AN ABSTRACT OF THE THESIS OF

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Circadian clocks are internal mechanisms regulating many physiological processes. Research suggests that the circadian clock may regulate repair of cellular damage caused by reactive oxygen species (ROS), but molecular pathways from the clock to ROS repair are not known. The gene *cncC*, known in mammals as *Nrf2*, and its repressor *keap1,* mediate the cellular responses to oxidative stress and activate genes such as glutathione-s-transferase (*gstD1*) and glutamate cysteine ligase (*Gclc*), which encode important detoxification enzymes. The question addressed in this study was whether clock genes regulate the response to oxidative stress in *Drosophila melanogaster* by stimulating expression of *cncC/Nrf2* and *keap1*, which then activate *gstD1* and *Gclc*. Expression profiles of *cncC*/*Nrf2*, *keap1*, *gstD1*, and *Gclc* were measured in flies with normal or disrupted clocks over a 24 h period. We show the circadian clock does not control transcription of *cncC/Nrf2* and *keap1*. In contrast, the effector genes *gstD1* and *Gclc* showed daily rhythms of expression, which were disrupted in clock mutants. These results suggest that the clock may modulate the expression of genes involved in ROS protection. This study provides new knowledge on ROS regulation, which is a rising health concern due to the cellular damage they cause.

Key words: circadian clock, oxidative stress, reactive oxygen species, glutathione

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Oxidative Stress Response Genes: A Circadian Connection?

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Marisa C. McAllister, Author

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1. Introduction and Background

1.1 Circadian rhythms

Circadian rhythms (from Latin *circa* meaning "about" and *dies* for "day") are fundamental biological processes that exist in a diverse array of organisms, including some bacteria, fungi, plants, and animals (Young and Kay, 2001). Circadian rhythms control many life functions, such as daily fluctuations in sleep and activity, foraging time, and hormone levels. These rhythms are the output of the circadian clock, a network of genes and proteins which generates "*circa*" 24 h cycles in most organisms. While rhythms are endogenously generated, in natural conditions they are synchronized (entrained) by exogenous factors called time givers, or Zeitgebers (ZT). The most prevalent exogenous factors are oscillations of temperature and light/dark (LD) cycles associated with the solar day. The fact that rhythms persist without these exogenous factors in constant laboratory conditions, such as continuous darkness (DD), demonstrates their endogenous nature (Allada et al., 2001).

1.2 Molecular mechanism of the circadian clock

The genetic basis of circadian clocks was intensely studied in model organisms such as the fruit fly *Drosophila melanogaster*, a powerful model used to study complex biological problems at the molecular level. In *D. melanogaster* circadian rhythms are

generated at the molecular level by a set of clock genes and proteins that work in two interlocked feedback loops (Figure 1). The first loop contains two transcriptional activators that are encoded by the genes *Clock (Clk)* and *cycle (cyc)*, which control the expression levels of the genes *period (per)* and *timeless (tim)* (Hardin, 2004). CLK/CYC protein dimers promote *per* and *tim* transcription by binding to the E-boxes in the promoter region of these genes (Young and Kay, 2001). During early evening the *per/tim* mRNA and PER/TIM proteins accumulate in the cytoplasm. Next, PER/TIM proteins form a heterodimer complex in the cytoplasm and enter the nucleus late at night to inhibit CLK-CYC mediated transcription of *per* and *tim* (Glossop et al., 2003). In the presence of light TIM protein is degraded, thus disrupting the PER/TIM dimer and resulting in synchronization of the molecular clock with exogenous light signals. Subsequent degradation of PER terminates inhibition of CLK-CYC mediated transcription, allowing the start of the next clock cycle. In addition to *per* and *tim*, CLK-CYC activates components of the second feedback loop; a transcriptional repressor, encoded by *vrille (vri)* and a transcriptional activator encoded by *Pdp1*, which control the expression of *Clk* (Cyran et al., 2003). Many similarities have been found between fly and mammalian clock genes (Stanewsky, 2003). The most studied circadian gene in *D. melanogaster* is *per*, which is conserved in the circadian clock of humans. This gene is necessary for daily rhythms in locomotor activity and may also control metabolic pathways.

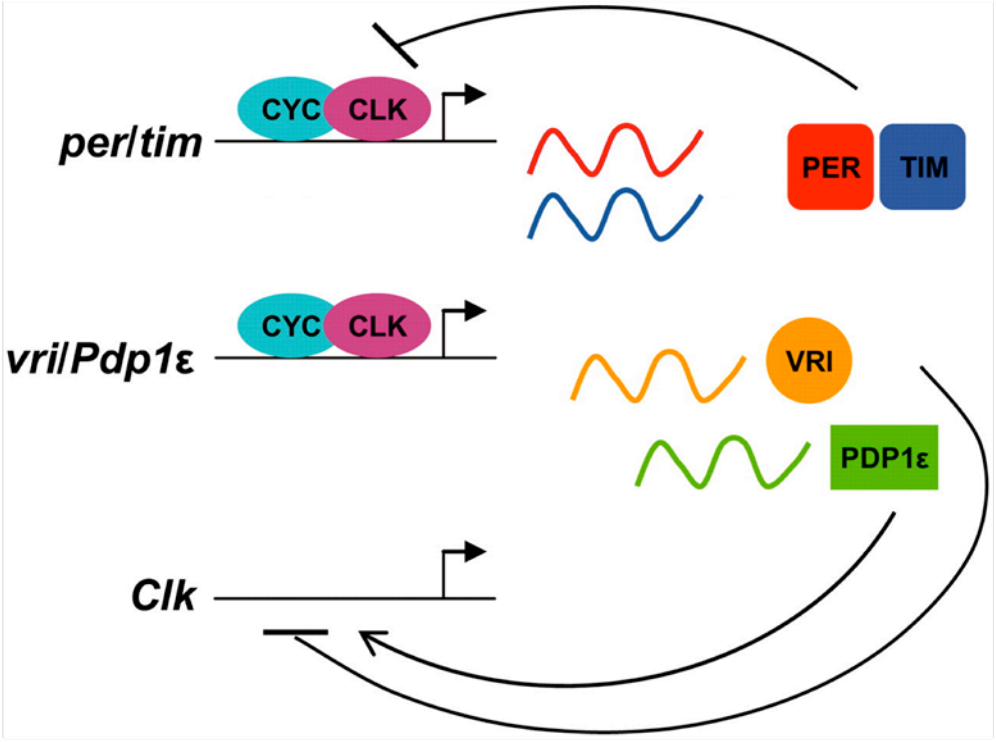

Figure 1. Molecular model of circadian clock in *D. melanogaster*
CLK and CYC form a heterodimer and bind to the E-box element of the circadian clock genes *per* and *tim* and activate their transcription during the day and early evening; as *per* and *tim* mRNAs peak, PER and TIM proteins accumulate, form a PER/TIM complex, and translocate into the nucleus to repress their own transcription during the late night. During the day, PER and TIM are degraded by light-dependent and independent pathways, thus allowing a new cycle of transcription to start. In another transcription-based loop, CLK/CYC activate transcription of *vri* and *Pdp1*; as VRI and PDP1 proteins accumulate, they translocate into the nucleus to inhibit and activate *Clk* transcription, respectively. Both VRI and PDP1 bind to sites in the *Clk* promoter. PDP1 accumulation lags behind that of VRI, resulting in rhythmic *Clk* transcription. Reproduced from (Zheng and Sehgal, 2008).

1.3 Do circadian clocks protect organisms from oxidative stress?

Clock genes modulate transcription of many clock-controlled output genes, as revealed by microarray studies of diurnal gene expression (McDonald and Rosbash, 2001). Among genes that appeared rhythmically expressed several were involved in the removal of reactive oxygen species (ROS) and repair of oxidative damage (Ceriani et al., 2002; McDonald and Rosbash, 2001). To understand the functional significance of these molecular rhythms, Dr. Giebultowicz's laboratory has studied the circadian regulation of the response to oxidative stress in *D. melanogaster*. Oxidative stress occurs when more ROS are produced than an organism is able to detoxify or remove (Valko et al., 2007). It was found that the time of day at which flies were exposed to hydrogen peroxide (H_2O_2) affected their ability to cope with this oxidative stressor. Flies exposed to H_2O_2 during the night had lower mortality rates than flies exposed during the day. To understand the significance of these rhythms flies with a disrupted *per* gene were investigated. Mutant flies with no functional *per* gene, (per^{01}) exhibited higher mortality at all times of day (Krishnan et al., 2008). In addition, flies with a normal clock showed a daily fluctuation in the level of ROS, while per^{01} flies had significantly higher and non-rhythmic ROS levels, and higher levels of oxidative damage to proteins (Krishnan et al., 2008). These data suggests a connection between the circadian clock and the genes controlling oxidative stress response.

Recently, several studies have determined that deteriorated circadian rhythms in humans over 65 can lead to night-time sleep disturbances and thus, a lower functioning ability during the day (Huang et al., 2002). In addition to the adverse affects on night-time sleep and day-time alertness there may be a connection between functional circadian

clocks and lifespan. A recent study in mice demonstrated that disrupting the circadian clock leads to a shortened lifespan (Antoch et al., 2008; Kondratov et al., 2006). When BMAL1 (a homolog of the fly *cyc* gene) was mutated, the mice had a shortened lifespan and symptoms of premature aging. The mice also had increased levels of ROS in some of their tissues (Kondratov et al., 2006). Similar results have been found in flies; it was recently reported that loss of the gene *per* resulted in accelerated aging in flies following a challenge of exogenous oxidative stress (Krishnan et al., 2009).

1.4 Reactive oxygen species, oxidative damage, and antioxidant defense systems

According to the free radical theory of aging, aging is a process that is at least partially caused by the build-up of ROS, which cause damage in biomolecules, such as nucleic acids, lipids, proteins, and carbohydrates. ROS have also been implicated in playing an important role in the development of neurodegenerative diseases, including Alzheimers's (AD), Parkinson's, mild cognitive impairment, amyotrophic lateral sclerosis, and Huntington's (Mariani et al., 2005). The brain is very susceptible to oxidative damage because of the high levels of polyunsaturated fatty acids, which are susceptible to lipid peroxidation, and lower levels of antioxidant defense systems. In addition to lipid peroxidation, there is a higher level of protein oxidation in aging adults (Jenner and Olanow, 1996). While excess ROS levels are detrimental to cell function, some levels of ROS are necessary, as these molecules are responsible for essential processes in organisms including cell signaling cascades and immune response.

At a molecular level, ROS are byproducts of normal cellular metabolism and are produced as a result of incomplete electron transfer in the electron transport chain during

aerobic respiration (Anderson, 2004). In addition to the naturally occurring ROS, organisms also develop ROS from exogenous sources, including UV light, ionizing radiation, chemicals, and toxic substances (Finkel and Holbrook, 2000). ROS are damaging to cells at high concentrations because of their negative impact on cell structures, nucleic acids, lipids, and proteins. Specifically, the hydroxyl radical can react with all of the components of DNA, which can cause permanent damage and this damage is often the first step in mutagenesis and aging (Valko et al., 2006). Hydroxyls can also react with proteins causing their oxidation, and with lipids, causing peroxidation (Valko et al., 2007).

Organisms have developed defense and repair systems to combat the damage caused by ROS. Important enzymes that detoxify ROS include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Valko et al., 2006). SOD catalyzes the reaction that converts a superoxide anion to hydrogen peroxide, which can then be broken down into water and oxygen by other defense enzymes, either catalase or GPx. Insects do not have GPx so they employ thioredoxin reductase to break down hydrogen peroxide and reduce glutathione disulfide (GSSG) (Kanzok et al., 2001). In addition to enzymatic defenses, organisms also possess non-enzymatic antioxidants including glutathione, ascorbic acid, α-tocopherol, carotenoids, and flavonoids (Valko et al., 2006).

1.5 KEAP1 and NRF2 signaling

Because high levels of ROS are detrimental to cells, several mechanisms evolved to "sense" the level of oxidative stress and induce protective enzymes. A leucine zipper

transcription factor, NRF2 (NF-E2-Related Factor 2) and its repressor, KEAP1 (Kelch-like ECH-Associated Protein 1) are part of an oxidative damage repair pathway that has been termed the NRF2-mediated defense response in mammals (Figure 2) (Itoh et al., 2004). This response includes the regulation of cellular redox homeostasis, cell growth and apoptosis, inflammatory response, and the ubiquitin-mediated degradation pathway. In the absence of oxidative stress, KEAP1 is bound to NRF2 and restricts it to the cytoplasm. KEAP1 also targets NRF2 for ubiquitination and degradation by the 26S proteasome to keep basal levels low. When the intracellular redox environment becomes pro-oxidizing, KEAP1 undergoes conformational modification because of its redox-sensitive cysteine residues and releases NRF2 to enter the nucleus (Zhang, 2006). Once in the nucleus, NRF2 coordinates the stress response through the cis-acting antioxidant response element (ARE) in the promoter region of target genes (Itoh et al., 2004). NRF2 binds to the ARE in tandem with the small MAF (Musculo-Aponeurotic Fibrosarcoma) protein (Itoh et al., 1997). The NRF2/MAF dimer activates thrioredoxins, glutathione-synthesizing enzymes, glutathione S-transferases, molecular chaperones, and proteasome subunits. A basal level of NRF2 activity is also present under non-stressed conditions. This allows cells to maintain a low level of antioxidant and detoxification genes at all times (Sykiotis and Bohmann, 2008).

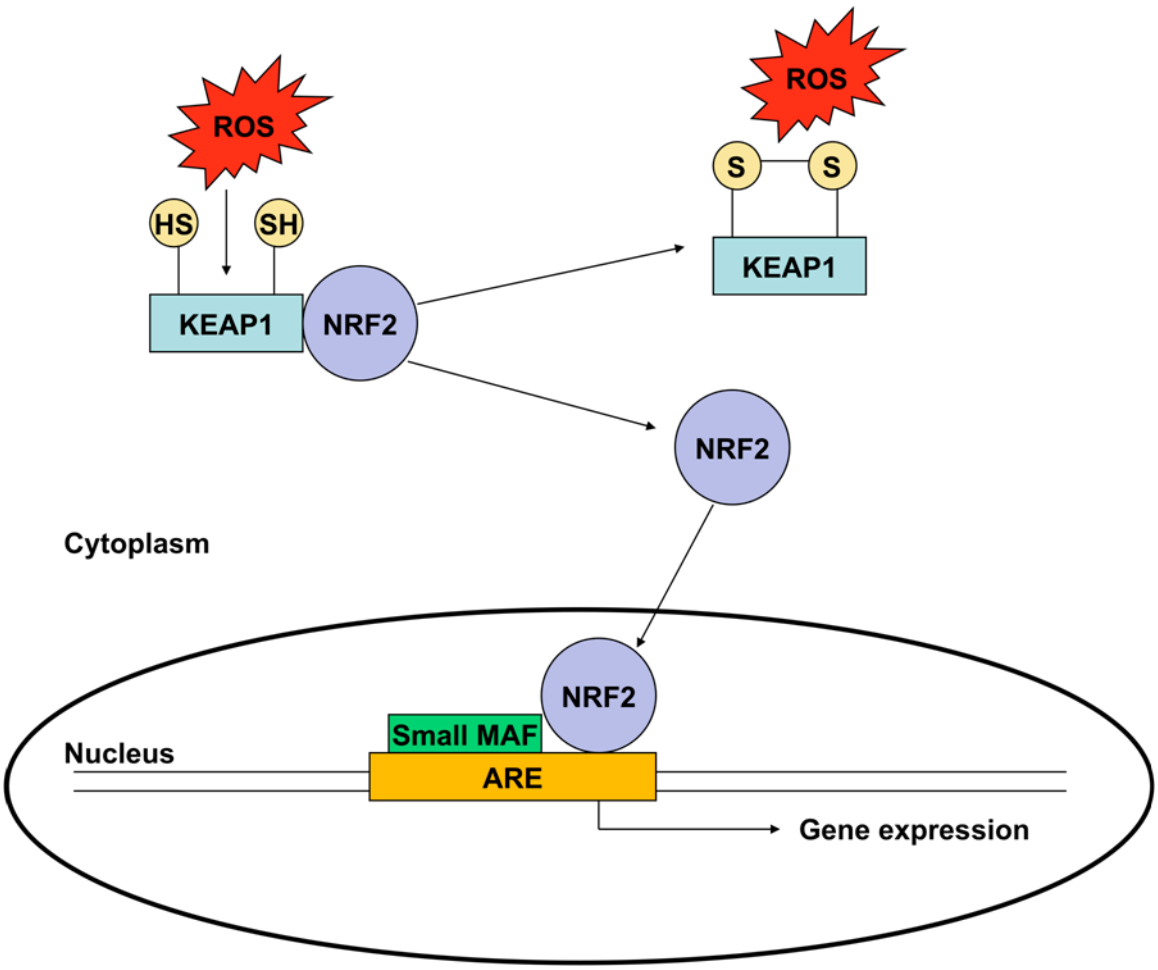

Figure 2. NRF2-mediated oxidative stress response pathway
In the absence of oxidative stress, KEAP1 is bound to NRF2 and restricts it to the cytoplasm. In the presence of oxidative stress, KEAP1 releases NRF2 to enter the nucleus and bind to the ARE element with small MAF protein. Together, NRF2 and MAF activate transcription of a number of detoxifying enzymes (Suryakant et al., 2009). Figure adapted from Kensler et al., 2003.

Homologs of mammalian *keap1* and *Nrf2* genes have been found in worms, insects, fish, and birds. In *D. melanogaster, Nrf2* is named *cncC* (*cap'n'collar*). These genes play important pro-longevity and antioxidant roles (Sykiotis and Bohmann, 2010). The N terminus of *cncC* is similar to the Neh2 domain of *Nrf2,* and *cncC* mRNA is highly expressed in the alimentary canal, which is similar to *Nrf2* expression in the mammalian digestive tract (Sykiotis and Bohmann, 2008). Hence forth *Nrf2* and the *D. melanogaster* homolog *cncC* will be referred to as *cncC/Nrf2*. Overexpression of *Keap1* inhibits CNCC/NRF2 activity and *cncC/Nrf2* knockdown decreases tolerance to oxidative stress in *D. melanogaster* (Sykiotis and Bohmann, 2008).

1.6 Glutathione and detoxification in *D. melanogaster*

One of the protective systems affected by KEAP1 and CNCC/NRF2 comprise Glutathione S-transferases (GSTs) and glutathione (GSH). GSH is the most abundant low-molecular weight thiol present in cells, (Wu et al., 2004) and has many physiological functions, including transport of amino acids, removal of xenobiotics from the cell, and maintaining the cellular redox state (Orr et al., 2005). GSH is readily oxidized by electrophilic substances to glutathione disulfide (GSSG). Low cellular GSH concentrations usually indicate oxidative stress, malnutrition, or other pathological conditions (Wu et al., 2004). Synthesis of GSH from glutamate, cysteine and glycine is controlled by two enzymes: glutamate-cysteine ligase, which is a heterodimeric enzyme consisting of a catalytic subunit (GCLC) and modulatory subunit (GCLM), and glutathione synthase. The activity of GCLC is the rate-limiting step in GSH *de novo* synthesis. CNCC/NRF2 can bind to the ARE, which is located upstream of the *Gclc*

gene, and activates its transcription (Li et al., 2009). In *D. melanogaster* overexpression of *Gclc* leads to increased GSH levels, which confers increased protection against oxidative stress, and extends lifespan. Conversely, inhibition of *Gclc* results in increased susceptibility to oxidative stress due to lower levels of GSH and complete knockout of *Gclc* is lethal (Luchak et al., 2007; Radyuk et al., 2009).

Glutathione S-transferases (GSTs) are found in many aerobic organisms and are an important part of detoxification and drug resistance pathways. GSTs are well researched in both mammals and insects (Ranson and Hemingway, 2005). GSTs conjugate a thiol group of glutathione to a compound with an electrophilic center, which helps to eliminate toxins from cells (Low et al., 2007). A specific GST of interest is *GstD1*, which has been shown to be important in detoxification of cells and resistance to the insecticide dichloro-diphenyl-trichloroethane (DDT) (Matzkin, 2008). *GstD1* is a known target of CNCC/NRF2 (Sykiotis and Bohmann, 2008). Furthermore, microarray data suggest that *GstD1* may be rhythmic in flies (Keegan et al., 2007).

1.7 Objectives

As discussed above (sec. 1.3), there is evidence that the circadian clock confers protection from oxidative stress in both flies and mammals, but molecular pathways from the clock to detoxifying mechanisms are not understood. The major objective of this thesis was to determine if the circadian clock in the model organism *D. melanogaster* regulated oxidative stress signaling genes *cncC/Nrf2* and *keap1* and genes involved in repair of oxidative stress *gstD1* and *Gclc*. *D. melanogaster* is an ideal model organism for this research because it has a large number of available mutants, and a sequenced

genome. In addition the genes regulating; the circadian clock and ROS detoxification are highly conserved between flies and mammals. I hypothesize that clock genes regulate the response to oxidative stress by stimulating rhythmic expression of *cncC/Nrf2* and *keap1*, or by directly regulating effector genes *gstD1* and *Gclc*. These alternative models are illustrated in Figure 3. This hypothesis was tested in experiments in which daily profiles of the mRNA expression levels were measured to determine if the genes were rhythmically expressed over a 24 h period and regulated by the clock genes *period* or *cycle*.

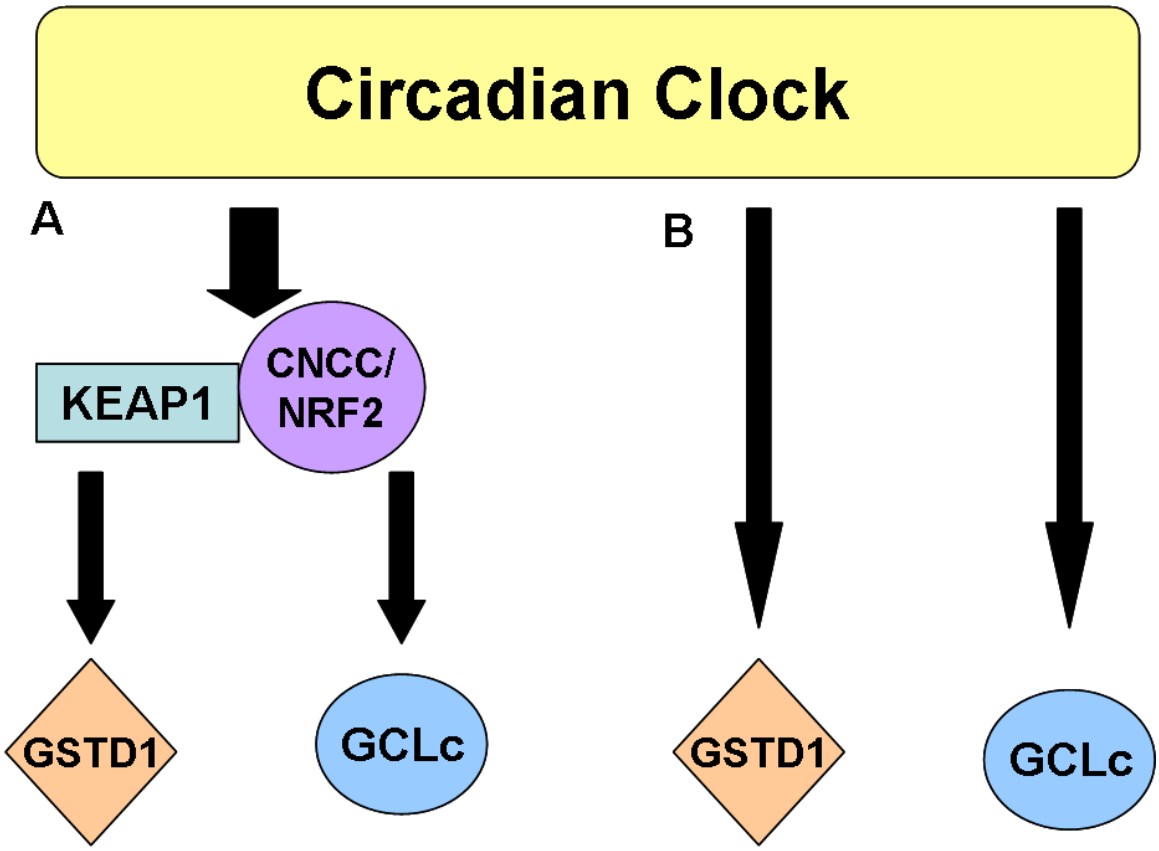

Figure 3. Hypothetical pathways of clock regulated response to oxidative stress A) Circadian clock genes regulate *gstD1* and *Gclc* transcription through rhythmic transcription of *keap1* and/or *cncC/Nrf2*. B) Clock genes could also influence the transcription of *gstD1* and *Gclc* independent of rhythmic *keap1* and *cncC/Nrf2* transcription.

2. Materials and Methods

2.1 Fly Rearing

Drosophila melanogaster were raised on a cornmeal-yeast diet at 25±1°C in 12 h light: 12 h dark cycles (LD). Lights were switched on at 9:00am, which is denoted as Zeitgeber Time (ZT) 0. Lights were switched off at 9:00pm, which is denoted as ZT 12. Wild-type Canton-S (CS) flies were used as reference stock with a normal circadian clock. Flies with a loss-of-function mutation in the genes *period* (per^{01}) (Konopka and Benzer, 1971), or *cycle* (cyc^{01}) (Rutila et al., 1998), were also used. Prior to the onset of experiments cyc^{01} (Beaver et al., 2010) and per^{01} mutants (Krishnan et al., 2008) were backcrossed to CS stock for at least 6 generations to equalize their genetic background. Flies were separated by sex 1-2 days after emergence, and were transferred to fresh diet vials 2 days before collections. Five-day old males were used for all experiments. For gene expression studies, 75 males of each genotype were collected at 4 hour intervals for 24 h, beginning at ZT 0 and ending at ZT 24; the samples were frozen at -80°C.

2.2 Quantitative Real-Time PCR (qPCR)

Fly heads and bodies were separated using a sieve and dry ice. Whole flies were placed into the sieve with dry ice and shaken vigorously for two minutes. Frozen flies collide with the dry ice and break at the most fragile point, the neck. The heads then fall through the holes of the sieve to a separate chamber where they are collected. The heads and bodies were then placed in separate microtubes with 200 µl of Tri Reagent (Sigma,

St. Louis, MO). Each sample was homogenized with a motorized pestle (Kontes, Vineland, NJ) for 2-3 minutes and then combined with chloroform (Sigma). The samples were then centrifuged at 12,000 g for 10 minutes at 4°C to separate the RNA, chloroform, and debris. The RNA containing fraction was removed to a separate microtube, combined with isopropanol (ISC BioExpress, Kaysville, UT) and mixed by inversion. Samples were centrifuged at 12,000 g for 10 minutes to precipitate the RNA and the supernatant was removed.

Columns and centrifugation were used to separate the RNA from any remaining contaminating DNA. Specifically, samples were purified using a Qiagen RNAeasy mini kit according to the manufacturer's protocol. The quantity and quality of RNA was determined spectrophotometrically in 2 µl aliquots of each sample using a nanodrop (Fisher, Pittsburgh, PA). After the quality of the RNA was verified, it was diluted to equal a concentration of 20 ng/µl in each sample. cDNA was synthesized using an iScript cDNA synthesis kit (Biorad, Hercules, CA) according to the manufacturer's protocol. Following synthesis cDNA was diluted to either 0.2 ng for use with *rp49, timeless (tim)*, *cncC/Nrf2,* and *gstD1* primers or 1 ng for use with *keap1* and *Gclc* primers. Each series of samples, representing a 24 h time course collected at 4 h intervals, was evaluated by running a plate with *rp49* and *timeless (tim)* primers. This provided positive control to verify that the cDNA was of high quality and that *tim* followed the expected gene expression patterns.

qPCR was carried out using an ABI Prism Step-One Plus real-time machine. Each reaction contained 3 µl of cDNA combined with 7 µl iTaq SYBR Green Supermix with Rox (Biorad, Hercules, CA) and 1 µl of 2.5 µM forward and reverse primer mix (IDT

Technologies, San Diego, CA). Each sample was run in triplicate in an ABI prism plate. Primers were designed for the target gene sequences using PerlPrimer 1.1.17 (Marshall, 2004) and the sequence of each primer is given below (Table 1). Each primer set was tested for optimal reaction temperature by running an optimization plate at 5 different temperatures. The genes *gstD1, cncC/Nrf2,* and *keap1* were run at 62°C and *Gclc* was run at 60°C. qPCR consists of 3 stages with different temperatures. The first stage is the holding stage at 95°C. During this stage the activation of the enzymes occurs, which prepares the cDNA for amplification. The next stage is the cycling stage at 62°C. In this stage the cDNA is synthesized and fluorescence levels are measured. The last stage starts at 95°C and decreases to 60°C and it is the melt curve stage. Finally qPCR for each gene of interest was further optimized by evaluating reaction efficiency for each primer set using cDNA template concentrations varying from 0.2 – 2.0 ng/ul.

Table 1: Primer sequences for qPCR

Gene	Forward Sequence	Reverse Sequence
Gclc	5'-ATG ACG AGG AGA ATG AGC TG-3'	5'-CCA TGG ACT GCA AAT AGC TG -3'
gstD1	5'-TCG AGG TGG CCA AAT TCG AGA TCA-3'	5'-ACC ACC TGT TCA CAT TGG CGT ACT -3'
cncC	5'-TCG GAG ATG ACG AGG AGG AGA GT-3'	5'-GCA TTG ATG ATC GCC TCC TGG T -3'
keap1	5'-CG CCA GCT CCA AGG GGT GTG T-3'	5' –CCA GAA AGG CAC AGC AGG CAT C -3'
rp49	5'-TGG TTT CCG GCA AGC TTC AAG ATG-3'	5' –ATG AAG TGC TTG GTG CGC TTC TTC-3'
tim	5'–GTG CTT CTG CTG AGG CGT TTC AAT-3'	5'-GGC GAA TGG TTT GAC ATC CAC CAA-3'

Plates were placed in the ABI Prism Step-One Plus real-time machine, which used SYBR green, a cyanine dye, to visualize the amount of amplified DNA. SYBR green dye binds to the minor groove of double stranded DNA and when excited by blue light, emits green light. The more DNA that is present after amplification, the brighter the signal that was emitted from the sample. The fluorescence signal was detected by the camera on the qPCR machine and quantified in the exponential phase, which can be graphed on a log scale to demonstrate a linear relationship. The exponential phase of qPCR is the phase in which a significant increase in PCR product correlates with the initial amount of target template. This phase began with the threshold cycle (C_T), which is the point when the machine detects an increase in the fluorescent signal over the background. The more copies of mRNA transcribed from the target gene that were initially present in the sample, the sooner a fluorescent signal is detected. A sample containing primers, master mix, and no cDNA, was run as a negative control on each plate to verify no contamination occurred. An additional check was completed to verify that all the PCR products for each primer set had the same melting point indicated by the fluorescence signal that a specific PCR product was generated.

Data were normalized to the ribosomal protein, *rp49,* which is constitutively expressed with no diurnal rhythm (Beaver et al., 2010). Differences in expression between time points were calculated relative to control samples at ZT 0. The delta delta C_T, or comparative C_T, method was applied to quantitate the results. C_T is the critical threshold at which a gene's fluorescence passes the threshold cycle. In this method the C_T values from the sample of interest were compared with the C_T values for the CS control.

2.3 Analysis of data

Data obtained from 3 independent experiments of different bio-replicates were averaged and displayed in graphical form. For statistical analysis, 1-way ANOVA was used to analyze the significance of differences in mRNA levels between time points for a single genotype. To compare two genotypes, 2-way ANOVA was used. This test determines if there is a significant difference in mRNA levels at a given time point between the two genotypes. As appropriate, t-tests comparing two different conditions were used. A 95% significance level was used for all statistics. All statistics and graphs were completed using GraphPad Prism 5 (San Diego, CA).

3. Results

3.1 Diurnal profiles of *cncC/Nrf2* and *keap1* mRNA levels

Recent research in the lab has demonstrated that the circadian clock gene *period* may be involved in protection from ROS. per^{01} mutants were more susceptible to oxidative stress at all times of day than wild-type flies. The latter showed a rhythmic response: they were more susceptible to oxidative stress at ZT 8 than ZT 20 (Krishnan et al., 2008). Since the sensitivity to oxidative stress is rhythmic in flies we asked if the circadian clock regulates genes known to be activated by oxidative stress, namely *cncC/Nrf2* and *keap1*. Gene expression levels were measured by qPCR in wild-type (CS^P) flies to determine whether the levels fluctuated in a daily rhythmic fashion. mRNA levels were examined independently in fly heads and bodies. To verify the quality and accuracy of the qPCR method *tim* mRNA expression levels were evaluated (Figure 4A). As expected *tim* mRNA levels peak at ZT 16 and are lower at ZT 0 and 24 (Glossop et al., 2003).

No circadian rhythm was detected for *cncC/Nrf2* mRNA expression in fly heads ($p>0.05$) (Figure 4B). As demonstrated by the relatively large standard error of the mean (SEM), the expression of this gene was variable at each time point and in each independent experiment (Figure 4B). Examination of *cncC/Nrf2* mRNA levels in the bodies of *Drosophila* also revealed no apparent circadian rhythm in expression ($p>0.05$) (data not shown).

Similar to *cncC/Nrf2*, no circadian rhythm was detected in *keap1* mRNA levels in wild-type *D. melanogaster* heads ($p>0.05$) (Figure 4C). There was a large SEM at all

time points and the mRNA levels appear to vary throughout the day. Examination of *keap1* mRNA levels in bodies of wild-type flies also revealed no apparent circadian pattern throughout the day ($p>0.05$) (data not shown).

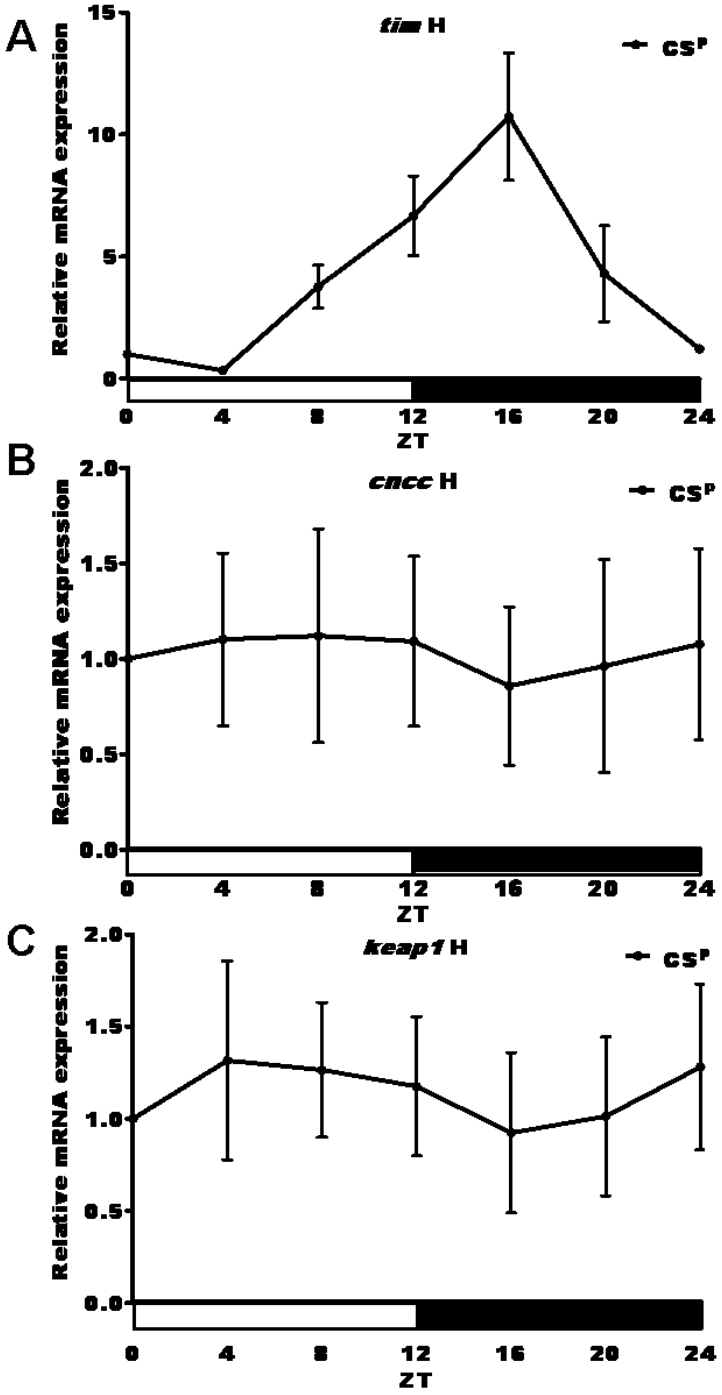

Figure 4. Diurnal profiles of *tim*, *cncC/Nrf2* and *keap1* mRNA levels in heads of wild-type flies

A) Profile of *tim* mRNA expression. B) There is no significant difference in *cncC/Nrf2* expression level over the 24 h day ($p>0.05$) C) There is no significant difference in *keap1* expression level over the 24 h day ($p>0.05$). Data shown in A and B represent average values obtained from 3 independent bio-replicates. Error bars represent the standard error of the mean (SEM).

3.2 Clock genes *per* and *cyc* do not regulate transcription of *cncC/Nrf2* and *keap1*

Altered expression patterns of many genes were detected in flies with a mutated clock, even if the genes did not show a diurnal rhythm in control flies (McDonald and Rosbash, 2001). While no apparent rhythm was detected in *cncC/Nrf2* or *keap1* mRNA levels, there was a possibility that the expression of *cncC/Nrf2* or *keap1* could be altered in clock mutants. Therefore, we measured expression levels of these genes in the heads of per^{01} and cyc^{01} flies at ZT 8 and ZT 20. The loss of *per* or *cyc* function did not significantly alter the expression patterns of *cncC/Nrf2* mRNA, as compared to wild-type flies ($p>0.05$) (Figure 5A). We further demonstrated that *keap1* mRNA levels were not significantly altered in per^{01} and cyc^{01} fly heads (Figure 5B). Taken together, these results suggest that *cycle* and *period* do not regulate steady state *cncC/Nrf2* or *keap1* mRNA levels.

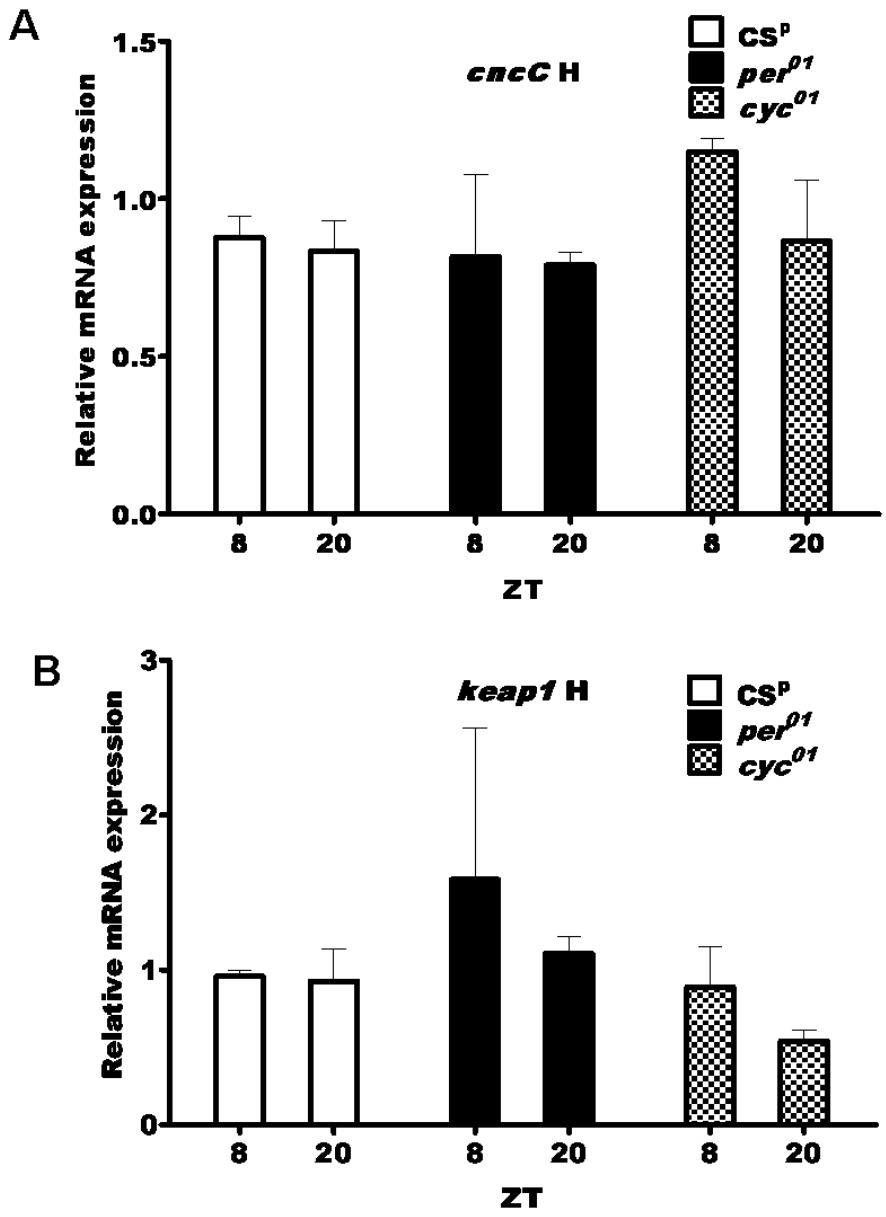

Figure 5. Expression of *cncC/Nrf2* and *keap1* mRNA in the heads of control and *per*^{01} or *cyc*^{01} mutant flies

A) There is no significant difference in expression level of *cncC/Nrf2* between ZT 8 and ZT 20 for CS^{p}, *per*^{01}, or *cyc*^{01} ($p>0.05$). There is also no significant difference between the expression level of CS^{p} at ZT 8 and *per*^{01} or *cyc*^{01} at ZT 8 ($p>0.05$). B) There is no significant difference in expression level of *keap1* between ZT 8 and ZT 20 for CS^{p}, *per*^{01}, or *cyc*^{01} ($p>0.05$). There is also no significant difference between the expression level of CS^{p} at ZT 8 and *per*^{01} or *cyc*^{01} at ZT 8 ($p>0.05$). Data shown in A and B represent average values obtained from 3 independent bio-replicates. Error bars represent the standard error of the mean (SEM).

3.3 Profile of *gstD1* mRNA levels throughout diurnal cycle

Since it has been established that there is a rhythmic sensitivity to oxidative stress in flies (Krishnan et al., 2008) we decided to determine if specific ROS protecting genes that are CNCC/NRF2 targets may be regulated by the clock independently of the CNCC/NRF2 and KEAP1 system. It was shown that expression of *gstD1* is activated by paraquat, an oxidative stress generator (Sykiotis and Bohmann, 2008). We first analyzed expression of *gstD1*, which is involved in cellular detoxification; it eliminates toxins by conjugating a thiol group of glutathione to a compound with an electrophilic center (Low et al., 2007). We detected a statistically significant circadian rhythm ($p<0.01$) in *gstD1* expression levels in heads of wild-type males (Figure 6A). The peak expression level was at ZT 8, that is, 8 hours after lights on. The trough in *gstD1* expression was observed 12 hours later at ZT 20 (Figure 6A). In another experiment, we measured *gstD1* expression in bodies of wild-type flies. The mRNA levels in the CS^p bodies appear to show a rhythmic tendency in an opposite phase of mRNA expression in heads. However, differences between time points were not statistically significant ($p>0.05$) (Figure 6B). These data suggests that the circadian clock may regulate *gstD1* transcription, but in a different manner in heads and bodies (see also discussion).

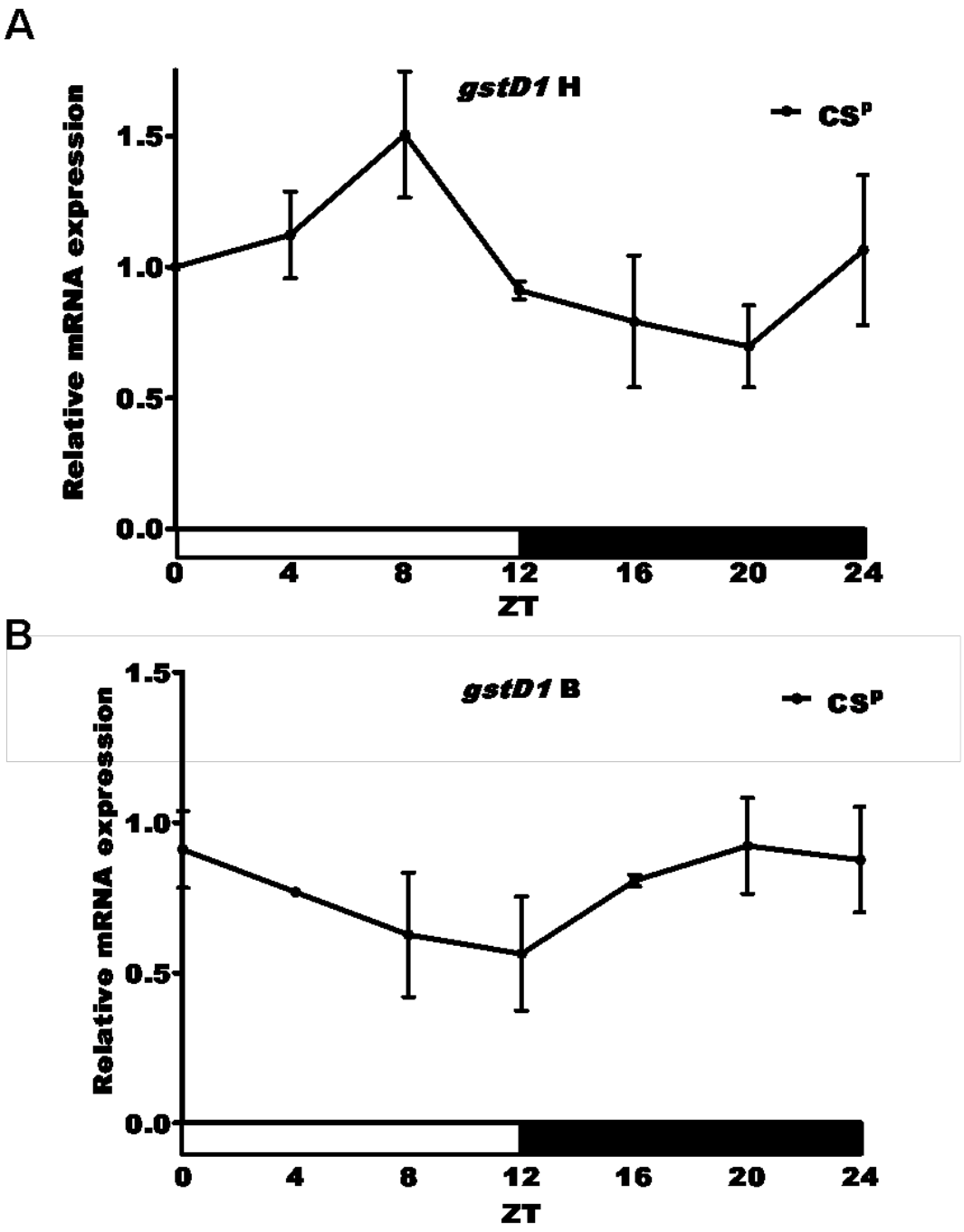

Figure 6. *gstD1* mRNA expression levels in heads and bodies of wild-type flies
A) There is a significant difference in expression level in heads over the 24 h day. ZT 8 is significantly different from ZT 12, 16, and 20 ($p<0.01$). There is a 2.15 fold difference between ZT 20 and ZT 8. B) Data represents average values obtained from 2 independent bio-replicates. There is no significant difference in expression level in bodies over the 24 h day ($p>0.05$). Data shown in A and B represent average values obtained from 3 independent bio-replicates. Error bars represent the standard error of the mean (SEM).

3.4 Clock genes regulate *gstD1* mRNA expression

Given the clear circadian expression pattern of *gstD1* in heads, we explored if the expression of this gene was altered when the clock genes *per* and *cyc* were mutated. Measuring *gstD1* mRNA expression in *per* and *cyc* null mutants allows us to investigate whether PER and CYC regulate the transcription of *gstD1*. In heads of wild-type flies, the *gstD1* mRNA level was significantly higher at ZT 8 than ZT 16, in agreement with the full circadian profile data (Figure 6A). This rhythm was abolished in per^{01} mutants: the expression level of *gstD1* mRNA was at the minimum level observed in wild type flies at ZT 20 ($p<0.05$). Similar low levels of *gstD1* mRNA were detected in heads of cyc^{01} mutants at ZT 8 and ZT 20. While cyc^{01} mRNA expression was slightly higher at ZT 8 than ZT 20, the difference was not statistically significant ($p>0.05$) (Figure 7). Taken together these data demonstrate that the circadian clock genes regulate the expression of *gstD1* in *D. melanogaster* heads.

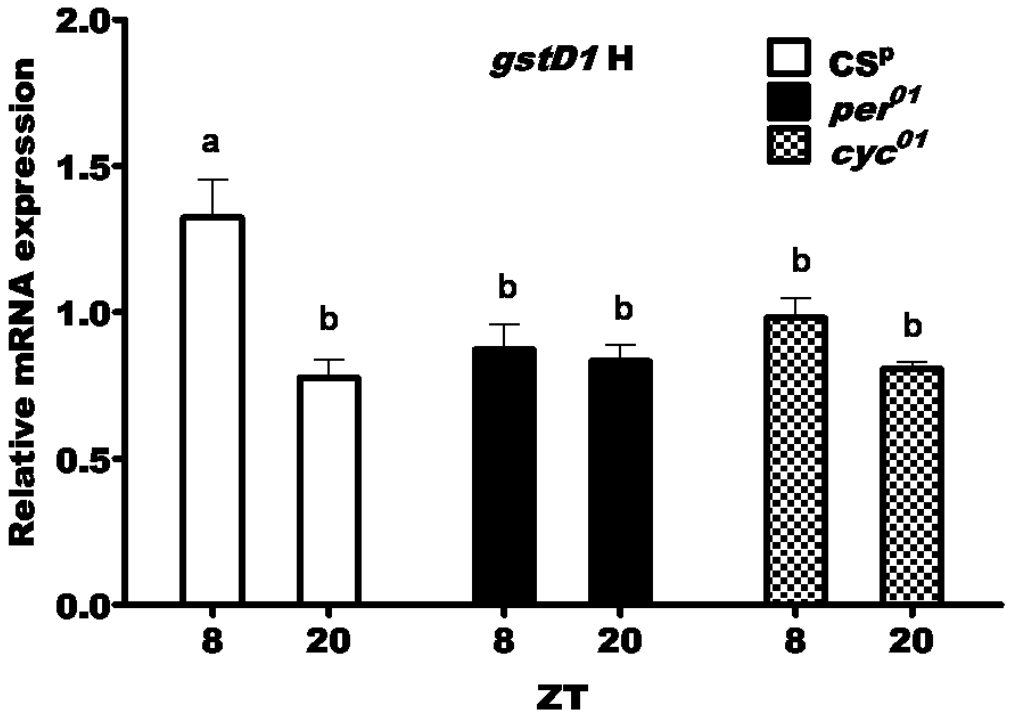

Figure 7. Comparison of expression of *gstD1* mRNA in the heads of control and *per*^{01} or *cyc*^{01} mutant flies

Data shown represents average values obtained from 3 independent bio-replicates. Error bars represent the standard error of the mean (SEM).There is a significant difference in expression level between ZT 8 and ZT 20 for CS^p, ($p<0.01$). There is also a significant difference between the expression level of CS^p at ZT 8 and both time points of *per*^{01} and *cyc*^{01} ($p<0.05$). Data set (a) is significantly different from data sets (b).

3.5 Daily profile of *Gclc* mRNA levels in wild type flies

Glutathione S-transferases, such as *gstD1*, require glutathione (GSH) to conjugate it with toxic compounds. GSH is synthesized by two subunits of glutamate cysteine ligase. The catalytic subunit (*Gclc*) is responsible for the rate-limiting step of GSH synthesis (Orr et al., 2005). Therefore, we tested whether the circadian clock might control *Gclc* mRNA expression. Diurnal pattern of *Gclc* mRNA levels was evaluated in the heads of wild-type flies and a significant circadian rhythm was detected ($p<0.05$). The peak in *Gclc* mRNA expression levels was at ZT 16, during the late night (Figure 8). The trough in expression was during daylight hours at ZT 4-8 (Figure 8). Examination of *Gclc* mRNA levels in bodies of wild-type flies revealed no apparent circadian pattern throughout the day. Since no circadian trend was apparent in the tissue from fly bodies, multiple repeats were not conducted (data not shown). Taken together, the *Gclc* expression patterns suggest that the circadian clock regulates *Gclc* expression in the heads of wild-type flies.

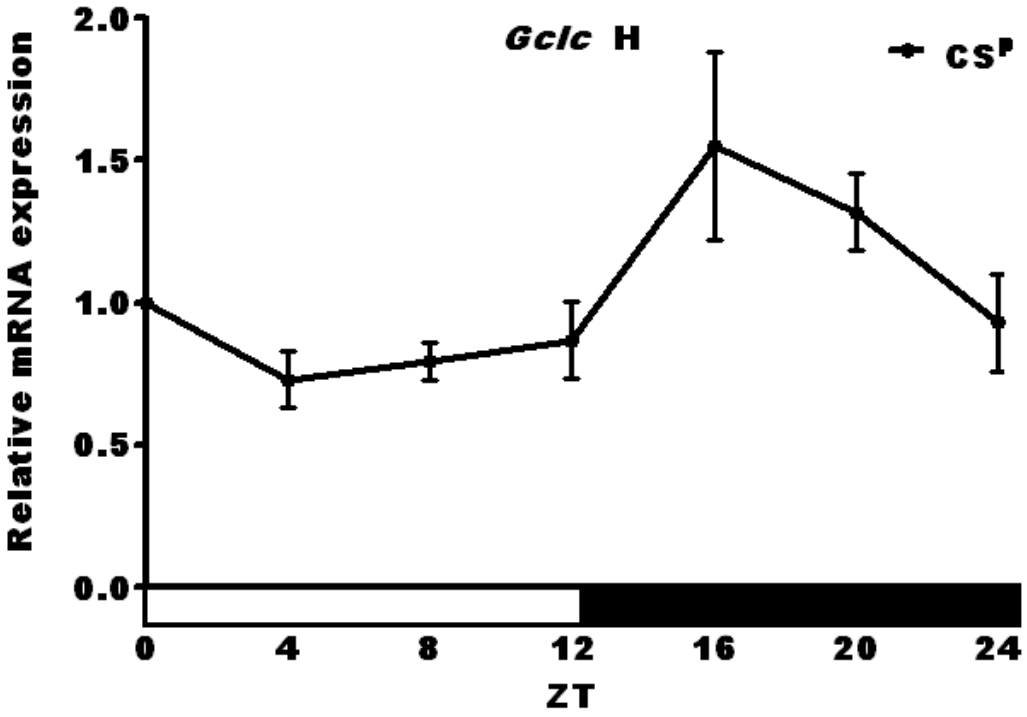

Figure 8. Diurnal profiles of *Gclc* mRNA levels in heads of wild-type flies
Data shown represents average values obtained from 5 independent bio-replicates. Error bars represent the standard error of the mean (SEM). There are significant differences in expression level over the 24 h day. Levels of mRNA at ZT 4 and ZT 8 are significantly different from mRNA levels at ZT 16 ($p<0.05$). There is a 2.14 fold difference between ZT 4 and ZT 16.

3.6 Circadian clock genes may directly regulate *Gclc* mRNA expression

Since a rhythm was detected in *Gclc* mRNA levels in wild-type heads, we tested whether the expression of this gene is altered in flies with a null mutation in clock genes *per* or *cyc. Gclc* expression in the heads of per^{01} flies was significantly higher than in the wild-type control at ZT 4 ($p<0.01$) (Figure 9). *Gclc* expression at ZT 16 was similar to ZT 4 in per^{01} heads, suggesting a relatively constant and high level of expression throughout the day in per^{01} flies. *Gclc* expression was altered in the opposite way in heads of cyc^{01} flies. The expression of this gene was low at both ZT 4 and ZT 16, such that the peak observed in wild-type flies was missing at ZT 16 in cyc^{01} mutants (Figure 9). Taken together these data suggest that the circadian clock regulates *Gclc* mRNA levels because expression is non-cycling in mutants. Furthermore, the consistently high level of *Gclc* mRNA in per^{01} and low level in cyc^{01} suggests that the positive limb of the clock regulates rhythmic transcription of the *Gclc* gene (see discussion).

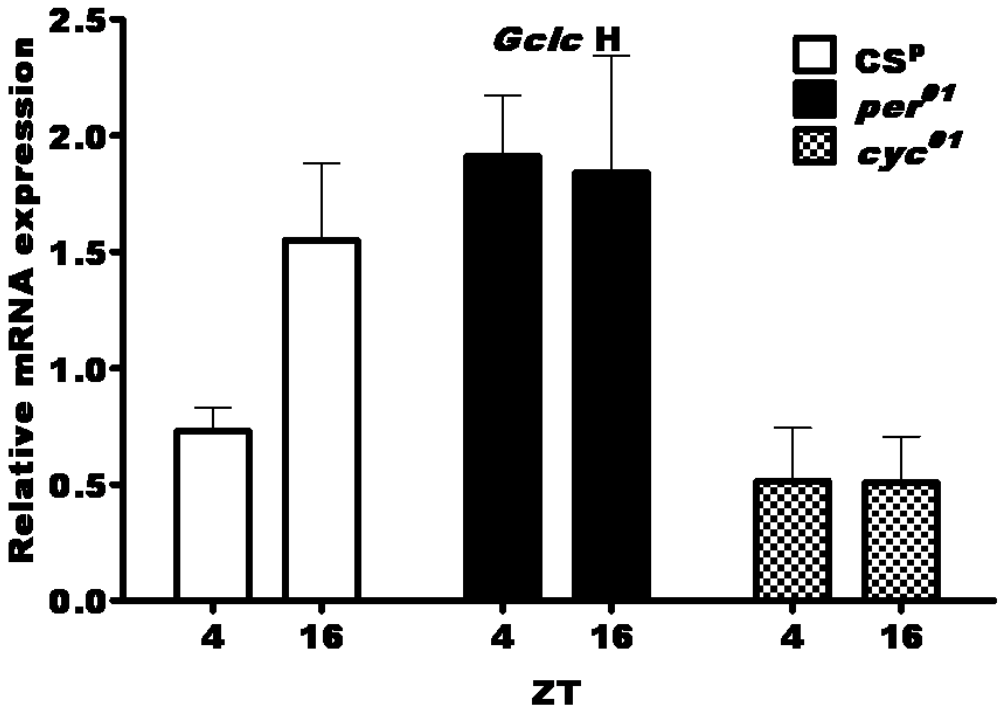

Figure 9. Circadian clock regulation of *Gclc* mRNA expression levels
Data shown represents average values obtained from 3 independent bio-replicates. Error bars represent the standard error of the mean (SEM). There is a significant difference in expression level between ZT 4 and ZT 16 for CS^p, ($p<0.05$). There is also a significant difference between the expression level of CS^p at ZT 4 and per^{01} at ZT 4 ($p<0.01$). Finally cyc^{01} is significantly lower than per^{01} at both ZT 4 and ZT 16 ($p<0.01$).

4. Discussion

4.1 Circadian clock genes do not regulate oxidative stress response genes *cncC/Nrf2* and *keap1*

In this study, we explored the molecular basis of the previously reported circadian susceptibility of *D. melanogaster* to oxidative stress. From a number of independent experiments it was determined that circadian clock genes do not regulate the expression of oxidative stress response genes *cncC/Nrf2* and *keap1* (Figure 10C). The mRNA expression levels were variable throughout the day in both heads and bodies of wild-type flies, but a circadian rhythm was not detected. Our data does not exclude that proteins encoded by *cncC/Nrf2* and *keap1* could act in a rhythmic fashion. Since a diurnal rhythm in ROS levels has been described (Krishnan et al., 2008), a rhythmic change in association between KEAP1 and CNCC/NRF2 heterodimer could facilitate the latter to enter the nucleus and affect target genes (Figure 10A) This possibility remains to be tested experimentally.

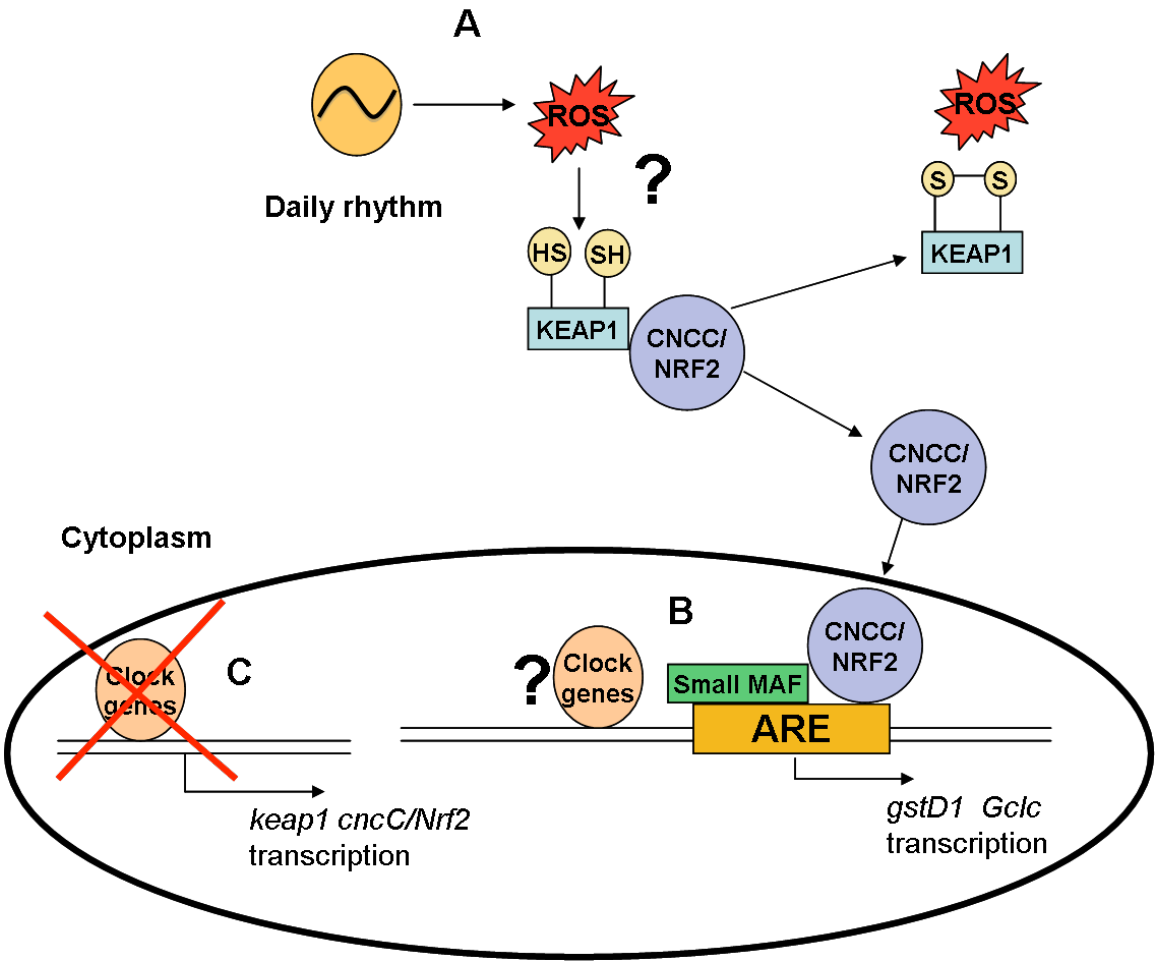

Figure 10. Diagram representation summarizing obtained results
A) A daily rhythm in the levels of ROS was observed in flies (Krishnan et al., 2008). This rhythm could facilitate a change in association between the KEAP1 and CNCC/NRF2 heterodimer that would allow CNCC/NRF2 to enter the nucleus and affect target genes. B) Rhythmic transcription of *gstD1* and *Gclc* suggests that clock genes regulate their transcription. C) Clock genes do not regulate the transcription of *keap1* and *cncC/Nrf2*.

4.2 Transcription of *gstD1* is under circadian control

Despite the lack of rhythm in *cncC/Nrf2* and *keap1*, a clear circadian rhythm was detected in *gstD1* mRNA levels, a gene whose transcription is known to be activated by CNCC/NRF2 (Figure 10B). The rhythm in *gstD1* occurred with a peak at ZT 8 and a trough at ZT 20. This expression pattern independently confirms previous microarray research that suggested a rhythm in *gstD1* (Keegan et al., 2007). This rhythm suggests that *gstD1* is under the control of the circadian clock.

By studying flies with a null mutation in clock genes we obtained a second line of evidence for clock control of mRNA levels of *gstD1*. The rhythm in *gstD1* expression was abolished in both per^{01} and cyc^{01} flies. In both mutants, the expression was significantly reduced from the peak expression levels of CS^{p} at ZT 8. These data are consistent with the recent research demonstrating increased susceptibility to oxidative stress in per^{01} flies (Krishnan et al., 2008).

The significant decrease in *gstD1* mRNA in clock mutant flies suggests that clock genes regulate *gstD1* transcription. Given the mechanism of the circadian clock (Figure 1) and the similar decline observed in mRNA levels of *gstD1* in both per^{01} and cyc^{01}, it is likely that clock genes indirectly control this effector gene. While we excluded transcriptional rhythm in *cncC/Nrf2* and *keap1* as mediators of clock effects, it remains to be determined whether other clock- controlled transcription factors could regulate *gstD1* expression. It has recently been reported that the clock can indirectly regulate transcription of other effector genes via *Pdp1* expression (Beaver et al., 2010). An important next step would be to measure GSTD1 protein levels under similar conditions to determine if they follow the same rhythmic pattern.

It has been suggested that other glutathione S-transferases, including those in the Delta and Epsilon families, have similar rhythmic expression patterns to *gstD1* in *D. melanogaster* heads. The Epsilon types peak during the morning hours followed by the Delta types during the early afternoon (Wijnen and Young, 2006). It should be noted that all expression peaks are during the daylight hours. This further supports the findings on increased susceptibility to oxidative stress in per^{01} flies. The expression of these glutathione S-transferases may be increased during daytime in order to prepare the flies for the increased oxidative stress incurred from increased daytime activities.

While *gstD1* expression was cycling in fly heads, it did not have a significant rhythm in bodies. The brain is especially susceptible to oxidative damage because of its high oxygen utilization, the presence of redox-active metals, and the content of polyunsaturated fatty acids (Valko et al., 2007). The circadian control of *gstD1* expression in heads could be a mechanism to provide increased resistance to high levels of ROS in the brain. With regards to bodies, it must be noted that many different organs, such as the gut, fat body and Malpighian tubules contain functional clocks (Giebultowicz et al., 2000). Although clock genes oscillate with the same phase in all fly peripheral tissues (Giebultowicz, 2001), it is possible that output genes oscillate with a tissue specific phase, resulting in non-rhythmic average expression.

4.3 Circadian control of *Gclc*

A distinct circadian rhythm was observed in *Gclc*, a gene whose transcription is activated by *cncC/Nrf2* (Li et al., 2009). Here we show a rhythm in *Gclc* mRNA levels, which was lower at ZT 4 and ZT 8 and peaked at ZT 16. This rhythm suggests that *Gclc*

may be under the control of the circadian clock. The expression levels in mutant flies suggest direct circadian control of *Gclc* transcription. Expression in flies lacking the *per* gene was consistently high at ZT 4 and ZT 16 similar to peak levels in wild-type flies. In contrast, expression of *Gclc* mRNA in flies lacking the *cyc* gene was consistently low at both at ZT 4 and ZT 16, remaining at the level of the wild-type minimum at ZT 4. A gene that is expressed at a high level in the per^{01} flies and at a low level in the cyc^{01} flies is very likely controlled by the positive limb of the circadian clock. When the *per* gene is absent, CYC/CLK transcriptional activity is never inhibited so genes that are direct targets of CYC/CLK have constitutively high expression. If the gene's transcription is regulated by CLK/CYC proteins, then when *cyc* is absent the target gene is expressed at very low levels. The loss of rhythm in the mutants suggests positive limb circadian control of this oxidative stress repair gene.

While both *gstD1* and *Gclc* genes work together to combat excess ROS in the fly, based on their expression in mutants they may be regulated differently by the clock. *gstD1* is non-cycling and lower in the absence of both *per* and *cyc*, whereas *Gclc* is also non-cycling in mutants but higher in *per* and lower in *cyc*. If the two genes were regulated in the same manner by the clock, then the expression pattern should be more similar in mutants.

An interesting and surprising finding of this study is that mRNA expression pattern peaks at different times of the day in *gstD1* and *Gclc*. While *gstD1* peaks at ZT 8, *Gclc* peaks at ZT 16. A possible explanation for this difference in peak expression is the relationship between glutathione production and glutathione utilization. More specifically, *gstD1* and *Gclc* may cycle out of phase because *gstD1* requires GSH to

function, and GSH is synthesized by *Gclc* (Wu et al., 2004). As *gstD1* and other gst's mRNA levels peak in the late day, their proteins are also likely to increase and may use up GSH at this time. A subsequent increase in *Gclc* during the night would replenish GSH levels when the flies are most active. To determine whether GSH synthesis is clock controlled will require examining diurnal patterns of GCLc protein and GSH levels.

To the best of our knowledge, the circadian control of *Gclc* has not been reported before. This is an important finding, because of the crucial role of *Gclc*-encoded enzymes in ROS homeostasis. It has been reported that over-expression of *Gclc* extends lifespan in *D. melanogaster* by up to 50% by providing increased resistance to oxidative stress (Luchak et al., 2007; Orr et al., 2005). Complete knockout of *Gclc* is lethal (Radyuk et al., 2009). *Gclc* is an essential gene in the process of combating oxidative stress. It has previously been observed that flies are more susceptible to oxidative stress, and have a higher mortality, at ZT 8 than ZT 20 (Krishnan et al., 2008). The peak of *Gclc* expression at ZT 16 was observed near the same time of day when oxidative stress effects were found to be lower in wild-type flies and also corresponds with lower amounts of mitochondrial ROS (Krishnan et al., 2008).

Higher expression of *Gclc* was observed in per^{01} flies. This does not appear to be protective based on previous research demonstrating increased mortality in *per* mutants (Krishnan et al., 2008). The data presented here suggest that flies lacking the *per* gene likely have additional deleterious effects that cannot be made up for by the increased levels of *Gclc*. In addition, the benefits of *Gclc*, such as lifespan extension are only possible when all other systems are working properly. Though the expression of *Gclc* in per^{01} flies is at a higher level, it is likely that it is not beneficial to the organism because

of general dysregulation of metabolic processes that are known to occur in clock-disrupted organisms (Reddy and O'Neill, 2009). This could be explored in the future by testing whether or not lifespan extension could be achieved by overexpression of *Gclc* in per^{01} flies.

4.4 Circadian clocks and aging

The important knowledge gained in this study concerning rhythmic expression of *gstD1* and *Gclc* may have future implications for investigation of aging in mammals. *Drosophila* and mice have genetically similar circadian clocks (Panda et al., 2002) and homologous genes are involved in oxidative stress protection in flies and mammals. When BMAL1, a homolog of the fly *cyc* gene, was knocked out the mice had shortened life spans and symptoms of premature aging. The mice also had increased levels of ROS in some of their tissues (Kondratov et al., 2006). Similar results have been found in flies; loss of function of the gene *per* resulted in accelerated aging and decreased functionality in flies following a challenge of exogenous oxidative stress (Krishnan et al., 2009). These results indicate that *D. melanogaster* and mammals with mutant clock genes have a phenotype of premature aging and/or age related diseases. The accumulation of ROS in the cells and tissues of these organisms is one possible reason to explain the premature aging in mutants. It has been determined that the species that have longer life spans have more efficient oxidative repair mechanisms, indicating the importance of oxidative repair genes (Valko et al., 2007). The rhythmic expression of *gstD1*and *Gclc* suggests that the circadian clock has the potential to repair ROS thus uncovering a possible molecular mechanism to explain the observed phenotypes. This potential mechanism, along with

previous research demonstrating decreased lifespan in *per* mutants (Krishnan and Giebultowicz, 2009), suggests that functional circadian clocks may prevent premature aging in flies by preventing age-related accumulation of detrimental levels of ROS.

4.5 Implications for health and diseases

Circadian rhythms are important coordinators of many life functions and their disruption contributes to many diseases (Reddy and O'Neill, 2009). Our data provide novel information on how the circadian clock may aid antioxidant defense systems. ROS have been implicated as a key player in numerous chronic diseases, including cardiovascular disease, cancer, Alzheimer's disease, and Parkinson's disease. Decreased glutathione levels have been linked with all of these diseases (Valko et al., 2007). Cancer incidence is also associated with increased ROS; individuals with disrupted circadian clocks, such as in swing shift workers, have an increased rate of tumor formation (Antoch et al., 2008). Data presented here suggests that the circadian clock can modulate the repair of oxidative stress caused by ROS. Thus it is worth investigating if manipulating the clock can decrease the incidence of disease, improve overall health, and promote healthy aging.

4.6 Future Directions

This data raises many new questions concerning the circadian regulation of oxidative stress repair. Now that it has been demonstrated that circadian clock genes regulate oxidative stress repair genes *gstD1* and *Gclc* at the mRNA level, the expression

profile of protein levels of GSTD1 and GCLC should be determined using a western blot. The daily expression profiles of the other glutathione S-transferases in the Delta and Epsilon families should be measured to determine if the circadian clock regulates all enzymes of this type. *per* and *cyc* could also be over-expressed to determine if this would change the mRNA expression of *gstD1* and *Gclc*. It would also be interesting to investigate mRNA expression in aging wild-type flies to measure if levels of *gstD1* and *Gclc* have changed. All of these experiments could provide further insights into the regulation of *gstD1* and *Gclc* by the circadian clock.

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