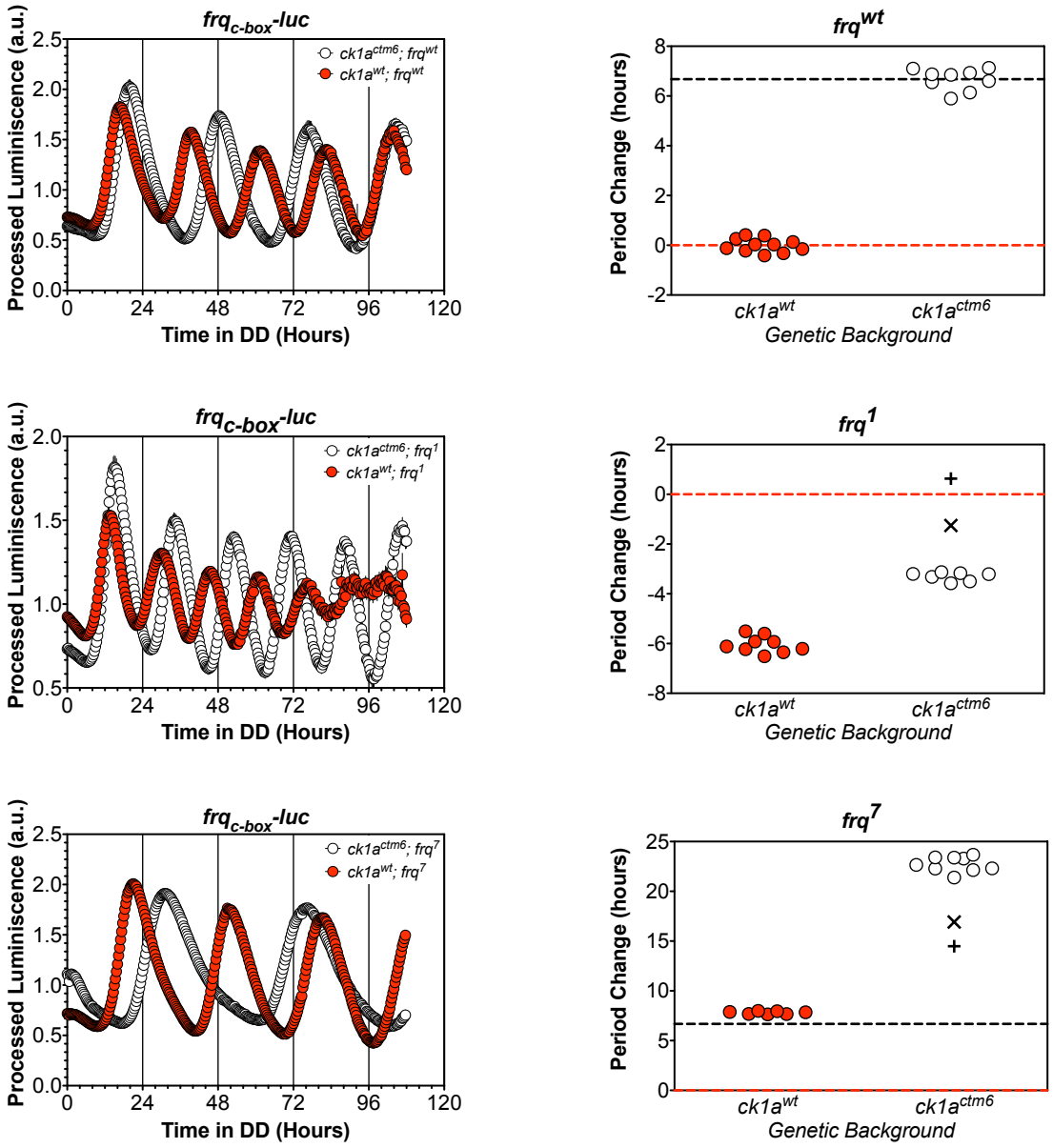

Figure 2.9. *ck1a*^{ctm6} interacts synergistically with *frq* alleles.
The left column shows the *frq*$_{c\text{-}box}$*-luc* luciferase traces of the strains of the indicated genotypes. The right column summarizes period changes respect to the WT (---) and the *ctm-6* mutants (---). The graphs also include the predicted period according to an additive (+) or multiplicative (×) model. From top to bottom the following *frq* alleles were examined: *frq*^{wt}, *frq*^{1}, *frq*^{7}.

Thus, the *frq*^{1} *ck1*^{ctm6} combination, produces an altered period of ~18 hours, which corresponds to a decrease of ~4 hours compared to WT, and far from the 0 to -1 hours of period change expected from the models. An even more remarkable result was observed in the strains containing the *frq*^{7} *ck1*^{ctm6} allele combination: a strong period change, that adds ~23 hours to the *wild-type* period, producing a strain with a 45 h clock, one of the longest periods reported in Neurospora (LAKIN-THOMAS and BRODY 1985) or, to our knowledge, in any other circadian system. Trying to interpret the observed synergistic effects, one could hypothesize that it is associated to the position of the changed amino acid in these *frq* alleles, as both are localized in FRQ's FCC domain, a key region for the interaction between FRQ and CK1a (HE *et al.* 2006a; QUERFURTH *et al.* 2007; BAKER *et al.* 2009).

The same synergistic effect was observed when *ck1*^{ctm6} was analyzed in conjunction with *frq*$^{\Delta C\text{-}term}$, a FRQ allele that lacks part of its C-terminus (Figure 2.10). The deleted region, from aa. 889 to 989, contains several phosphorylation sites which, when mutated, lead to short periods while also exhibiting changes in FRQ stability, suggesting that this region relates to a phase-specific phosphorylation process (BAKER *et al.* 2009). As recent studies show, the stability of the FRQ protein is not the mechanism leading to period determination (LARRONDO *et al.* 2015; ODE and UEDA 2017; ODE *et al.* 2017). Therefore, it is likely that the effect observed in a *ck1*^{ctm6} *frq*$^{\Delta C\text{-}term}$ is not related to FRQ stability but instead to additional alterations in the normal phase-specific phosphorylation process that dictates period (MONTENEGRO-MONTERO *et al.* 2015).

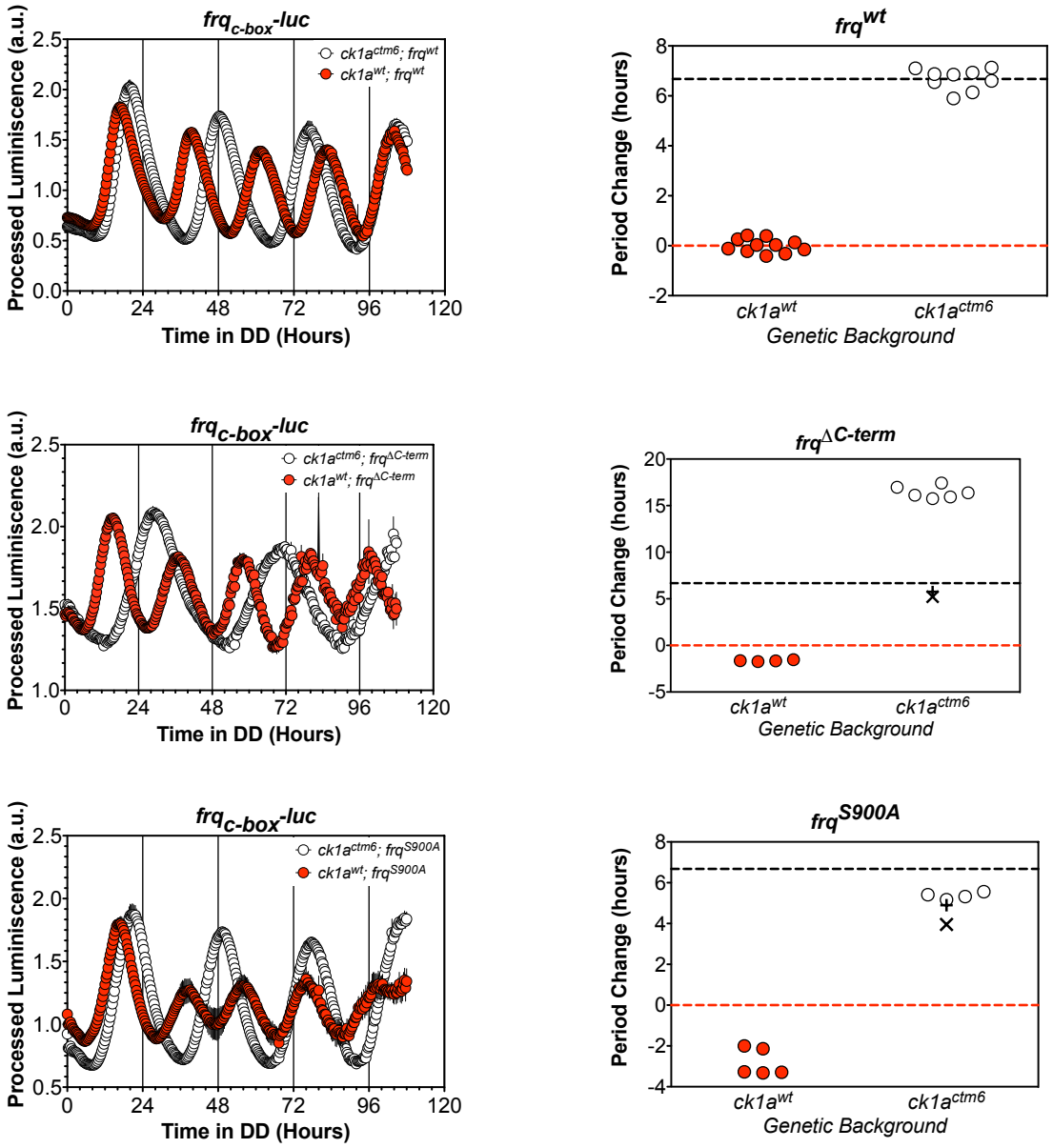

Figure 2.10. *ck1a*^{ctm6} interacts differentially with *frq* alleles that lack particular phosphorylation sites.

The left column shows luciferase traces of the strains of the indicated genotypes monitored using a *frq*$_{c\text{-}box}$*-luc* reporter. The right column summarizes period changes respect to the WT (---) and the *ctm-6* mutants (---). The graphs also include the predicted period according to an additive (+) or multiplicative (×) model. From top to bottom the following *frq* alleles were examined: *frq*^{wt}, *frq*$^{\Delta C\text{-}term}$, *frq*^{S900A}.

Interestingly, for the *frq*^{S900A} *ck1a*$^{ctm\text{-}6}$ combination, a period increase of ~5 hours, was observed (Figure 2.10), a result close to the predicted period in an additive model scenario, indicating independence between both mutations. The *frq*^{S900A} allele is a mutant strain for a phosphorylation site near to the PEST-2 domain of FRQ protein (BAKER *et al.* 2009), a region located inside the deleted region of *frq*$^{\Delta C\text{-}term}$. This relationship reveals that not all the phosphorylation sites inside the C-terminus of FRQ are related to the activity or interaction with CK1a.

DISCUSSION

A standard feature in every circadian system is the progressive phosphorylation of their core elements during the day. This time-dependent regulation from hypo- to hyper-phosphorylated states, in positive and negative elements is known to be a critical process for proper time-keeping (YOUNG and KAY 2001; MIZOGUCHI *et al.* 2006; GALLEGO and VIRSHUP 2007; MARKSON and O'SHEA 2009). In eukaryotes, phosphorylation of the negative elements has been shown to be a critical process in clock period-determination (ODE and UEDA 2017). It has been stated in the general literature that progressive phosphorylation marks the degradation of the negative element, and therefore, that a causal relationship between negative element half-life and period exists (RUOFF *et al.* 2005; BLAU 2008; CHIU *et al.* 2008; SYED *et al.* 2011; BROWN *et al.* 2012). However, recent evidence from Neurospora and mouse showed that the degradation of the negative elements *per se* is not determinant for clock function (LARRONDO *et al.* 2015; ODE *et al.* 2017). Thus, the main effect of phosphorylation events is not to achieve protein turnover but instead it appears to allow proper timing of clock function by permitting progressive changes of the negative elements. Thus, the speed of the clock depends of the timing and sequence of certain phosphorylation events, whereas the stability of the negative element is a consequence (and not a causative effect) of how fast/slow the clock runs (ODE and UEDA 2017). The positive arms in these feedback loops are also regulated by phosphorylation, and affect the activity of the transcriptional complex when one of the

members in the heterodimer change from a hypo- to a hyperphophorylated state (EIDE *et al.* 2002; SCHAFMEIER *et al.* 2005; KIM and EDERY 2006; SAHAR *et al.* 2010). This phosphorylation makes the transcriptional complex unstable in a manner that depends on the negative element dependent manner (HE *et al.* 2006a; YU *et al.* 2006; TAMARU *et al.* 2015).

Among the kinases involved in this process we highlight the role of casein kinases 1 (CK1), a large family of evolutionary conserved eukaryotic kinases, with a myriad of protein substrates and different cellular functions in several model organisms (GROSS and ANDERSON 1998; KNIPPSCHILD *et al.* 2005; CHEONG and VIRSHUP 2011). The circadian role for these proteins has been characterized in each eukaryotic clock model (REISCHL and KRAMER 2011; ODE and UEDA 2017), and despite the evolutionary distance, remarkable conservation exists through all the CK1 proteins among different eukaryotic systems from fungi to human (CHEONG and VIRSHUP 2011). The highly functional conservation between Ck1 proteins has been shown several times including equivalent mutations from one ortholog in others, replicating the same circadian phenotypic effect across the different circadian models (HE *et al.* 2006a; FAN *et al.* 2009; VAN OOIJEN *et al.* 2013). At a molecular level, Ck1 is probably the most relevant conserved element in the whole structure of the eukaryotic clocks, highlighting it as a keystone in the circadian evolution (TAUBER *et al.* 2004; FAN *et al.* 2009; ROSBASH 2009; REISCHL and KRAMER 2011; BROWN *et al.* 2012; ODE and UEDA 2017)

Several natural and induced mutations in this gene family have being identified by forward genetic screens across varied circadian model organisms throughout the years (RALPH and MENAKER 1988; KLOSS *et al.* 1998; PRICE *et al.* 1998; JONES *et al.* 1999; LOWREY *et al.* 2000; SURI *et al.* 2000; PREUSS *et al.* 2004). The knowledge obtained from them has given us a complete understanding of the time dynamics of the circadian clocks (XU *et al.* 2005; VANSELOW *et al.* 2006). Thanks to deep-sequencing techniques currently available, the mapping process required for these forward genetics approaches is now much faster than years ago (SMITH *et al.* 2008; NIU *et al.* 2017). Using these tools, we identified *ck1a*^{ctm6}, a novel SNP affecting a crucial transversal member of eukaryotic circadian clocks. At a structural level, this mutation is localized in the activation loop of the kinase protein, showing no resemblance to other mutation characterized for members of this family (REISCHL and KRAMER 2011). Most of the characterized Ck1 mutation correspond to catalytic alterations tested *in vitro* (TOH *et al.* 2001; XU *et al.* 2005; MUSKUS *et al.* 2007), but the circadian phenotypes are counterintuitive, showing either long or short circadian period with a reduced catalytic function (PREUSS *et al.* 2004; MENG *et al.* 2008; YU *et al.* 2009). Recent evidence in mammals highlights the role of the phosphorylated sites to activate the function of CK1δ are relevant to circadian function, including autophosphorylations process, principally in the variable C-portion of the kinase (CEGIELSKA *et al.* 1998; RIVERS *et al.* 1998; GIETZEN and VIRSHUP 1999; ENG *et al.* 2017).

The data obtained from inhibitor assay supports the hypothesis of a non-catalytical effect in CK1a caused by the *ctm-6* mutation (Figure 2.3). For the broad range, inhibitor 6-DMAP the data were consistent with what has been observed previously (LIU *et al.* 2000; LARRONDO *et al.* 2015), and is supported by the saturation curve observed using the specific and robust inhibitor PF-670462 (MENG *et al.* 2010; VAN OOIJEN *et al.* 2013). In both cases, the drug does not cause any differential effect between WT and CK1a^{ctm-6}. That was not the case for the reagent IC261, also a selective CK1δ/ε inhibitor, which works by an ATP-competitive mechanism (BEHREND *et al.* 2000; MASHHOON *et al.* 2000). Although this inhibitor has been shown to work in several clock systems, it had not been tested in terms of circadian period in Neurospora, although there was evidence supporting its effect over CK1a in the context of WC-2, but not FRQ phosphorylation (QUERFURTH *et al.* 2007). Our results failed to reveal important period lengthening using this drug on WT cells, although it showed a counterintuitive effect in *ck1a*^{ctm6}, causing a perceptible period shortening. IC261, in contrast to PF-670462, which caused a secondary effect in the cell cycle process, to causing a rapid induction of prometaphase arrest and subsequent apoptosis in multiple cancer cell lines. This activity is due to the binding to tubulin with an affinity similar to that for colchicine, a potent inhibitor of microtubule polymerization (BEHREND *et al.* 2000; CHEONG *et al.* 2011). This secondary activity of the IC261 over the cell cycle could explain the differential effect of this reagent in *ck1a*^{ctm6}. As is well known, CK1 isoforms are related to the cell cycle progression (JOHNSON *et al.* 2013; SCHITTEK and SINNBERG 2014), and a variety of evidence argues in favor of the coupling and synchronization of both oscillatory processes (MATSUO *et al.* 2003; PREGUEIRO *et al.* 2006; HONG *et al.* 2014; LIU *et al.* 2017). Even when we analyzed

the roles of the transcription factors identified in Chapter 1, the shortest (NCU06145) and longest (NCU08999) period change associate with mutations affecting cell cycle (Chapter 1). To entirely discard the possibility that a catalytically deficiency in CK1a caused by the *ctm-6* mutation, or its potential associations with cell cycle, further studies are required.

To further elucidate the effects of the *ctm-6* mutation in CK1a function, we looked into genetic interactions when combining $ck1a^{ctm6}$ with different *frq* natural and artificial alleles (Figure 2.1.). The synergistic genetic interactions of $ck1a^{ctm6}$ with *frq* alleles containing mutations on the FCD, argues of a likely effect of the CK1a mutation in perturbing the physical interaction between this enzyme and FRQ. In other circadian systems, the interaction of Ck1 with the negative element is postulated to be part of Ck1 also acting as a scaffold to recruit other kinases (TOH *et al.* 2001; YU *et al.* 2009). It is known that CK1a and FRQ form a complex (GORL *et al.* 2001; HE *et al.* 2006a; QUERFURTH *et al.* 2007) and that FRH, a dead-box containing RNA helicase, plays an important role as a nanny, or as a scaffold that allows FRQ to interact progressively with different proteins and kinases. These dynamics between FRQ and FRH also allow the controlled access of CK1a to different phosphorylation sites in FRQ, which in return mediate the subsequent interaction with, and inhibition of, WCC (LAUINGER *et al.* 2014; CONRAD *et al.* 2016). Indeed, we know that the effects of the CK1a are on phosphorylating both the positive and negative arms of the circadian oscillator (HE *et al.* 2006a; QUERFURTH *et al.* 2007; TANG *et al.* 2009).

Based on our results, it seems that the *ck1a*^{ctm6} mutation does not affect the catalytic activity of CK1a, although this must be confirmed by *in vitro* assays. Instead, it seems more likely that the period alteration is caused by deficient interactions in the FRQ-FRH-CK1a complex and with that the recruitment of active kinases to progressively act over FRQ. In addition, this could also impact the activity of CK1a over the clock positive element WCC. Follow up studies using this mutant allele in combination with additional alleles and synthetic strategies, could provide us with answers to pending question about the critical steps of period-determination in eukaryotic circadian systems (ODE and UEDA 2017). Indeed, it is anything but fascinating that the speed-limiting step of a 24-h process relies on such a fast reaction such as phosphorylation; a dynamical conundrum between a 24-h scale systemic process and a millisecond-scale assortment of biochemical reactions (AKIYAMA 2012)

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CONCLUDING REMARKS

The overarching goal of this thesis was to gain a better understanding of the molecular circuitry underlying the *N. crassa* circadian clock, a model system for circadian biology. Through both forward and reverse genetic approaches, we identified novel regulators and characterized hitherto unknown interactions in this complex molecular network. In all, we identified new TFs that are key for the proper operation of the clock, expanding our knowledge of circadian regulators in this system. Further, we developed a complete library of clock reporter strains in different genetic backgrounds lacking known TFs. Such a resource can now be further explored to study both the effect of different environmental perturbations and the role of these regulators in the clockworks. In addition, and despite unveiling TFs that are required for proper clock functioning in *N. crassa*, our study highlighted the overall robustness of the system, further supporting that disruption of only a few key actors can severely impact the clock. Lastly, we identified a novel spontaneous mutation in CK1a, a conserved kinase known to play a role in different circadian systems, which, contrary to previously reported clock-affecting variants in this fungus, is not located within its known catalytic domains, and we propose that it does not affect its catalytic activity. These results may lead to a different way of viewing the function of this kinase in circadian systems. Overall, our study provides new insights into the molecular workings of circadian clocks in eukaryotes.

PROJECTIONS

The available *ΔTF frq$_{cbox}$-luc* collection, spanning 171 different TFs, constitutes a valuable resource that will expedite new screenings contemplating particular single and combined environmental perturbations (nutrients, pH, etc.). The latter will help further unveiling a dynamic transcriptional network supporting robust and compensated clock function. We are working on the first manuscript based on this data named "Global Screening of Transcription Factors involved in the period regulation of *Neurospora crassa* circadian clock", formatted as a *mutant screen report*, a fast and easy format available in the journal "Genes, Genome, Genetics", guessing a submission in the first half of 2019.

Finally, this thesis shed light into potential novel components and interactions of the Neurospora circadian clock. From the previous screen, we identified a possible role of CBF1 and WCC in response to Temperature changes, which when further dissected, can provide new insights into temperature signal processing by the Neurospora clock. The unveiling of the mechanisms behind this regulation would be relevant to explain characteristic circadian phenomena such as temperature resetting and temperature compensation of the clock. Also, by serendipity, we found and described the new *ck1^{ctm-6}* allele, which leads to unprecedented period lengthening (particularly in combination with *frq* alleles), which renders the field with new tools to better understand

mechanisms for CK1 in eukaryotic period determination. Both work are being continued in our lab, expecting to produce two additional publications from this thesis.