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# Trimethylated Lysine 4 at Histone 3 Shows the Same Circadian Rhythm at Promoters of Diversely-Expressed Genes in *Chlamydomonas Reinhardtii*

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TRIMETHYLATED LYSINE 4 AT HISTONE 3 SHOWS THE SAME CIRCADIAN  
RHYTHM AT PROMOTERS OF DIVERSELY-EXPRESSED GENES IN  
*CHLAMYDOMONAS REINHARDTII*

A Thesis  
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Western Kentucky University  
Bowling Green, Kentucky

In Partial Fulfillment  
Of the Requirements for the Degree  
Master of Science

By  
Robyn Wilson

August 2016

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Circadian clocks are biochemical mechanisms that allow eukaryotic and some prokaryotic organisms to coordinate their physiology with daily environmental changes. It enables organisms to increase their fitness by taking advantage of beneficial environmental conditions while also avoiding or restricting certain sensitive processes during harsh conditions. Similarly, post-translational histone modifications allow eukaryotic organisms to regulate gene expression in response to environmental or developmental factors. Some post-translational modifications of histones are associated with active transcription while others are associated with repressed transcription depending upon the location, type and degree of modification. Trimethylation of lysine 4 on the N-terminal tail of histone H3 (H3K4me3) near a gene's promoter has been linked to active transcription of that gene in several organisms. The purpose of the current study was to investigate whether the amount of H3K4me3 at promoters of three specific genes shows a circadian rhythm in *Chlamydomonas reinhardtii*, a unicellular green alga. Two of the genes had previously been shown to display a circadian rhythm of expression with opposite phase (LHCBM6 and JMJD6-like2), while the third gene is constitutively expressed (RACK1). Quantitative PCR was used to determine the amount of immunoprecipitated H3K4me3 over a circadian cycle. It was hypothesized that



H3K4me3 amount at the JMJD6-like2 and LHCBM6 promoter would show a circadian rhythm with a phase correlating directly with the phase of each gene's rhythm of expression. Conversely, the H3K4me3 amount at the RACK1 promoter was predicted to not show a circadian rhythm, as the gene is constitutively expressed. Instead, results showed that H3K4me3 amount exhibits a circadian rhythm with identical phase for all three genes. ANOVA confirmed that the rhythms were not significantly different between the three genes. General histone H3 amount at promoters did not show a circadian rhythm across any of the three genes. Since recent genome-wide studies in mouse liver revealed a circadian rhythm of H3K4me3 amount with identical phase at the promoter of many genes with diverse expression, the findings presented here suggest that *C. reinhardtii* might show a similar global regulation of rhythmic H3K4me3 as in mice and that, therefore, this feature has been preserved during eukaryotic evolution.

## CHAPTER I

### Introduction

#### Circadian Clock

Eukaryotes, and some prokaryotes, have internal clocks that sense the passing of time and regulate biochemical processes on a cycle of approximately 24 hours (Ishiura et al. 1998). There are three main elements of a basic circadian clock: an input pathway, a central pacemaker and an output pathway (Eskin 1979). Circadian clocks revolve around a central pacemaker that maintains an oscillation period of about 24 hours, even in the absence of environmental cues. The output pathway processes temporal data from the pacemaker and transmits them to biochemical and physiological processing stations in the organism where the data are manifested as circadian rhythms. The input pathway resets and entrains the central pacemaker to daily environmental cues like light or temperature changes, allowing the clock to remain in synchrony with the environment.

Circadian rhythms are processes that display an endogenous period of approximately 24 hours. Circadian rhythms can increase an organism's fitness by allowing the organism to anticipate the daily changes in its environment and to prepare for them. For example, plants coordinate the production of components necessary for photosynthesis before dawn in order to take advantage of the sunlight (Hennessey and Field 1991). Similarly, some organisms phase UV-sensitive cellular processes, like DNA synthesis, to occur during the night to reduce the occurrence of deleterious mutations (Johnson 2010). This finding led to the "escape from light" hypothesis, a hypothesis that helps explain the evolution of circadian clocks. The hypothesis postulates that in the harsh, early environment on earth, the advantage of phasing UV-sensitive cellular processes to the

nighttime allowed evasion of thymine dimers and DNA mutations, and thus increased fitness (Pittendrigh 1993). The molecular mechanism behind circadian clock function is based on clock genes forming interlocking transcription-translation feedback loops that maintain the nearly 24-hour period of a circadian rhythm. In general, the process begins when positive regulators induce transcription of clock genes. The protein products of these clock genes accumulate within a cell and, upon reaching a threshold concentration, can act as negative regulators by inhibiting the transcription of the genes affected by the positive regulators (Sahar and Sassone-Corsi 2012).

### The Histone Code

An epigenome consists of the multitude of chemical compounds that may result in heritable changes to the activity of the genome that are not caused by direct changes to the DNA sequence. The cells of a multicellular organism possess the same genomic DNA sequence, but through epigenetic regulation of gene expression the cells can exhibit varying phenotypes. An epigenome maintains stability within a cellular state while also being dynamic enough to respond to environmental and developmental cues through modification of the chromatin landscape. The known mechanisms involved in these changes are DNA methylation on CpG sites, post-translational histone modifications, and chromatin remodeling (Sahar and Sassone-Corsi 2012). Heritability and reversibility of these changes in gene activity vary depending on the type of epigenetic mechanism involved in their regulation.

In eukaryotic organisms, DNA is wrapped around histone proteins to compact, protect, and regulate expression of the DNA. This structure of DNA and its

organizational proteins is called chromatin. The histone code is a hypothesis stating that covalent histone modifications act as a language that can be read by other proteins, leading to regulation of DNA-templated processes like transcription and replication (Strahl and Allis 2000). Highly specific enzymes that add post-translational modifications to the N-terminal tails of histone proteins extend the information potential past the limits of DNA sequence alone. The addition or removal of histone modifications induces a change in affinities for chromatin-associated proteins as well as chromatin structure (Jenuwein and Allis 2001).

Like any code, the histone code requires “writers” and “readers” (Ruthenburg et al. 2007). There are a wide variety of writers and readers that contribute to the nearly endless potential for unique combinatorial phenotypes among cells containing identical DNA sequences. Writers of the histone code are the enzymes that are responsible for transferring a specific post-translational modification to an amino acid residue of a histone protein. For example, histone methyltransferases act as writers of the histone code by transferring methyl groups to lysine or arginine side chains. Readers are proteins that recognize and often bind to these post-translational modifications. An example of a reader is heterochromatin-associated protein-1 (HP1), a protein that contains a chromodomain that recognizes and binds to methylated lysine 9 residues on the N-terminal tails of histone H3 (Jacobs et al. 2001).

Alteration of chromatin structure is one method of transcription regulation. When chromatin is more compact, the genes present on the DNA in that region are less likely to be transcribed due to physical inability of transcription factors to bind to the DNA. Rhythmic alteration of chromatin state can be accomplished by regulation of the enzymes

that add or remove modifying groups to histone proteins. Specific post-translational modifications are associated with either active or inactive transcription. For example, the addition of three methyl groups to histone H3 at the lysine 4 position (H3K4me3) is associated with active transcription of the associated DNA in *Arabidopsis thaliana* and mice liver tissue (Santos-Rosa et al. 2002, Fischle et al. 2003, Malapeira et al. 2012). Similarly, mono-methylated histone H3 at lysine 4 is associated with silenced euchromatin and inactive transcription of associated DNA in *Chlamydomonas reinhardtii* (van Dijk et al. 2005) and other organisms. Chromatin remodeling is a central factor in the transcription-translation feedback loops that assists in keeping plasticity in circadian clocks (Aguilar-Arnal and Sassone-Corsi 2013, Sahar and Sassone-Corsi 2012).

### *Chlamydomonas reinhardtii*

*Chlamydomonas reinhardtii* is a eukaryotic, unicellular green alga. It has been utilized as a model organism for studies on the subjects of cilia and flagella assembly, motility, metabolism and chloroplast assembly (Harris 1989). *C. reinhardtii* has two anterior flagella, which are similar to flagella and cilia of other eukaryotes including animals, and a chloroplast similar to the chloroplast of plants. The 1.21 Mb nuclear genome sequence was published in 2007 (Merchant et al. 2007), and the mitochondrial and chloroplast genomes have also been sequenced (Vahrenholz et al. 1985, Maul et al. 2002). Furthermore, there is an abundance of protocols specifically optimized for use with *C. reinhardtii*.

*C. reinhardtii* is a classic model organism for studies on circadian clocks. Forty-five years ago *C. reinhardtii* was shown to exhibit a circadian rhythm of phototaxis, or

directional movement in response to a light source (Bruce 1970). Maximum cell accumulation in a light beam occurred during subjective day, i.e. the portion of the endogenous circadian cycle that corresponds to the light phase during constant conditions. The rhythm was even shown to exist in constant conditions in outer space without time cues (Mergenhagen and Mergenhagen 1987). Since then, circadian rhythms of chemotaxis, cell division, UV sensitivity, adherence to glass and expression for many genes have been characterized in this species of green algae (Mittag et. al 2005). Recording circadian rhythms of phototaxis has even been automated to allow for easy measurement of period and phase changes as a result of experimental manipulation (Gaskill et al. 2010). *C. reinhardtii* is an exceptional eukaryotic model organism because not only does it share the highly conserved chromatin structures that plants and animals possess, but it is also very simple and inexpensive to maintain in the lab.

### Question and Hypothesis

The aim of this project was to determine if the amount of histone H3 lysine 4 trimethylation (H3K4me3), a post-translational modification associated with active gene expression, shows a circadian rhythm at rhythmically expressed genes in *C. reinhardtii*. To test this, two *C. reinhardtii* genes that are expressed with a circadian rhythm, but with opposite phases, were chosen along with a constitutively expressed control gene. The two rhythmically expressed genes were JMJD6-like2 and LHCBM6. In mammals, JMJD6, which contains a JmjC domain, acts as a histone arginine demethylase (Chang et al. 2007). LHCBM6 (formerly known as CABII-1 or LHCB1) functions in *C. reinhardtii* as a component of the light-harvesting complex of photosystem II in photosynthesis

(Jacobshagen et al. 1996). Peak expression of JMJD6-like2 amount, at the level of mRNA in *C. reinhardtii*, occurs during the middle of subjective night (S. Jacobshagen, unpublished), while peak expression of LHCBM6 occurs during the middle of subjective day (Jacobshagen and Johnson 1994). RACK1, which stands for receptor of activated protein kinase C 1, is a constitutively expressed gene (Matters and Beale 1995) that was used as a control. It was expected that the amount of H3K4me3 would show a circadian rhythm at genes that are expressed with a circadian rhythm, with the peak occurring at about the same time as the peak of gene expression. Further, it was expected that H3K4me3 amount would not show a circadian rhythm in the non-rhythmically expressed RACK1 gene. The hypothesis is based on similar findings in mouse liver tissue, where the rhythmically expressed albumin D element-binding protein gene (*dbp*) was shown to exhibit a rhythm of H3K4me3 at its promoter under diurnal 12 h light/12 h dark cycle conditions (Ripperger and Schibler 2006). The peak of the H3K4me3 rhythm occurred during the middle of the day, just about 2 hours later than the peak amount of the mRNA rhythm. The hypothesis is also based on similar findings in the model plant *Arabidopsis*, where H3K4me3 at the promoter of all three rhythmically-expressed clock genes CCA1, LHY and TOC1 shows a circadian rhythm under constant light (Song and Noh 2012) but with different phases. The mRNA amount rhythms for CCA2 and LHY, as well as the H3K4me3 rhythms at their promoters, show their peak amount at about subjective dawn, whereas for TOC1 the mRNA amount and H3K4me3 rhythms show their peaks during early subjective night.

## CHAPTER II

### Materials and Methods

#### *Chlamydomonas reinhardtii* Strains and Growth Conditions

The strain of *Chlamydomonas reinhardtii* utilized in this study was CC-125, a wild-type strain that was obtained from the *Chlamydomonas* Resource Center ([www.chlamycollection.org](http://www.chlamycollection.org)). Stock cultures were maintained under 12-hour light/12-hour dark cycles on YA agar slants (Harris 1989). Working cultures were inoculated from slants and grown autotrophically in 50 ml of 0.3 HSM (Harris 1989) in 125 ml Erlenmeyer flasks on an orbital shaker (Innova 2100, New Brunswick Scientific) at 150 rpm under 12-hour light/12-hour dark cycles. For probe sonication optimization, one liter *C. reinhardtii* liquid cultures were directly inoculated from slants and grown in 0.3 HSM on an orbital shaker at 150 rpm under 12-hour light/12-hour dark cycles to  $\sim 1.8 \times 10^6$  cells/ml. For Covaris ultrasonicator optimization, *C. reinhardtii* liquid culture was grown similarly but to  $1.3 \times 10^6$  cells/ml and then 20 ml aliquots were harvested. Final samples were 500  $\mu$ l in volume, containing  $\sim 2.6 \times 10^7$  cells.

For circadian samples, 1 L of 0.3 HSM was inoculated at 10,000 cells/ml from a working culture and placed in an incubator with constant bright light from two sides provided by four 24 inch, 20 W plant and aquarium fluorescent light bulbs ( $\sim 80 \mu\text{mol photons m}^{-2} \text{ sec}^{-1}$  total) at 19°C. All cultures in the incubator were bubbled with air from an aquarium pump that was attached to a sterile filter followed by a 1 ml glass pipette, which in turn was inserted into the top of each 1 L glass bottle containing the culture. When the cultures reached  $1.3\text{-}2 \times 10^6$  cells/ml (late logarithmic phase), the glass bottle was wrapped in aluminum foil to grow in darkness for 12 hours. During these 12 hours,



the 2 incubator lamps were wrapped in filter paper to reduce the light intensity from  $\sim 80 \mu\text{mol photons m}^{-2} \text{ sec}^{-1}$  to  $\sim 10 \mu\text{mol photons m}^{-2} \text{ sec}^{-1}$  total. Exactly 12 hours after being wrapped in aluminum foil, the foil was removed and the cultures released into the constant dim light conditions. Aliquots of  $2.6 \times 10^7$  cells were harvested at regular intervals and placed in 50 ml plastic Falcon tubes, at which point the ChIP procedure began. Four samples were collected during each of the nine harvest times that were spaced at three-hour intervals starting at 12 hours into the constant dim light conditions.

During the first circadian experiment, there was a slight change in procedure. The filter paper was wrapped around the glass bottle containing *C. reinhardtii* culture instead of the lamps to achieve dim light ( $10 \mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ ). By changing to directly wrapping the lamps in filter paper during the second and third circadian experiment, the potential for light contamination during harvest was greatly lowered during the later procedure.

Cell concentrations were estimated using a Hy-Lyte Ultra Plane hemocytometer (1/10 mm deep) and microscope to regularly count cells (Harris 1989). One milliliter aliquots were collected from liquid cultures and 13.5  $\mu\text{l}$  of 0.25% iodine in ethanol was added to stop the movement of the cells for ease of counting. To the hemocytometer, 13.5  $\mu\text{l}$  of the iodine-fixed culture was added to each side for counting. Cells in each viewing field of 1 sq. mm were counted, multiplied by  $1 \times 10^4$  and averaged to determine the current estimated cell concentration (cells/mL).

## Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described for *C. reinhardtii* (Strenkert et al. 2011) but with some modifications. Four aliquots of the *C. reinhardtii* culture containing  $\sim 2.8 \times 10^7$  cells were collected per time point using an electronic pipette pump and transferred to 50 ml Falcon tubes (Corning, 21008-951). A volume of 0.3 HSM needed to raise the total volume to exactly 20 ml was also added to each tube. Before addition of culture, 37% formaldehyde was placed in each tube for immediate crosslinking at a final formaldehyde concentration of 0.35%. Upon addition of *C. reinhardtii* culture to the tubes containing formaldehyde, tubes were placed on a Daigger Mini Shaker to incubate for 10 minutes at a setting of 525 rpm and then 1 ml of 2.5 M glycine was added to each sample for quenching. Tubes were returned to the shaker for an additional 5 minutes of shaking at 525 rpm. Next samples were centrifuged at 3220 x g for 2 minutes at 4°C. Supernatants were carefully removed and disposed. Pellets were resuspended in 1 mL KH buffer (20 mM Hepes-KOH, pH 7.6, 80 mM KCl), and transferred to 2 ml microfuge tubes. Samples were then centrifuged in a microcentrifuge for 2 minutes at 17000 x g and 4°C. Supernatants were disposed and pellets were resuspended in 500 µl lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) with 2.5 µl protease inhibitor cocktail (Sigma P9599) added just before use. Samples were frozen at -80°C and thawed just before sonication. Samples were sonicated using the Covaris S220 ultrasonicator in a volume of 130 µl for 24 minutes, if not otherwise stated.

One 130 µl aliquot of Covaris-sonicated *C. reinhardtii* per time point was combined in a 2.5 ml screw-top microfuge tube with 120 µl lysis buffer containing 0.6 µl protease inhibitor cocktail added just before use. To each of these tubes, 250 µl ChIP

buffer (1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.0) was added. Samples were centrifuged for 20 sec at 17000 x g and 4°C. Supernatants were transferred to 2.5 ml screw-top microfuge tubes containing prepared antibody solutions for the first of the two sequential immunoprecipitations. Antibody solutions consisted of either 15 µl anti-H3 antibody (Abcam 1791, 1 mg/ml), 5 µl anti-cryptochrome antibody solution (Reisdorph and Small 2004) or 5 µl of 1X PBS control solution without antibody, each mixed with 10 µl BSA (Sigma A9418, 10 mg/ml) in lambda DNA<sub>soni</sub> solution. Lambda DNA<sub>soni</sub> solution was prepared by mixing 185 µl lambda DNA (Promega D1501, 539 µg/ml) with 815 µl ChIP buffer and probe sonicating it at 50% amplitude, 4 times with 10 sec on/off pulse using a Sonics and Materials Incorporated VCX130 ultrasonic processor and a 3 mm standard probe.

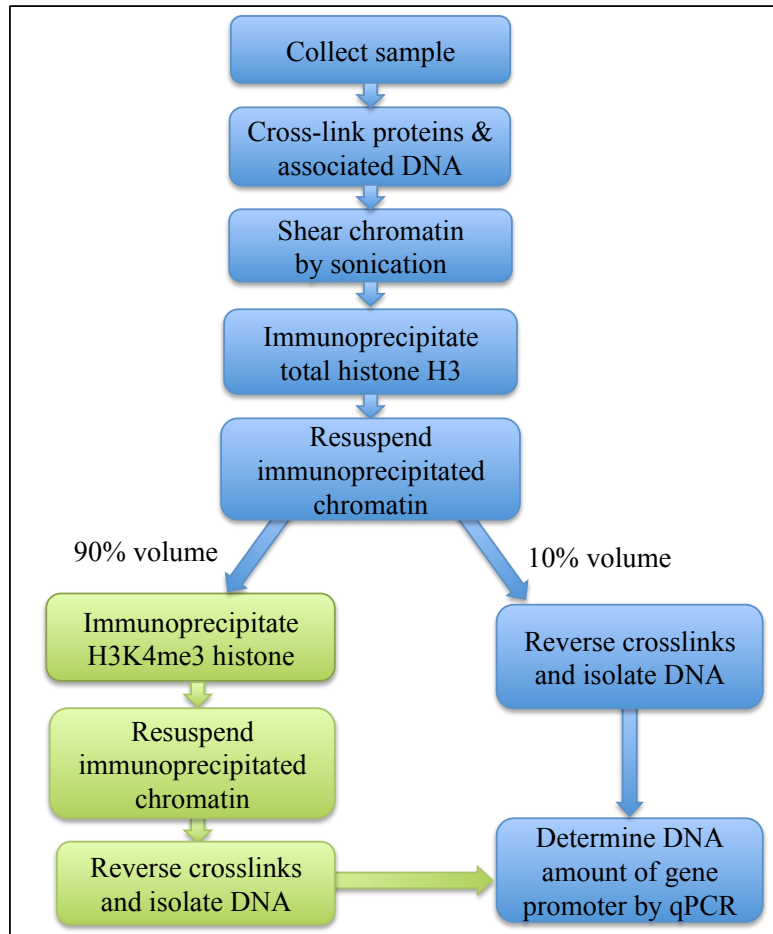
After addition of the antibody, samples were placed on a rotation wheel for 1 hour at 4°C. Samples were then centrifuged for 20 sec at 17,000 x g and 4°C and the supernatants carefully transferred to 2 ml microfuge tubes containing 120 µl protein A sepharose bead solution. Protein A sepharose bead solution was prepared by adding 50 mg protein A sepharose (Sigma P3391) to 1 ml ChIP buffer, then incubating the mixture at 4°C for 30 minutes. After that, beads were centrifuged at 1000 x g for 3 minutes then washed twice with 500 µl ChIP buffer. Beads were then resuspended in 250 µl ChIP buffer and 250 µl lambda DNA<sub>soni</sub> solution.

Tubes containing the samples and sepharose bead solution were placed on a rotation wheel and slowly rotated overnight at 4°C. Next, tubes were centrifuged for 3 min at 1000 x g and 4°C and supernatants were discarded. The loose pellets of beads were washed once with 1 ml Washing buffer 1 (150 mM NaCl, 0.1% SDS, 1% Triton X-

100, 2 mM EDTA pH 8.0), then 1 ml Washing buffer 2 (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0), then 1 ml Washing buffer 3 (250 mM LiCl, 1% Nonidet P40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) (Strenkert et al. 2011), and twice with 1 ml TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Then, 250  $\mu$ l Elution buffer (0.1 M NaHCO<sub>3</sub>, 1% SDS) was freshly prepared and added to each sample and incubated for 15 minutes at 65°C. Samples were centrifuged for 3 minutes at 1000 x g and 21°C and the supernatant was transferred to a fresh tube. Elution was repeated and the two supernatants were combined for each sample. Fifty microliters of each 500  $\mu$ l sample were transferred to a fresh tube and stored at -80°C. These 50  $\mu$ l aliquots constituted the first of the sequential precipitation, the anti-H3 precipitated samples, the first of the anti-cryptochrome samples or the first of the no-antibody samples. The remaining 450  $\mu$ l samples were immunoprecipitated again in exactly the same way but with 10  $\mu$ l anti-H3K4me3 antibody (Abcam 8580, 0.9 mg/ml), 5  $\mu$ l anti-cryptochrome antibody or 5  $\mu$ l no-antibody control solution mixed with 10  $\mu$ l BSA in lambda DNA<sub>soni</sub> solution and 120  $\mu$ l sepharose A beads. The steps of the ChIP procedure are illustrated in Figure 1.

### *Controls*

Two controls were chosen for the ChIP experiments: a no-antibody control (1X PBS) and an anti-cryptochrome antibody control (Reisdorph and Small 2004) that was kindly provided by Garry Small (North Dakota University at Vermillion). The particular *C. reinhardtii* cryptochrome that served as antigen for the anti-cryptochrome antibody is called CPH1 (Small et al. 1995) and belongs to the plant cryptochrome family. In the first



**Figure 1: Flow chart for the sequential ChIP procedure.** A sample of *Chlamydomonas reinhardtii* culture was collected, cross-linked with formaldehyde, cells lysed and chromatin sheared by sonication, and the first immunoprecipitation performed with anti-histone H3 antibody. After resuspension of the precipitated chromatin, 10% was removed, cross-links reversed and DNA isolated. The remaining 90% was used as the input for the second immunoprecipitation with anti-H3K4me3 antibody. Isolated DNA from both of the sequential precipitations was quantified for particular genomic regions using qPCR. Control samples were immunoprecipitated with an anti-cryptochrome antibody twice in a row using the same sequential ChIP procedure. Additional control samples were subjected to the sequential ChIP procedure but with no antibody.

of the two sequential precipitations, a sample from every other harvest time point (12, 18, 24, 30, and 36 h into constant dim light) was harvested and immunoprecipitated with the no-antibody control solution, while a sample from every time point was harvested and immunoprecipitated with the anti-cryptochrome antibody. In the second of the sequential precipitations, there was a sample from every other harvest time point (12, 18, 24, 30, and 36 h into constant dim light) that was immunoprecipitated again with the no-antibody control solution and a sample from every time point that was immunoprecipitated again with the anti-cryptochrome antibody.

#### *Reversion of crosslinks*

To the 50  $\mu$ l anti-H3 precipitated samples, 450  $\mu$ l elution buffer was added, bringing the volume of the anti-H3 precipitated samples to 500  $\mu$ l just like the anti-H3K4me3 precipitated samples. To reverse DNA-protein crosslinks, 50  $\mu$ l of 5 M NaCl was added to each sample and the samples were incubated overnight at 65°C. After incubation, proteins were removed from the samples by adding 10  $\mu$ l 0.5 M EDTA (pH 8.0), 20  $\mu$ l 1 M Tris-HCl (pH 8.0) and 2.1  $\mu$ l proteinase K (10 mg/ml) then incubated for 1 hour at 55°C.

#### *DNA extraction*

After incubating for one hour at 55°C, DNA was extracted once with 500  $\mu$ l phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) then extracted once with 500  $\mu$ l chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 50  $\mu$ l 3 M Na-acetate (pH 5.2), 2.5  $\mu$ l glycogen (Sigma G1767, 2.5  $\mu$ g/ $\mu$ l) and 1 ml 100% EtOH, then

incubated for 3 hours at -20°C. Next, samples were centrifuged for 20 minutes at 17,000 x g and 4°C. Pellet was washed with 500 µl 70% EtOH (stored on ice), then centrifuged again at 17,000 x g and 4°C for 15 minutes. Pellets were air dried and resuspended in 20 µl TE buffer (pH 8.0). The 20 µl samples were mixed well, then 10 µl of each sample was removed and stored at -80°C for potential future sequencing. The remaining 10 µl was mixed with 70 µl TE buffer (pH 8.0) and stored at -80°C for qPCR.

### Shearing of Chromatin

*C. reinhardtii* cells were sonicated to lyse the cell walls and to shear the crosslinked chromatin. Sonication time, intensity and sample volume were varied to result in DNA fragments averaging 200 bp.

### *Probe sonication*

Initially, probe sonication with a Sonics Vibra Cell sonicator (VCX130) was optimized as the primary source of sonication. Ultimately, probe sonication was not used for any circadian samples. Probe sonication was used for optimization of the chromatin immunoprecipitation method. Probe sonication was performed with a Sonics Vibra Cell sonicator (VCX130) in the WKU Biotechnology Center. Cells were sonicated in an ice bath in volumes of 500 µl at 60% amplitude, 40 times with 20 second on/off pulse, unless otherwise stated. *C. reinhardtii* cells were sonicated after they had been harvested, crosslinked with formaldehyde, quenched with glycine, washed with KH buffer, resuspended in 500 µl lysis buffer and frozen at -80°C as described before.

### *Covaris Adaptive Focused Acoustics (AFA) ultrasonication*

The University of Louisville Genomics Core Facility generously allowed access to their Covaris S220 ultrasonicator for comparison between the Covaris and probe sonication. For each sonication sample, 130  $\mu$ l of thawed, crosslinked culture was added to 130  $\mu$ l microtubes with fibers (microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm, Covaris 520045). Covaris TruChIP yeast chromatin shearing protocol for low cell concentration for 130  $\mu$ l volume was followed, with time of ultrasonication exposure being varied for optimization. Duty cycle was set to 2%, intensity was set to 3, peak incident power was set to 105 watts, and water bath was adjusted to 4°C.

### Extraction of *C. reinhardtii* Genomic DNA

Genomic DNA was used to create standard curves for analysis and quantification of qPCR results. To extract genomic DNA, cells were grown to  $6.85 \times 10^6$  cells/ml in 200 ml TAP medium (Harris 1989). To each of two 250 ml screw cap centrifuge bottles, 95 ml of culture was added and centrifuged for 5 min at  $3800 \times g$  and 21°C in a Sorvall RC-5C+ centrifuge. Supernatants were discarded, aside from approximately 10 ml, which was retained in order to resuspend the cells. Cells were resuspended using a 10 ml serological pipette and transferred to 30 ml Corex tubes. Cells were centrifuged for 5 minutes at  $12,000 \times g$  at room temperature. Supernatant was discarded, and pellet was resuspended in 10 ml CTAB Lysis buffer (2% hexadecyltrimethylammonium bromide (Sigma, H9151), 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl). Tubes were incubated with shaking for 1 h at 65°C in a water bath. Next, 10 ml



phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) was added to each tube and tubes were shaken well but not too vigorously under a chemical hood. Samples were incubated for another 10 min at 65°C, shaken again under a chemical hood, then centrifuged for 15 min at 12,000 x g, and 16°C.

The upper aqueous phase was transferred to a fresh tube using a 1 ml micropipette and disposable tips that had been shortened by about 0.5 cm to prevent shearing of the genomic DNA. Ten ml chloroform/phenol/isoamyl alcohol (25:24:1, pH 8.0) was added to each tube and tubes were carefully shaken to mix. Tubes were centrifuged for 15 min at 12000 x g at 16°C. The upper phase was transferred to fresh 15 ml Corex tubes. Isopropanol was added at 0.6 times the upper phase volume, and the tubes were shaken to mix. Mixtures were incubated on ice for 30 minutes, then centrifuged for 15 min at 12,000 x g, and 4°C. Supernatants were discarded and pellets were air dried. Pellets were resuspended with shortened 1 ml tip in 600 µl TE (pH 8.0) and transferred to 1.5 ml microfuge tubes. Next, 10 µl of 10 mg/ml RNase A (ThermoFisher EN0531), 60 µl 3M Na-acetate (pH 5.3) and 1 ml ethanol was added to each tube. Samples were incubated on ice for 30 minutes, then spun for 10 min at 16000 x g and 4°C. Supernatant was discarded and pellets air dried, then resuspended in 200 µl TE and RNase A solution (600 µl TE buffer pH 8.0, 1 µl 10 mg/ml RNase A). Samples were stored at -80°C.

### Characterization of Isolated DNA

#### *Gel electrophoresis*

To determine fragment sizes, isolated DNA after sonication was separated on a 1.5% agarose gel of 10 cm length (Fisher Biotech Mini-Horizontal Electrophoresis

System, Fisher Scientific) with TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA). The gel contained 0.5 µg/mL ethidium bromide. A 100 bp DNA ladder (NEB N3231) was loaded into one well on each gel. Gels were run for 60 minutes at 100 V. Gel results were imaged under UV light using a digital imaging system (FluorChem HD2 from Alpha Innotech) in the WKU Biotechnology Center. Gel electrophoresis of genomic DNA was performed in the same way except that a 0.7% agarose gel was used and two ladders were added to the gel, 0.5 µg lambda DNA cut with HindIII and 10 µl 1 kb ladder (NEB N3232).

#### *Capillary electrophoresis*

To test the efficacy of sonication with higher sensitivity and resolution, DNA was also separated using the Agilent 2100 Bioanalyzer with High Sensitivity DNA kit (5067-4626) according to the manufacturer's instruction. The sensitivity of the instrument was too low for analysis of DNA samples after ChIP. All tests were therefore performed with DNA isolated directly after sonication.

#### *Spectrophotometric measurements*

For optimization of crosslinking by formaldehyde, 1 µl of each sample after ChIP was applied to a Nanodrop 2000 spectrophotometer (Thermo Scientific) and DNA concentrations determined by their absorbance at 260 nm. Sample concentrations were then standardized to account for the sequential precipitation and therefore differing input between H3 and H3K4me3 samples.

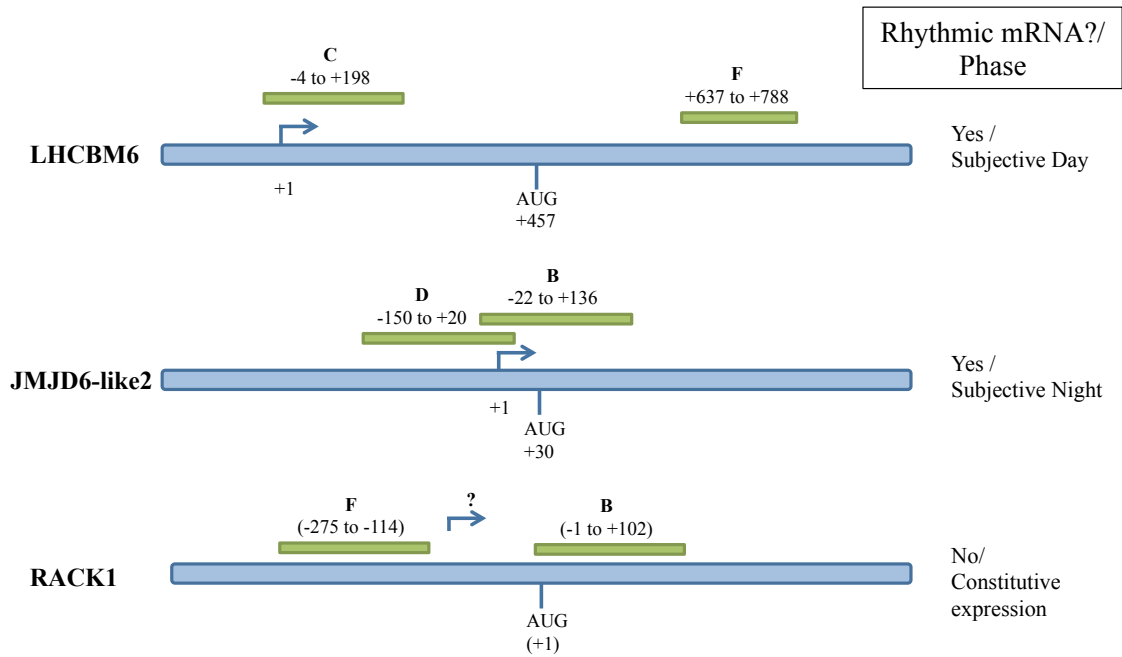
## *Quantitative PCR*

*C. reinhardtii* gene sequences were obtained from the NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/nucleotide>). Primers were designed using Primer3Plus ([http://biotools.umassmed.edu/bioapps/primer3\\_www.cgi](http://biotools.umassmed.edu/bioapps/primer3_www.cgi)) with default settings, and ordered from Integrated DNA Technologies. Primers were designed for each of the three genes investigated in this study: RACK1 – Receptor of Activated Kinase C 1 (Gene ID: 5723548, Protein ID: XP\_001698065), JMJD6-like 2 – Jumonji domain-containing protein 6-like2 (Gene ID: 5716618, Protein ID: XP\_001690890), and LHCBM6 – Chlorophyll a-b binding protein of Light Harvesting Complex II type I (Gene ID: 5720993, Protein ID: XP\_001695353). Multiple primer pairs were designed to produce amplicons near the transcription start site of each gene. The primers were tested with anti-H3 and anti-H3K4me3 chromatin immunoprecipitated DNA. Two primer pairs that yielded a detectable amplicon after quantitative PCR (qPCR) were selected for each gene (Table 1). Two primer pairs were chosen per gene to target different positions near the promoter region of each gene. Each amplicon's specific location is illustrated in Figure 2.

After ChIP, qPCR reactions were carried out in 96 well optical reaction plates (Applied Biosystems 4306737). Each 15  $\mu$ l reaction contained 7.5  $\mu$ l 2X Power SYBR Green PCR Master Mix (Applied Biosystems 4367659), 5.5  $\mu$ l of a ChIPped DNA sample and 2  $\mu$ l of a primer pair master mix containing 600 nM forward and 600 nM reverse primer. Optical reaction plates were sealed with optical film, gently vortexed on low speed to mix and then centrifuged briefly. A 7300 Real Time PCR System (Applied Biosystems, ver. 1.4) was used to run amplification reactions and dissociation curves. Amplification reactions were designed to run for 10 minutes at 95°C, followed by 45

<b>Gene-specific amplicon</b>	<b>Primer sequences</b>	<b>Amplicon size (bp)</b>	<b>Relative amplicon location</b>
JMJD6-B	F: TTATCCTTGTCGGGGCAGTT	159	Before and into 5' UTR, first exon and first intron
	R: AACCCAGACTTCCCAATCG		
JMJD6-D	F: TGTGAACACGTCGGCTTAAA	171	Before and into 5' UTR
	R: TGGTTCAGACTCGAGCACAG		
LHCBM6-C	F: TGTACTGTTTGGCGCTTTTG	203	5' UTR
	R: GTACTGGGGTTTTGGTCTCG		
LHCBM6-F	F: GCACATTTAAGCCGCGGG	152	Second exon
	R: CTTGGCAGCGGTCTTCTTG		
RACK1-B	F: AATGGCCGAGACTCTGACTC	103	First exon
	R: GGACAGGAGGGTGTGGAC		
RACK1-F	F: GCAATGCCATGAAGCTATGA	162	Before first exon
	R: GGCCCGCAGATAAAGATGTA		

**Table 1: Primer pairs for genes of interest.** Several primer pairs were designed and tested for each gene of interest, i.e. JMJD6-like2, LHCBM6 and RACK1. Two primer pairs with melting curves containing single peaks, indicating single amplicons, were chosen for each gene. Relative amplicon locations and sizes are noted for each primer pair.



**Figure 2: Location of amplicons on target genes.** The green bars show the relative locations and sizes of each of the two amplicons for each gene of interest. The time of peak expression is also listed next to each gene.

cycles of 15 seconds at 95°C and 1 minute at 60°C. Two technical replicates for each DNA sample from ChIP and for each dilution of genomic DNA were tested per reaction plate. Non-template controls were included as well.

### Standardization of ChIP Data

qPCR was used to quantify the amount of immunoprecipitated DNA fragments containing the targeted regions at each time point. To account for non-specific binding, the concentration of DNA precipitated with anti-cryptochrome antibody was subtracted from the concentration of DNA precipitated with anti-histone H3 antibody at the corresponding time point. Similarly, the concentration of DNA precipitated with anti-cryptochrome antibody during the second of the sequential precipitations was subtracted from the concentration of DNA precipitated with anti-H3K4me3 antibody at the corresponding time point. Standardization of immunoprecipitated DNA data was performed separately for each individual time series determined for a particular gene, primer pair and experiment. Standardization was executed in Mathematica, which took each precipitated DNA mass value in a 9 point time series, subtracted the mean of the series from it before dividing it by the standard deviation of the series: Standardize [list] in Mathematica "is effectively  $(\text{list} - \text{Mean}[\text{list}]) / \text{StandardDeviation}[\text{list}]$  for nonzero StandardDeviation [list]".

### Statistical Analysis

Statistical analysis of DNA mass associated with H3 and H3K4me3 was performed by multivariate ANOVA through the Mathematica statistics program. The

analyses investigated relationships between experiments, time points, genes and gene-specific amplicons.

Results from the protein A sepharose bead and antibody amount optimization experiments were analyzed by ANOVA through the R statistics program. Cell concentration data for the circadian experiments were analyzed by a two-tailed t test in Excel.

## CHAPTER III

### Results

#### Optimization of ChIP Procedure

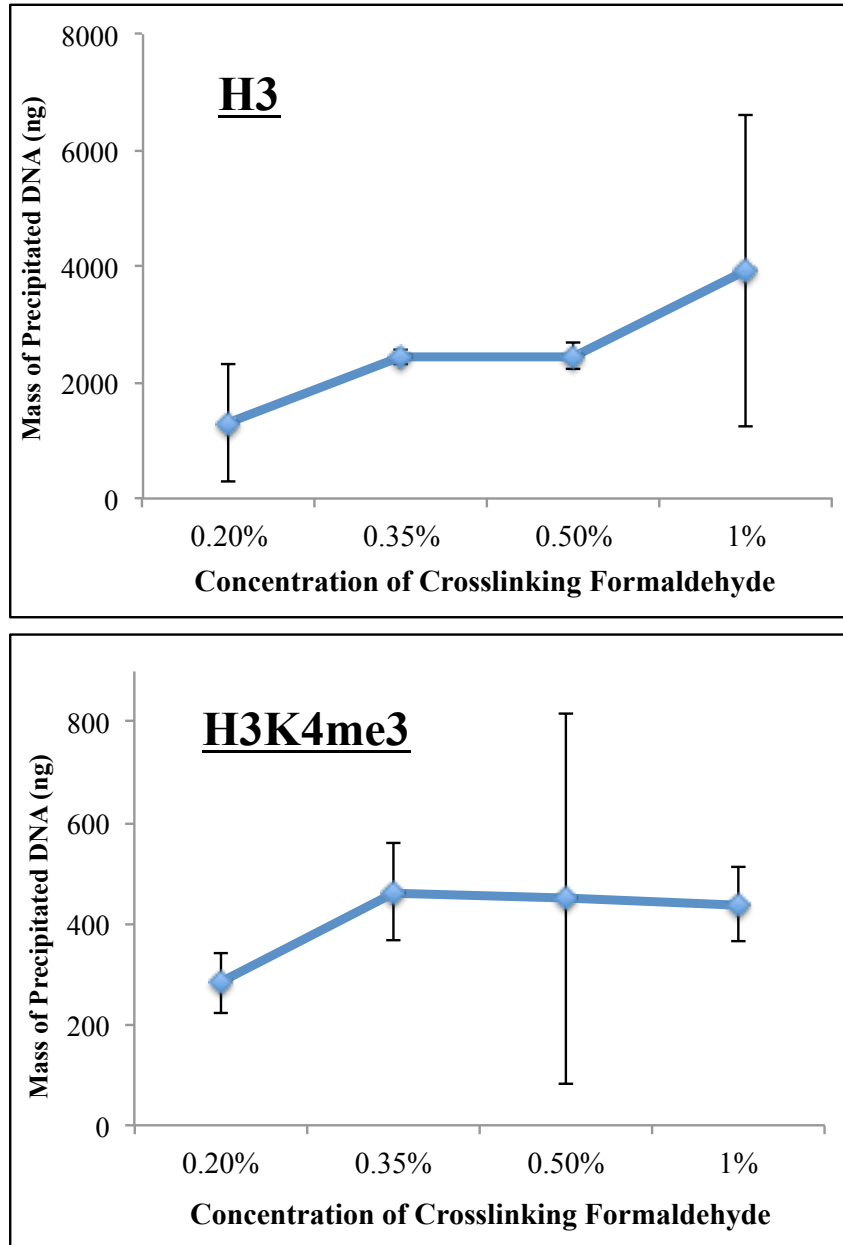
##### *Formaldehyde concentration for crosslinking*

A previous publication on the ChIP method with *C. reinhardtii* has suggested crosslinking with a final concentration of 0.35% formaldehyde (Strenkert et al. 2011). An experiment was performed to determine whether this was also the optimal concentration for crosslinking under the conditions used in this study. Crosslinks need to be strong but reversible. Four different concentrations of formaldehyde were tested: 0.2%, 0.35%, 0.5% and 1.0%. Two replicate samples from the same *C. reinhardtii* culture were subjected to a sequential ChIP after being crosslinked for each concentration of formaldehyde. Results for both the H3-precipitated samples and the H3K4me3-precipitated samples showed that crosslinking with 0.35% formaldehyde already yielded high amounts of precipitated DNA that did not increase further with higher formaldehyde concentrations (Fig. 3). Although the H3 samples that were fixed with 1.0% formaldehyde showed a much higher amount of DNA, the data point also showed a high variability as indicated by a large error bar and therefore was not considered reliable. For all subsequent ChIP experiments, 0.35% formaldehyde was used to crosslink samples.

##### *Shearing of chromatin by probe sonication*

A previous study utilizing *C. reinhardtii* suggested that chromatin of 200 bp was optimal for ChIP (Strenkert et al. 2011). Larger fragments result in reduced resolution while smaller fragments result in increased breaks in the region of the amplicon. Also,





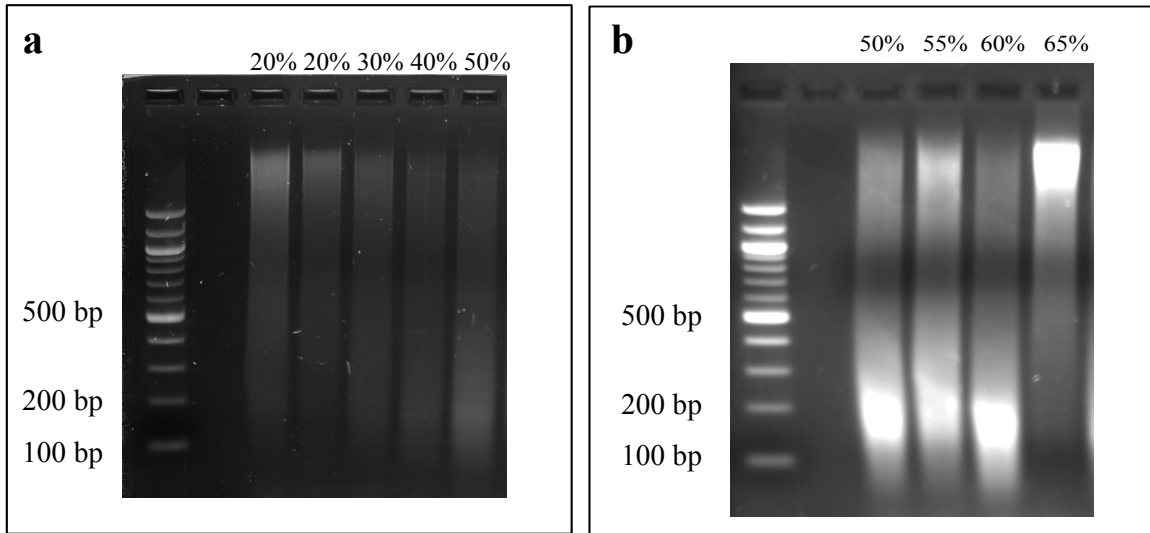
**Figure 3: Optimization of formaldehyde concentration for crosslinking.** Four concentrations of formaldehyde were tested and the amount of total DNA isolated with ChIP was determined using the absorbance at 260 nm. Results are depicted for ChIP with the antibody against total histone H3 (top panel) and the H3K4me3 modification (bottom panel). Error bars show one standard deviation across two technical replicates.

extended sonication may cause increased denaturation of the target protein. When probe sonication was tested, the amplitude was first varied between 20% and 50% to determine the setting that would produce the desired fragment length (Fig. 4a). Sonication at 50% amplitude produced DNA fragments closest to 200 bp, but the range of fragment sizes showed high variability.

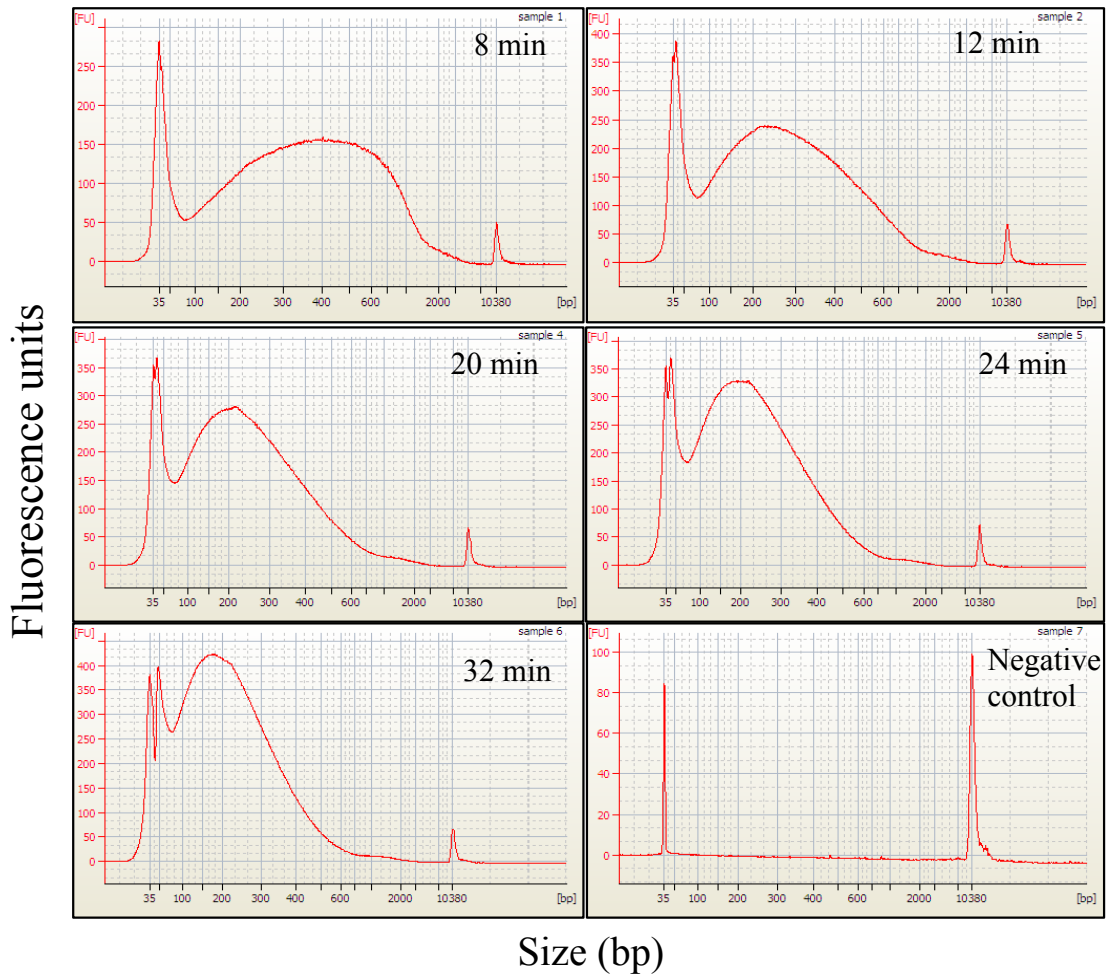
In a second optimization experiment, the amplitude was varied between 50% and 65% in 5% increments (Fig. 4b). Sonication at 65% was too harsh resulting in foaming of the sample and incomplete shearing of the chromatin. Sonication at 60% produced fragments around the desired 200 bp with the least fragments of other sizes. Therefore, an amplitude setting of 60% was used for all further samples that were sonicated with the probe sonicator. These were the samples for antibody and protein A bead optimization during ChIP (Fig. 6).

#### *Shearing of chromatin by Covaris sonication*

Crosslinked samples of *C. reinhardtii* were exposed to Covaris S220 sonication for 8, 12, 16, 20, 24 or 32 minutes. Crosslinks were reversed and DNA was isolated as previously described. DNA was separated using the Agilent 2100 Bioanalyzer with Agilent High Sensitivity DNA kit. Covaris sonication exposure for 24 minutes produced the greatest number of DNA fragments around 200 bp (Fig. 5). Samples sonicated for 8, 12 and 20 minutes produced a majority of DNA fragments larger than the target size of 200 bp. Sonication for 32 minutes resulted in a majority of DNA fragments smaller than 200 bp. Therefore, 24 minutes was chosen as the optimal duration for Covaris treatment.



**Figure 4: Separation of probe-sonicated DNA by electrophoresis.** *C. reinhardtii* cells were crosslinked, then sonicated using the Sonics Vibra Cell probe sonicator as described in Materials and Methods. All samples were sonicated 40 times for 20 seconds, with a 20 second “off” pulse between the “on” pulses. The amplitude was first varied from 20% to 50% (a). Sonication at 50% amplitude yielded fragments that were closest to 200 bp in length, but fragment sizes showed high variability. Samples were then sonicated from 50% to 65% amplitude (b), which showed that sonication with 60% amplitude produced fragments around 200 bp.



**Figure 5: Separation of DNA from Covaris-sonicated *C. reinhardtii*.** After sonication in the Covaris S220 for varying periods of time, DNA was extracted from the samples and separated using the Agilent 2100 Bioanalyzer with Agilent High Sensitivity DNA kit. Fluorescence units signify the relative amount of DNA. A negative control was included on the same Agilent chip that contained no DNA.

### *Immunoprecipitation*

An experiment was performed to determine optimal volumes of antibody and protein A beads during ChIP. DNA immunoprecipitated with various volumes of antibody and beads was quantified with qPCR using the RACK1B primer pair (Fig. 6). Tests were performed with two independent ChIP samples per condition and two technical qPCR replicates per sample. After the initial immunoprecipitation with anti-histone H3 antibody, qPCR showed that samples treated with 15  $\mu$ l antibody and 120  $\mu$ l protein A beads resulted in the lowest cycle time (Fig. 6, left panel). After a consecutive immunoprecipitation with anti-histone H3K4me3 antibody, qPCR showed that samples treated with 10  $\mu$ l antibody and 120  $\mu$ l beads resulted in the lowest cycle time (Fig. 6, right panel). Therefore, these optimal volumes for anti-histone H3 antibody and anti-H3K4me3 antibody, respectively, were used in further experiments.

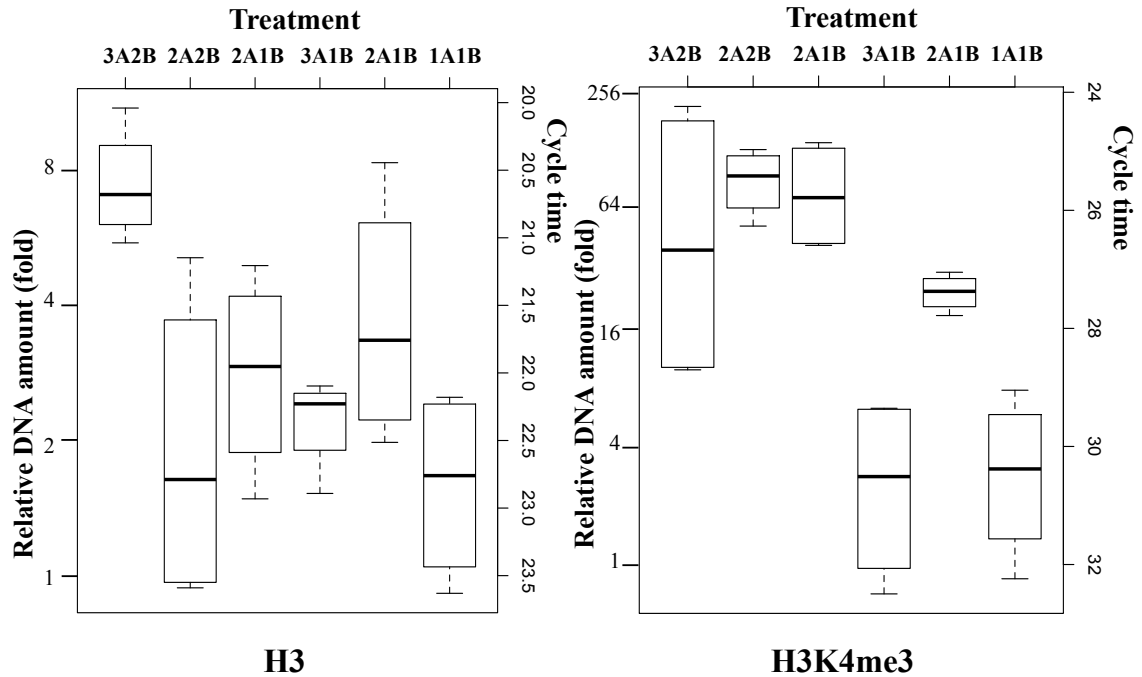
### *Genomic DNA*

*C. reinhardtii* genomic DNA was isolated for use as mass reference in qPCR. Characterization by gel electrophoresis (Fig. 7) indicated one single band, confirming that the genomic DNA was successfully isolated and not degraded during the isolation procedure.

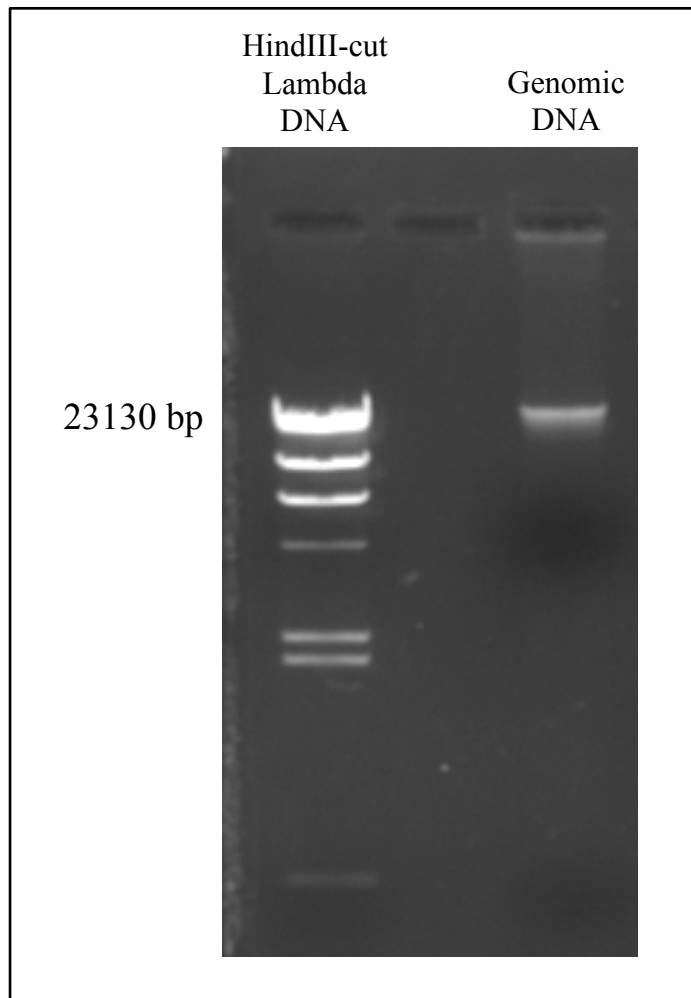
## Results of Circadian ChIP-qPCR Experiments

### *Cell concentration*

Cell concentration was assessed at three times throughout each of the three circadian experiments: 1) before the 12-hour dark pulse, 2) at the time of the first



**Figure 6: Cycle times of qPCR with ChIP samples designed for optimization of antibody and protein A bead volume.** Chromatin immunoprecipitation was performed with either 5  $\mu$ l (1A), 10  $\mu$ l (2A) or 15  $\mu$ l (3A) antibodies and either 60  $\mu$ l (1B) or 120  $\mu$ l (2B) of protein A beads. After ChIP, DNA was isolated and quantified using qPCR with the RACK1B primer pair. Average cycle time of two technical qPCR replicates for each of two independent ChIP samples is depicted for antibody against total histone H3 (left panel) and the H3K4me3 modification (right panel). Relative DNA amount is indicated on the left axis: With each cycle time decrease by an increment of one, the original DNA concentration in a sample is assumed to double. Bars indicate the minimum and maximum cycle times for each treatment. Boxes contain the middle 50% of the data points with the bold line representing the median cycle time.



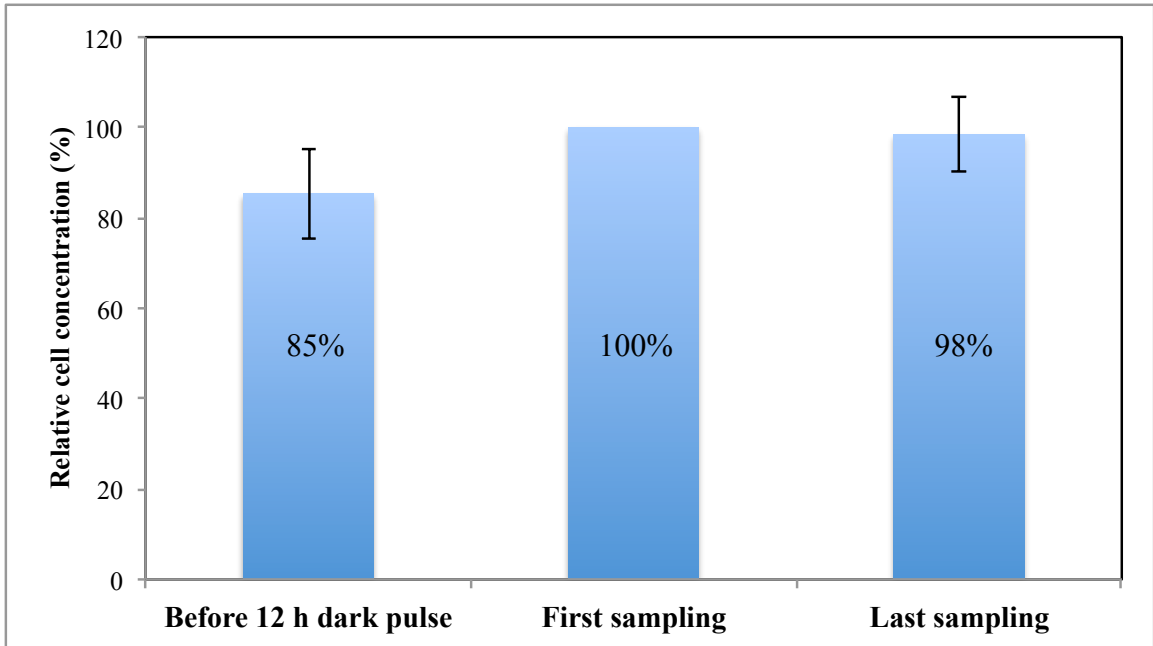
**Figure 7: Characterization of genomic DNA by electrophoresis.** Genomic DNA was isolated from *C. reinhardtii*, then run on a 0.7% agarose gel. A total mass of 1  $\mu\text{g}$  genomic DNA was loaded in the right lane of the gel as well as 0.5  $\mu\text{g}$  of HindIII-cut Lambda DNA in the left lane.

sampling, which took place 12 hours into the following constant dim light, and 3) at the time of the last sampling, which took place 36 hours into constant dim light. For each independent experiment, the concentration at the time of the first sampling was set to 100% and the other two measurements were expressed relative to that concentration. The relative concentrations of all three independent experiments were then averaged (Fig. 8). The relative average cell concentration before the 12-hour dark pulse was lower than at the time of the first sampling (85% versus 100%) indicating that there was some growth occurring before the first harvest time, although the increase in cell number was not statistically significant ( $p = 0.1240$  in a 2-tailed t test). There was no statistically significant growth between the first and last harvest time (100% versus 98%) ( $p = 0.0818$  in a 2-tailed t test).

### *Histone H3*

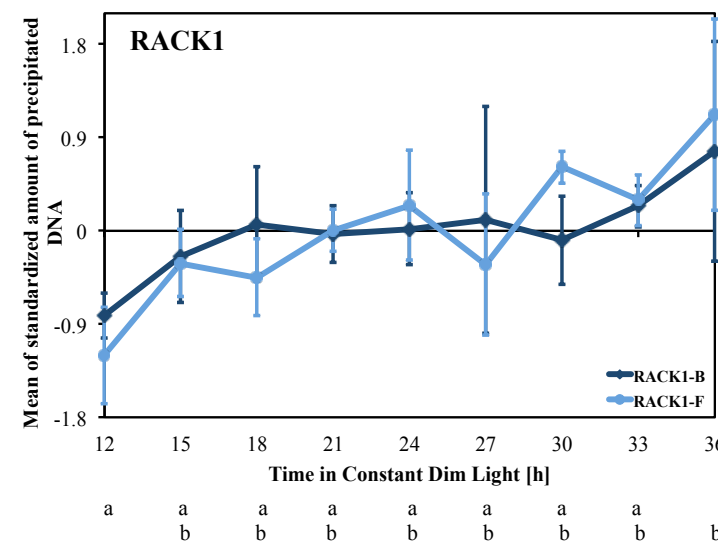
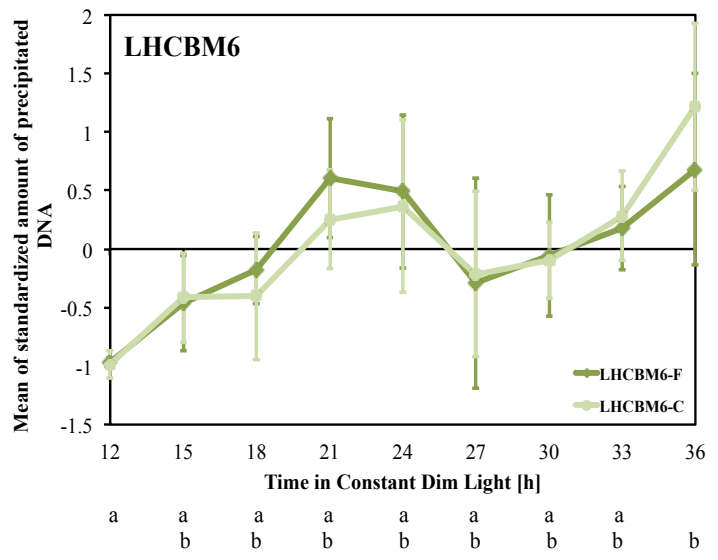
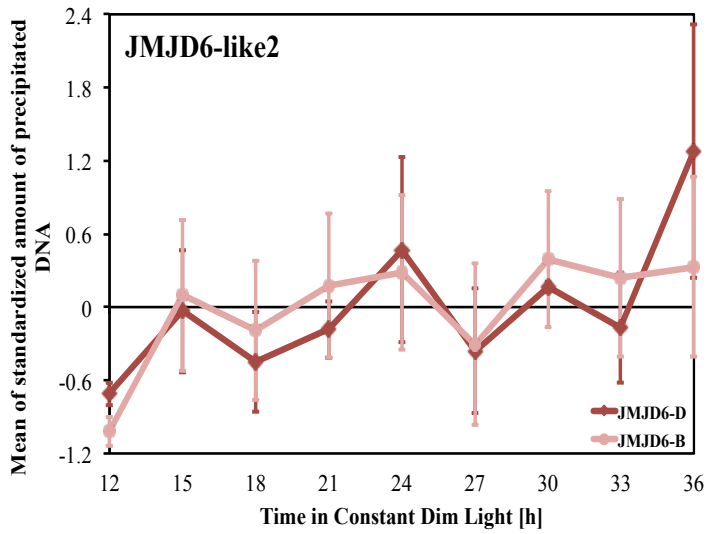
DNA mass that was precipitated with anti-H3 antibody was adjusted for DNA mass precipitated with the control anti-cryptochrome antibody, standardized, and then averaged for the three independent circadian experiments (Fig. 9). The pattern of this DNA mass over a circadian cycle suggests that total histone H3 does not show a circadian rhythm for any of the genes or amplicons. The pattern rather indicates a noisy and slightly increasing amount of total histone H3. ANOVA showed that there was no significant difference between the three independent experiments or between the two amplicons that were tested for each gene. ANOVA also showed that for JMJD6-like2 there was no significant difference between any of the time points. For both LHCBM6 and RACK1, the only significant difference occurred between hour 12 and hour 36, with





**Figure 8: Average cell concentration during circadian experiments.** Cell

concentration was measured at three time points during the circadian experiments: before the 12 h dark pulse, at the time of the first sampling (12 hours into constant dim light) and at the time of the last sampling (36 hours into constant dim light). The concentration at the time of the first sampling of each experiment was set to 100%. The “before 12 h dark pulse” and “last sampling” concentrations of each experiment were expressed relative to their respective concentration at the time of the first sampling, then averaged in the graph above. The error bars indicate one standard deviation of three independent experiments. A 2-tailed t test showed that there was no significant difference between the concentration before 12 h dark pulse and first sampling ( $p = 0.1240$ ) or between the concentration at the first sampling and the last sampling ( $p = 0.0818$ ).



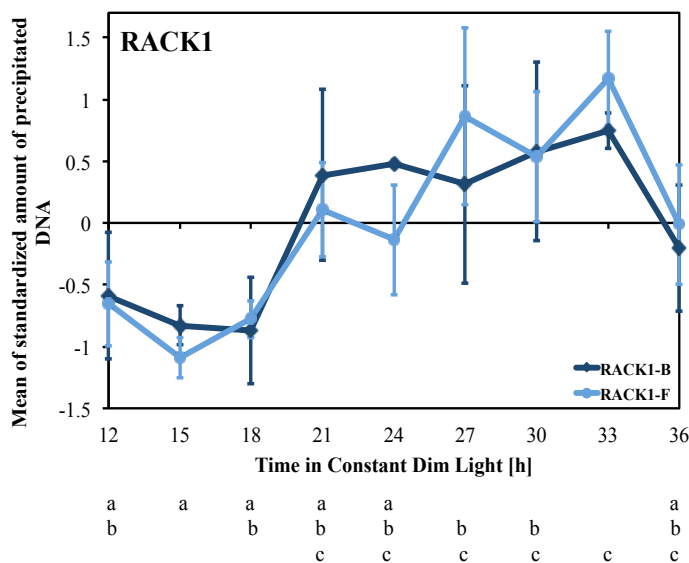
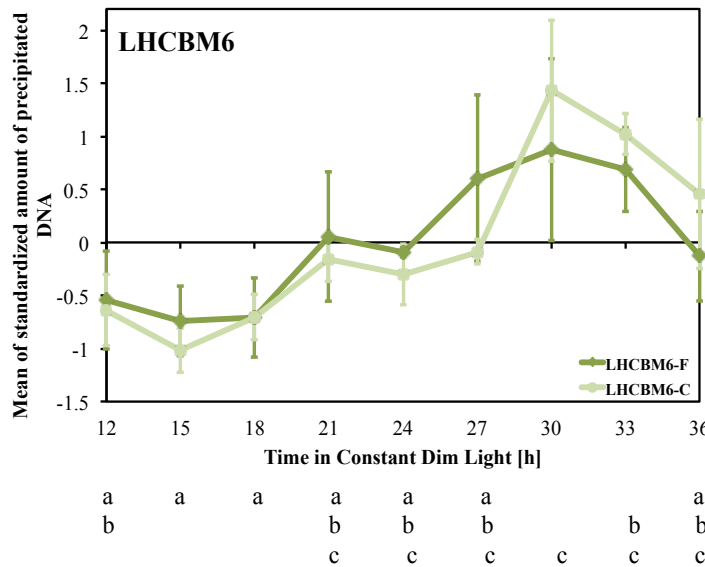
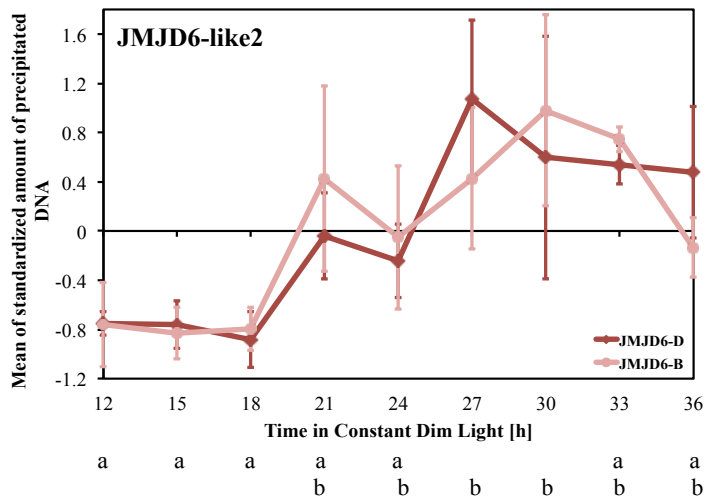
**Figure 9: Amounts of DNA precipitated with anti-H3 antibody over the course of a circadian cycle for each of the three genes investigated.** Mass of DNA precipitated with anti-H3 antibody at each time point was determined using qPCR for the three genes JMJD6-like2, LHCBM6 and RACK1. DNA mass was adjusted for DNA mass precipitated with the control anti-cryptochrome antibody and then standardized using the “standardize” function in Mathematica. Mean standardized DNA mass of the three independent circadian experiments is shown for each time point. Each graph depicts the result for one gene with a separate line for each of the two amplicons investigated. Beneath each graph, the result of an ANOVA is shown indicating the time points at which DNA amounts were significantly different. There were no significant differences between time points for the JMJD6-like2 gene. Error bars indicate one standard error of the mean for the three independent experiments.

the DNA mass at hour 36 being higher than at hour 12 (Fig. 9).

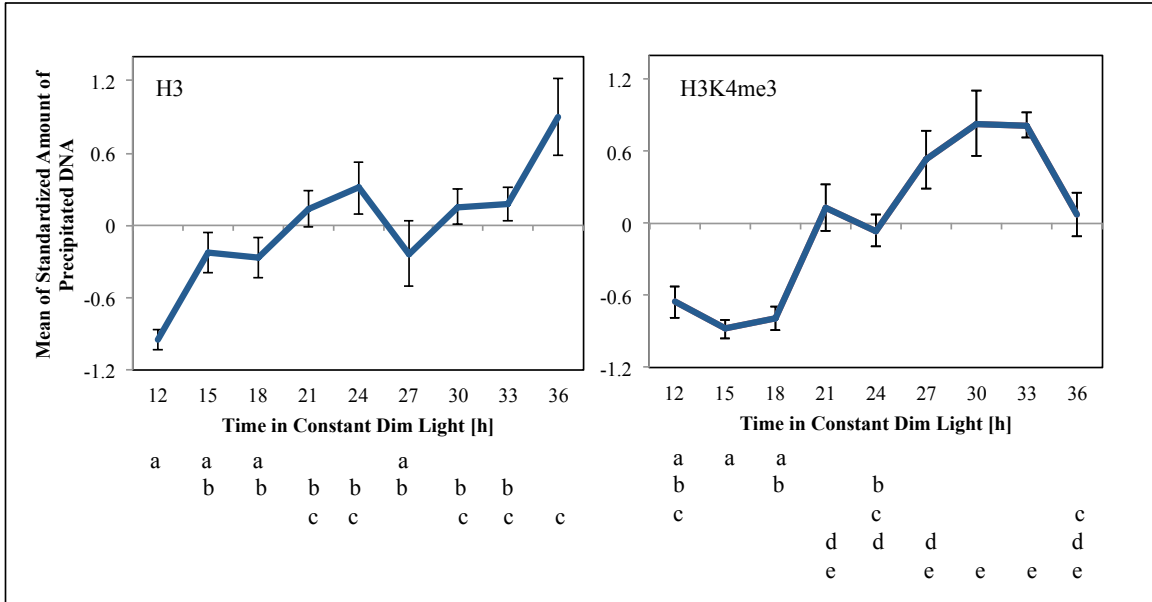
The anti-H3 antibody-precipitated DNA masses were then combined for all three target genes (Fig. 11). ANOVA showed that there was no significant difference between genes, amplicons or experiments. There were, however, significant differences between time points that were somewhat more extensive than when analyzing each gene individually. These differences suggest that total histone H3 does not show a circadian rhythm but rather a general increase over time.

#### *Histone H3K4me3 modification*

Chromatin from the anti-H3 antibody precipitation was subsequently precipitated with anti-H3K4me3 antibody. The DNA mass from this second precipitation was adjusted for DNA mass of the second precipitation with the control anti-cryptochrome antibody, standardized, and then averaged for the three independent circadian experiments (Fig. 10). The H3K4me3 DNA mass over a circadian cycle suggests quite a different pattern than that for total histone H3. Though noisy, it suggests a circadian pattern for H3K4me3 at each of the genes and amplicons. Furthermore, it suggests a circadian rhythm with the same phase for each gene and amplicon, where the peak occurs at 27 to 30 hours of dim light or in the middle of subjective day and the trough occurs at 15 to 18 hours of dim light or in the middle of subjective night. An ANOVA showed that there was no significant difference between the three independent experiments or between the two amplicons that were tested for each gene. The ANOVA also showed that there were significant differences between some of the time points for each of the three target genes. For the JMJD6-like2 gene, there were significant differences between



**Figure 10: Amounts of DNA precipitated with anti-H3K4me3 antibody over the course of a circadian cycle for each of the three genes investigated.** Mass of DNA precipitated with anti-H3K4me3 antibody from the anti-H3 antibody-precipitated chromatin in a sequential manner was determined at each time point using qPCR for the three genes JMJD6-like2, LHCBM6 and RACK1. DNA mass was adjusted for DNA mass precipitated with the control anti-cryptochrome antibody in a sequential manner and then standardized using the “standardize” function in Mathematica. Mean standardized DNA mass of the three independent circadian experiments is shown for each time point. Each graph depicts the result for one gene with a separate line for each of the two amplicons investigated. Beneath each graph, the result of an ANOVA is shown indicating the time points, at which DNA amounts were significantly different. Error bars indicate one standard error of the mean for the three independent experiments.



**Figure 11: Amount of precipitated DNA with anti-H3 antibody and anti-H3K4me3 antibody over a circadian cycle for all three genes combined.** The standardized mass of precipitated DNA as depicted in Fig. 9 and 10 are shown for each time point as the average for all experiments, all amplicons and all genes. Beneath each graph, the result of an ANOVA is shown indicating the time points at which DNA amounts were significantly different. Error bars show one standard error of the mean for the three independent experiments, the three genes and the two amplicons per gene (mean of 18 values per time point).

hours 12-18 (low amounts) and hours 27-30 (high amounts). For the LHCBM6 gene, the pattern of significant differences was somewhat more complex, with an additional group of similar time point values in the intermediate amount range that was distributed between the low and high ranges, i.e. for hours 12, 21-27 and 33-36. For the RACK1 gene, the pattern of significant differences was generally similar to that of LHCBM6 with a group of intermediate time point values as well and with broadly the same distribution.

When DNA masses that were precipitated with anti-H3K4me3 antibody were combined for all three target genes, the circadian pattern became even more pronounced (Fig. 11). Rather than showing a general increase as was seen for total histone H3, the pattern for H3K4me3 amount immediately decreases with a trough at around hour 15, then increases with a peak at around hour 30, before finally decreasing again at the end of the circadian cycle. The pattern of H3K4me3 amount for the investigated genes is in accordance with a circadian rhythm. ANOVA revealed that there was no significant difference between the three target genes, the experiments or the amplicons. The only kind of significant difference occurred between some of the time points with a pattern that is even more complex than for any of the genes individually.

#### *Additional no-antibody control for circadian ChIP data*

In addition to the anti-cryptochrome antibody that was used as control in the ChIP experiments (Fig. 9-11), a no-antibody control was also tested, although this control was collected for only 5 of the 9 time points. The mass of DNA that was precipitated with anti-H3 antibody and H3K4me3 antibody was standardized by subtracting the mass of DNA precipitated with the no-antibody control at each respective time point. There was

no significant difference between the results of subtracting DNA mass values obtained for the no-antibody control and of subtracting DNA mass values obtained for the anti-cryptochrome antibody control. The anti-cryptochrome antibody control was used for final statistical analysis because there was an anti-cryptochrome sample collected at every time point.

## CHAPTER IV

### Discussion

#### Same Circadian Rhythm of H3K4me3 in Diversely-Expressed Genes in *C. reinhardtii*

This study was designed to quantify the H3K4me3 histone modification at the promoter of three genes showing different expression patterns in *C. reinhardtii*. The amount of modification was determined in a sequential ChIP that involved precipitation of total histone H3 first. The experiments revealed that the amount of total histone H3, when tested under circadian conditions of constant dim light, does not show a circadian rhythm in any of the three promoters (Fig. 9 and 11). However, evidence suggests that the amount of H3K4me3 does show a circadian rhythm at each of the promoters (Fig. 10 and 11). The rhythms detectable at all three promoters show an identical phase with the peak amount of H3K4me3 occurring around 30 hours in constant dim light, which represents the middle of subjective day.

The results of the experiments reported here show that over a 24 hour period there is a general increase in the amount of total histone H3 at the three analyzed gene promoters (Fig. 9). Yet, in the first study to analyze histone modifications over the course of a day (Ripperger and Schibler 2006), a low amplitude rhythm in histone H3 occupancy within the *dbp* locus was detected in liver tissue of mice under diurnal 12-hour light/12-hour dark cycle conditions. As expected, the trough of this rhythm aligned with the time of highest *dbp* mRNA abundance. The rhythm in H3 occupancy was detectable but dampened in regions upstream and downstream from the *dbp* gene. In a more recent study, which was conducted under constant conditions instead of a 12-hour light/12-hour dark cycle, similar to the study reported here, H3 amount did not display a rhythm



(Valekunja et al. 2012). The study utilized liver tissue from mice that was harvested in constant dim red light after a 12-hour light/12-hour dim red light entrainment and a further 24-hour period of constant dim red light. Since the findings reported here also do not show a rhythm, collectively, the results suggest that a low amplitude rhythm in H3 is only detectable under diurnal conditions.

The general increase in the amount of total histone H3 at the promoters of all three genes over a circadian cycle was unexpected. Since the same culture volume was used for each time point within an experiment, it was originally assumed that the increase in H3 was related to an increase in cell number over time. However, the cell concentration remained constant from the time of the first sampling to the last sampling with no significant difference between the two sampling times (100% versus 98.4%,  $p=0.0818$ , see Fig. 8). Therefore, the increase in H3 suggests that there was an increase in histone occupancy at the targeted regions with no detectable rhythm in the measured time frame.

H3K4me3 is a histone modification that is often associated with active transcription when in close proximity to promoter regions (Fischle et al. 2003). Therefore, it was hypothesized that greater amounts of H3K4me3 would be detected at about the time of peak mRNA abundance of the two rhythmically expressed genes, JMJD6-like 2 and LHCBM6. Since the genes show opposite phases of their mRNA abundance rhythms, the H3K4me3 rhythms would also show opposite phases. Similarly, it was hypothesized that the amount of H3K4me3 would remain constant across the circadian cycle for the constitutively expressed gene RACK1. Surprisingly, results from the three circadian experiments indicate that the amount of H3K4me3 shows not only a

circadian rhythm across all three genes investigated but also that each rhythm exhibits the same phase (Fig. 10). The peak of the rhythm occurs during the middle of subjective day, which correlates with the expression pattern of the LHCBM6 gene but is opposite in phase to that of the JMJD6-like2 gene and an entirely different pattern when compared to the expression of the RACK1 gene.

A circadian rhythm of H3K4me3 was reported for the first time in 2006 (Ripperger and Schibler 2006). The study determined the amount of H3K4me3 at the rhythmically expressed albumin D element-binding protein gene (*dbp*) under diurnal conditions of 12 h light/12 h dark cycles in mouse liver. The authors found that H3K4me3 shows a rhythm at regulatory elements around the transcription start site that parallels the rhythm in *dbp* expression. Both the *dbp* mRNA amount and the H3K4me3 amount at the *dbp* promoter are highest during the middle of the day. This finding, together with findings for other histone modifications, led the authors to suggest that parts of the *dpb* locus changes from a euchromatin-like to a heterochromatin-like state every day through reversible post-translational modifications. While these results suggest a close relationship between gene expression patterns and patterns of H3K4me3 amount at the promoter of the respective genes, more recent reports challenge this assumption (Koike et al. 2012, Valekunja et al. 2012).

Similar to the results of the study presented here, recent findings suggest that H3K4me3 rhythms are not necessarily related to the expression pattern of an affected gene. A recent genome-wide ChIP study in mouse liver in constant dark surprisingly found not only that quite a number of genes show a circadian rhythm of H3K4me3 at their promoters but the vast majority of these rhythms also show the same phase, i.e. a

peak during the middle of subjective night (Koike et al. 2012). This general H3K4me3 rhythm is closely paralleled by a general rhythm in recruitment of RNA polymerase II to transcription preinitiation complexes, with RNA polymerase II occupancy peaking at about the same time. Yet determination of transcription initiation complexes instead of preinitiation complexes revealed that rhythms of RNA polymerase II as part of these complexes show no preferred phase but are evenly distributed throughout the circadian cycle. The authors also measured intron- and exon-based RNA amounts of the entire transcriptome in an effort to distinguish initial unspliced pre-mRNA as an indicator of transcription, from mature mRNA as an indicator of mRNA abundance. Comparing their datasets, they found that many genes with H3K4me3 rhythms at their promoters do not show intron cycling, or rhythms in amount of unspliced pre-mRNA, and therefore do not show a rhythm of transcription (Koike et al. 2012).

Another recent study on mouse liver (Valekunja et al. 2012) confirmed part of the results. The authors also found many genes in the genome, both rhythmically expressed and not, exhibiting a circadian rhythm of H3K4me3 at their promoters with nearly all of them showing a peak in the middle of subjective night. Only 23% of these genes had been reported to show a circadian rhythm in mRNA amount and only 33% of genes with a reported circadian rhythm in mRNA amount also showed a circadian rhythm of H3K4me3 at their promoters. The authors determined that loci that did not undergo rhythmic histone methylation were 3.16-fold less likely to show circadian transcription. Interestingly, the results also showed a rhythm of H3K9me3 amount in antiphase to the rhythm of H3K4me3 amount for a few genes. This modification has been correlated with repression of gene expression. Overall, these findings suggest that the circadian clock, in

mammals at least, regulates global transcriptional poise and chromatin state, but that the final outcome of expression for individual genes depends on additional measures.

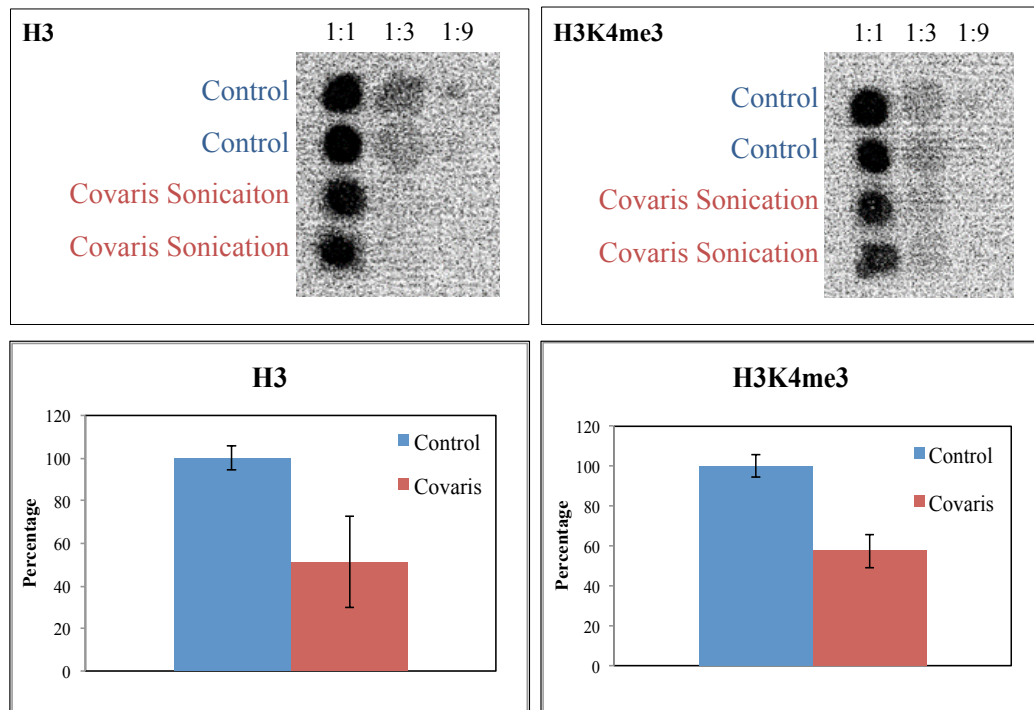
### Optimization of Techniques

A technique that is commonly used to investigate histone patterns at particular DNA regions is chromatin immunoprecipitation (ChIP). A published procedure of ChIP for *C. reinhardtii* was followed (Strenkert et al. 2011), but several aspects of the ChIP technique were successfully optimized for this study. One consideration of a ChIP experiment is the fixing agent, which in the case of this study was formaldehyde stabilized with methanol. The fixing agent crosslinks DNA-binding proteins to the DNA so that both are precipitated with a single antibody. The formaldehyde used in the experiments was from a fresh bottle that had been opened for the first time at the start of this study to reduce the risk of a lowered effective concentration due to prolonged exposure to air and light. Methanol acts as a preservative and it also increases the permeability of cells, which increases efficiency of fixation. Some ChIP protocols call for using formaldehyde that contains no methanol (Khoja et al. 2012) while others do not specify. To ensure that over-fixation was not occurring, the fixation procedure was optimized to determine the lowest formaldehyde concentration that still yielded the most precipitated DNA (Fig. 3). Fixing cells in media can also result in the formation of cell aggregates, which leads to some cells being more or less fixed than other cells. During fixation, samples were placed on a shaker to prevent aggregate formation.

Previous studies have shown that it is important to recognize the relationship between fixation and sonication and to optimize these two steps simultaneously

(Strenkert et al. 2011, Khoja et al. 2012). Over-fixation of chromatin can lead to samples requiring more intense sonication to shear the chromatin. Histones are small proteins that are not easily susceptible to degradation by sonication, but destruction of histone epitopes can take place if exposed to sonication for too long. Covaris Adaptive Focused Acoustics (AFA) ultrasonicators are typically considered superior to probe sonicators because they offer continuous temperature control and shear to more precise ranges, leading to more reproducible results. They also do not require direct probe-sample contact, which reduces the risk of sample contamination. The truChIP Low Cell chromatin shearing protocol by Covaris showed that epitope integrity was preserved when  $3 \times 10^6$  HeLa cells in 130  $\mu\text{l}$  were sonicated for up to 12 minutes. The undergraduate Richard Dawson tested the integrity of H3 and H3K4me3 epitopes after sonication for 24 minutes with the Covaris truChIP Low Cell chromatin shearing protocol (Fig. 12). He found that about 50% of epitopes were still recognized by the anti-H3 and anti-H3K4me3 antibodies after Covaris treatment (R. Dawson and S. Jacobshagen, unpublished results) and therefore that this length of sonication was acceptable.

Altogether, three independent circadian experiments were carried out. The first of the three experiments varied slightly from the other two. For the first experiment, the tissue serving as neutral density filter was directly wrapped around the glass bottle containing the *C. reinhardtii* culture, rather than around the lamps, before harvest began. The amount of light reaching the culture was about  $10 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$  in both cases. However, the decision to wrap the lamps instead of the bottle came after it was realized that wrapping the bottle led to some possible light contamination during harvest due to the top foil being removed from the culture bottle. When the lamps were wrapped,



**Figure 12. Dot blot and quantitative analysis of histone integrity after sonication.**

Dilutions of *C. reinhardtii* samples treated for 24 minutes with Covaris sonicator to shear the chromatin were loaded on a membrane. Control samples were treated with probe sonication (Sonics VCX130) 4 times for 10 seconds just to lyse the cells before dilutions were blotted on the membrane. The membrane was incubated with anti-H3 (top left) and anti-H3K4me3 (top right) antibodies to detect the presence of either histone H3 or H3K4me3. Quantitation was performed using a FluorChem HD2 gel imager with quantitation software (Alpha Innotech). The bar graphs show control 1:1 and 1:3 dilutions averaged for each antibody then set to 100%. Covaris-treated 1:1 and 1:3 diluted samples were then expressed relative to the control and averaged. Error bars show one standard deviation of two replicate samples for each of two dilutions. (R. Dawson and S. Jacobshagen unpublished results)

it was also easier to view the level of the culture and there was no potential for light contamination because all other lights in the room were off. Multivariate ANOVA revealed that there was no statistically significant difference between the three experiments, confirming that the different ways to dim the light did not significantly influence the outcome. The possible light contamination every three hours during harvest of the first experiment might have been either too low or at intervals too short to cause significant changes to the circadian physiology of *C. reinhardtii*.

To control for possible nonspecific antibody interactions during ChIP, DNA mass that was immunoprecipitated with the control anti-cryptochrome antibody in the first of two sequential precipitations was subtracted from DNA mass precipitated with the anti-H3 antibody. Similarly, DNA mass that was immunoprecipitated with the control anti-cryptochrome antibody in the second of two sequential precipitations was subtracted from DNA mass precipitated with the anti-H3K4me3 antibody. Interestingly, there was no significant difference in the results for either H3 or H3K4me3 when subtracting DNA mass obtained with a control ChIP using no antibody in place of the control anti-cryptochrome antibody. It suggests that both serve as valid controls in these experiments.

### Implications and Further Studies

The findings presented here suggest that *C. reinhardtii* might show a similar global regulation of rhythmic H3K4me3 as was shown in mammals and that therefore this feature has been preserved during eukaryotic evolution. Since a green alga like *C. reinhardtii* is evolutionarily quite removed from mammals like mice, it suggests that this genome-wide regulation of H3K4me3 would have evolved early and consequently is

expected to fulfill a fairly important function in the cell or organism. Whether such global regulation of chromatin modification also occurs in other eukaryotic organisms is not clear yet. The plant model *Arabidopsis* has so far not been investigated on a genome-wide scale. What is known about *Arabidopsis* is that H3K4me3 rhythms can have different phases, at least at the promoters of several clock genes (Song and Noh 2012, Malapeira et al. 2012). Investigations of *Arabidopsis* in particular would provide insight into the potential evolution of global regulation of H3K4me3, because it is an organism that is multicellular like mammals but closely related to green algae.

The surprising discovery of H3K4me3 rhythms in *C. reinhardtii* that occur at the promoters of both rhythmic and non-rhythmic genes and that have the same phase warrants further analysis. Only a portion of the isolated DNA from the ChIP samples was used in this study, meaning that some of the isolated DNA is available for further investigation. The remaining precipitated DNA could be deep-sequenced to investigate patterns in H3K4me3 amount and general H3 amount across the entire genome.

Another potential direction would be the exploration of histone modifications known to be associated with inactive transcription, such as H3K9me2. Similar experiments could be performed through ChIP with antibodies that would specifically target H3K9me2. It would be fascinating to see if the amount of this repressive mark shows a rhythm in antiphase to the rhythm of H3K4me3 amount, as has previously been shown for the *dbp* gene in mouse liver (Ripperger and Schibler 2006). Similarly, rhythms in the amount of acetylation of lysine 9 on histone H3 (H3K9ac), a modification associated with active transcription, could be investigated through similar means using *C. reinhardtii*. The amount of the H3K9ac modification was shown to correspond to the



phase of H3K4me3 amount pattern in mouse liver at the *dbp* locus (Ripperger and Schibler 2006). In more practical terms, understanding the rhythm that a histone modification might show is especially important in determining predispositions for and improving treatment of diseases linked to certain histone modifications, such as Huntington's and Parkinson's diseases (Peña-Altamira et al. 2013), autoimmune diseases (Gupta et al. 2015), cancers (Sawan and Herceg 2010) and more. There could also be implications for disorders linked to disrupted circadian rhythmicity ranging from jet lag and sleep disorders to metabolic syndromes and cancers (Takahashi et al. 2008).

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