Title: The Clock:cycle complex is a major transcriptional regulator of *Drosophila* photoreceptors that protects the eye from retinal degeneration and oxidative stress

Running title: Clock activity in *Drosophila* photoreceptor neurons

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Abstract:

The aging eye experiences physiological changes that include decreased visual function and increased risk of retinal degeneration. Although there are transcriptomic signatures in the aging retina that correlate with these physiological changes, the gene regulatory mechanisms that contribute to cellular homeostasis during aging remain to be determined. Here, we integrated ATAC-seq and RNA-seq data to identify 61 transcription factors that showed differential activity in aging *Drosophila* photoreceptors. These 61 age-regulated transcription factors include two circadian regulators, Clock and cycle, that showed sustained increases in activity during aging. When we disrupted Clock activity in adult photoreceptors, we observed changes in expression of 15 – 20% of genes including key components of the phototransduction machinery and many eye-specific transcription factors. Using ATAC-seq, we showed that loss of Clock activity leads to changes in activity of 31 transcription factors and causes a progressive decrease in global

24 levels of chromatin accessibility in photoreceptors. Supporting a key role for Clock-dependent
 25 transcription in the eye, disruption of Clock activity in photoreceptors also induced light-
 26 dependent retinal degeneration and increased oxidative stress, independent of light exposure.
 27 Together, our data suggests that the circadian regulators Clock and cycle act as neuroprotective
 28 factors in the aging eye by directing gene regulatory networks that maintain expression of the
 29 phototransduction machinery and counteract oxidative stress.

30 **Key words:** *Drosophila*, eye, aging, Clock, transcription, visual, photoreceptor, neuron.

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Introduction

One of the hallmarks of the aging eye, as well as many age-related eye diseases, is the loss of photoreceptor function and survival (1,2). The aging epigenome and transcriptome of cells in the retina undergo changes that correlate with decreased visual function and increased cell death (3–5). Importantly, disruption of epigenetic mechanisms is associated with the onset of age-related eye diseases, such as age-related macular degeneration (6,7), suggesting that transcriptional regulation contributes to the changes in homeostasis that are observed in the aging eye. However, we still have a basic understanding of how the molecular mechanisms that drive the age-associated changes in the transcriptome increase the risk of ocular disease with advanced age.

Transcription factors (TF) function as regulatory hubs of gene expression programs in a highly-tissue specific manner. While several conserved pathways contribute to changes in the aging transcriptome across tissues, such as the longevity-associated FOXO and Insulin axis (8–10), age-associated changes in gene regulatory networks can be highly specific to individual cell types (5,11). The emergence of bioinformatic and computational approaches that combine chromatin accessibility data with transcription factor binding sites (TFBS) has allowed researchers to interrogate unbiasedly how transcription factor activity changes at a genome-wide scale in different biological conditions by estimating changes in chromatin accessibility around TFBS (see (12) for review on chromatin accessibility data analysis). The majority of chromatin accessibility studies in the eye have focused on development in invertebrate (13) or vertebrate models (14), and TF activity in the developing eye has often been assessed based on transcriptomic-based co-expression inference rather than chromatin accessibility (15). Further, ATAC-seq analysis of retinal pigmented epithelium from patients with age-related macular degeneration identified global changes in chromatin accessibility at the onset of the disease state, suggesting that differential activity of regulatory elements strongly contributes to

the initial stages of age-associated ocular disease (16). Thus, identification of TFs with differential activity in the aging eye could provide insight into the mechanisms that contribute to the increased risk of retinal degeneration during aging.

Drosophila melanogaster, like humans, experience an age-dependent decline in visual function coupled with increased risk of retinal degeneration (3,17). Although flies possess a compound eye that differs anatomically from the vertebrate eye, much of the phototransduction machinery is conserved between flies and mammals, with the outer fly photoreceptors resembling vertebrate rods in function (18,19). Considering its relatively short lifespan, *Drosophila* provides a useful model system for studying the processes involved in normal aging within specific tissues, such as the eye (20). To profile the transcriptome and epigenome of specific cell types in the eye, we have developed a cell type-specific nuclei immunoenrichment technique that we have previously used to examine gene expression in aging photoreceptors (3,21,22). Here, we applied this technique to profile the transcriptome and chromatin accessibility landscapes of *Drosophila* photoreceptors across an extended time course into relatively old age. By integrating these aging data from photoreceptors, we identified 61 TFs with substantial changes in activity during aging, including increased activity of two of the master circadian regulators, Clock (Clk) and cycle (cyc). Using genetic approaches, we showed that these circadian regulators have an integral role in controlling gene expression and global levels of chromatin accessibility in photoreceptors, and that Clock activity is essential for the survival of photoreceptors that are exposed to the normal daily light cycle. Our data identify a novel neuroprotective role for the circadian clock in the *Drosophila* eye, and suggest that this role may become increasingly critical in advanced age to prevent retinal degeneration.

Results

Tissue-specific profiling of the photoreceptor nuclear transcriptome reveals significant changes during early and late aging

To profile the transcriptome of aging *Drosophila* photoreceptors, we used our recently improved tissue-specific nuclei immunoenrichment approach (22). Briefly, we tag nuclei using a Green Fluorescent Protein (GFP) fused to the “Klarsicht, ANC-1, Syne Homology” (KASH) domain, which anchors GFP to the outer nuclear membrane and allows for nuclei purification; GFP^{KASH} is expressed in outer photoreceptors under Rh1-Gal4 control, herein referred as Rh1>GFP^{KASH} (Fig. 1A). Similar to wild-type strains, such as OregonR and Canton-S (23), Rh1>GFP^{KASH} flies begin to show a substantial decline in viability after day 60, with 50% of male flies dying by day 70 (3). Thus, to generate a comprehensive aging dataset, we purified photoreceptor nuclei over 10-day windows from day 10 until day 60 and performed RNA-seq (Fig. 1B).

We assessed the overall variability of the RNA-seq samples using Principal component analysis (PCA). The RNA-seq samples clustered together with 48.3% of the variation separating the samples by age in a progressive manner (Fig 1C). We also observed a similar grouping by age using Pearson’s correlation analysis with high concordance between the three biological replicates (Pearson’s $r > 0.91$) (S1 Fig). These observations reveal progressive changes in transcription during aging in photoreceptors, similar to previous studies from our group that aged flies to day 40 (D40) (3). Differential expression analysis of each time point relative to day 10 (D10), the youngest state, revealed significant changes in gene expression during aging ($p\text{-adj} < 0.05$) (Fig. 1D-S1 table). Hierarchical clustering of the genes that were differentially expressed between any age and D10 revealed that 1412 and 982 genes showed progressive increases, or decreases in expression, respectively. Gene Ontology (GO) term analysis revealed that the genes that were age-upregulated (Clusters 2 and 3) were enriched for several processes including cytoplasmic translation and double strand break repair (Fig. 1E). In contrast, the genes that were age-downregulated (Cluster 5) were enriched for neuronal processes, such as cognition and regulation of nervous system development. Although 75% of the differentially expressed genes (2394 out of 3156) exhibit progressive changes, we also

identified two clusters with expression changes only during early aging (D20; Clusters 1 and 4). We note that while D20 was separated by age from the other samples along PC1, it was also separated from its nearest time points (D10 and D30) along PC2 (Fig. 1C), consistent with these early and transient changes in gene expression in aging photoreceptors. Interestingly, Cluster 4, which contained genes that showed an early decrease in transcription but increased levels again at later time points, was enriched for protein folding. These age-associated transcriptomic signatures are consistent with our previous observations in aging photoreceptors and with aging studies from other tissues (3,4).

Photoreceptors undergo substantial changes in transcription factor activity during aging

To identify mechanisms that contribute to the age-associated transcriptomic changes in photoreceptors, we sought to evaluate changes in TF activity. TFs regulate transcriptional outputs of their targets by acting as activators or repressors of gene expression at the transcriptional level. Importantly, TF activity can be affected by several factors, including protein levels, localization, and post-translational modifications (24,25). Because of the technical complexity of isolating intact photoreceptors for proteomic studies, and the relatively low protein abundance of TFs in the eye (26), we used our RNA-seq data to assess how transcript levels of genes associated with the GO category “DNA-binding transcription factor activity, RNA polymerase II-specific” (GO:0000981) changed during aging. Notably, 23% of TFs showed significant differential expression during aging at the nuclear transcript level (Fig. 2A), suggesting that differential TF activity could contribute to the aging transcriptome of photoreceptors. To identify TFs with differential activity during aging, we used *diffTF*, a software package that analyzes TF activity based on differences in aggregate ATAC-seq signal around predicted/validated TF binding sites, or TFBS (27). To do this, we first profiled the chromatin accessibility landscape of aging photoreceptors at the same time-points as for RNA-seq. PCA of ATAC-seq samples revealed that 69.7% of the variation could be explained by age (Fig. 2B),

and Pearson correlation analysis showed high concordance between biological replicates. Additionally, accessible peak annotation revealed a stable distribution of peaks through-out aging (S2A Fig). Together with the RNA-seq aging time course, these data indicate that the chromatin accessibility and transcriptional landscape of photoreceptors undergo progressive changes during aging. We then used *diffTF* to compare all time points relative to D10. The TFBS used for this analysis were generated by CIS-BP, which provides a comprehensive dataset of experimentally validated TFBS (28). We also examined *de novo* and known motifs identified using Homer (29) (Fig. 2C). We took advantage of the aging RNA-seq time series data to perform *diffTF* analysis in integrative mode, enabling us to discard TFs that were not expressed in adult photoreceptors. Using this approach, we identified 61 TFs with significant differential activity during aging (FDR<0.001) (Fig. 2C), herein referred to as “age-regulated TFs”. Most of these age-regulated TFs showed continuous changes in activity with age, with nearly two thirds showing increased activity with age. We observed an almost two-fold increase in the number of age-regulated TFs identified at D50 and D60 relative to younger ages, suggesting that late aging is associated with distinct changes in gene regulatory networks (Fig. 2D). Hierarchical clustering of these age-regulated TFs by the mean weighted difference between each age comparison resulted in grouping of TFs that are known to associate *in vivo*, such as *Motif 1 Binding Protein* (M1BP), *DNA replication element factor* (Dref) and *Boundary element-associated factor of 32kD* (BEAF32), which bind topologically associating domains (Ramirez, 2018). We also identified *moira* (*mor*), which physically interacts with *similar* (*sima*) (30). Additionally, we identified *seven up* (*svp*) and *Pvull-PstI homology 13* (*Pph13*), which physically interact to regulate eye-specific transcriptional programs together with *eyeless* (*ey*) (31). Considering that *svp*, *ey* and *Pph13* show modest but significant increases in TF activity with age, our data also indicate that photoreceptor identity remains distinct even at advanced age in flies.

The stress response is transcriptionally regulated during aging across a broad range of tissues (32). Accordingly, we identified several TFs that are involved in the regulation of stress response genes as having differential activity with age including the *Drosophila* JUN-FOS complex that is formed by *Jun-related antigen (Jra)* and *kayak (kay)* (33), the *Drosophila* homolog of Hypoxia response factor HIF-1a, *similar (sima)* (34), *Heat shock factor (Hsf)* (35), and *Activating transcription factor-2 (Atf-2)* (36,37). We also identified *cropped (crp)*, which has previously been associated with aging through *in silico* analysis due to interactions with DNA repair pathways (38).

One of the most interesting changes in TF activity during aging was in the activity of the master circadian regulators *Clock (Clk)* and *cycle (cyc)*, which showed a progressive increase in TF activity during aging. CLK and CYC bind the same CACGTG motif upon heterodimerization, explaining the identification and clustering of both factors in the *diffTF* analysis, which uses DNA sequence motifs as the TFBS source (S2B Fig). The circadian clock is a molecular time keeper that controls rhythmic behaviors, such as locomotor behavior, which is coordinated by pacemaker neurons located in the brain (39). In *Drosophila*, many peripheral tissues also contain working clocks, including the fat body, Malpighian tubule cells, and the retina (40). Importantly, aging has been associated with changes in the circadian clock (41,42). We note that flies for the aging time course experiment were raised under a 12:12 hour light-dark cycle, and were harvested at relative Zeitgeber time (ZT) 6 +/- 1 hour (see methods), suggesting that the enrichment of Clk and cyc in the *diffTF* analysis is not simply due to a time-of-day bias in sample collection. Taken together, our data identify a subset of TFs that exhibit significant changes in activity during aging in photoreceptors, including TFs associated with stress response and circadian rhythm.

Clock regulates the transcriptional output of phototransduction genes in photoreceptors

Clock and cycle are basic Helix-Loop-Helix (bHLH)-TFs that form a heterodimer (CLK:CYC) to control rhythmic expression of their targets. Canonical targets of the CLK:CYC complex include core clock genes, such as *vri* (*vri*), *PAR-domain protein 1* (*Pdp1*), *timeless* (*tim*), *period* (*per*), and *clockwork orange* (*cwo*). However, Clk and cyc also regulate transcription of many other genes either directly or indirectly, and these genes are often referred as “Clock-output genes”, and can include tissue-specific genes (43,44). To evaluate the biological role of Clock-dependent transcription specifically in photoreceptors, we disrupted the CLK:CYC complex by expressing a dominant negative version of CLK (CLK^{DN}) specifically in outer photoreceptors under Rh1-Gal4 control. CLK^{DN} lacks the basic DNA binding domain, impairing its recruitment to DNA without disrupting its ability to form a heterodimer with cyc (Fig. 3A) (45). Expression of CLK^{DN} inhibits Clock-dependent transcription and rhythmic behaviors *in vivo* when expressed in pacemaker or antennal neurons (45). To facilitate nuclei immuno-enrichment for RNA-seq analysis, we co-expressed UAS-GFP^{KASH}. As a control, we profiled the transcriptome of photoreceptors that expressed LacZ, herein referred as Rh1>Ctrl. We collected both Rh1>CLK^{DN} and Rh1>Ctrl flies at ZT 9, when clock-dependent transcription is active (46,47), harvesting flies at D1 and D10 to study the progressive effect of disruption of the circadian clock in adult photoreceptors (Fig. 3B). We note that Rh1-Gal4 activity begins in the very late stages of pupal development (48); thus Rh1>CLK^{DN} flies have disrupted CLK:CYC activity in adult photoreceptors, but not in the developing eye.

Differential gene expression (DGE) analysis of Rh1>CLK^{DN} relative to Rh1>Ctrl at either D1 or D10 revealed a consistent downregulation of direct CLK:CYC targets such as *vri*, *per*, *tim* and *Pdp1* (Fig. 3C-S2A Fig). We also observed a significant upregulation of *Clk* itself, which we showed by qPCR reflects expression of *Clk*^{DN} rather than the endogenous wild-type *Clk* (S2B Fig). When we compared the control D1 and D10 flies, we did not observe differential expression of core clock genes, suggesting there is little change in the circadian clock in the

early stages of adult life. In addition, only 147 genes changed in control flies between D1 and D10, indicating there is relatively little change in gene expression in general at these early stages of adult life in photoreceptors. In contrast, when we disrupted CLK:CYC activity by expressing CLK^{DN}, we observed significant changes in expression of 15% and 22% of actively transcribed genes in photoreceptor at D1 and D10, respectively. These data demonstrate that sustained loss of CLK:CYC activity leads to progressive dysregulation of gene expression in photoreceptors at nearly a quarter of expressed genes.

To further identify direct versus indirect targets of CLK:CYC, we compared previously published high-confidence binding sites identified for Clk and cyc using ChIP-seq in *Drosophila* heads and bodies (49) with our list of accessible peaks obtained with ATAC-seq data from D10 photoreceptors. Genomic overlap analysis revealed that 10% of accessible peaks contained an experimentally identified Clk-cyc TFBS (Fig. 3D). Next, we annotated these photoreceptor-specific TFBS (n=1461) to their nearest transcription start site (TSS) and found that 1004 photoreceptor-expressed genes contained a potential Clk-cyc binding site. We refer to these 1004 genes as “Clock-bound genes” or CBGs (Fig. 3E), which we compared to genes that were differentially expressed in Rh1>CLK^{DN} relative to Rh1>Ctrl at either D1 or D10. We reasoned that if a gene is bound by Clk and is differentially expressed, then it can be classified as a “direct target” of CLK:CYC. Using these criteria, we identified 475 direct targets of Clock in photoreceptors. In contrast, we identified a far greater number of genes (2253) that are likely to be indirect targets of CLK:CYC regulation, at least based on the available ChIP-seq data from whole heads (49). Consistent with the predicted role of CLK:CYC as transcriptional activators, Clock direct targets were more likely to be downregulated relative to indirect targets upon expression of Clk^{DN} (S3C Fig). GO term analysis of direct CLK:CYC targets showed significant enrichment of genes associated with several biological processes, including phototransduction and circadian rhythm (Fig. 3F). However, both direct and indirect CLK:CYC targets were

enriched for processes including TF activity and eye development. This is consistent with previous reports that have proposed that CLK:CYC act at the top of a TF hierarchy, directly regulating transcript levels of key TFs in specific cell types, thereby indirectly regulating expression of their target genes (49,50). Notably, individual inspection of the TFs that were classified as direct CLK:CYC targets confirmed that several eye-specific TFs such as *ocelli* (*oc/otd*) and *eyeless* (*ey*) are bound at their promoters directly by Clk and cyc (Fig. 3G). To further identify the magnitude of changes in expression of these pathways upon disruption of CLK:CYC, we performed gene set enrichment analysis comparing CLK^{DN} vs Control at either D1 or D10 and obtained ridge plots showing the density of fold change for the genes associated with each pathway. Whereas upregulated genes showed significant enrichment of several biological processes, including TF activity, downregulated genes were associated with several phototransduction-associated pathways, including deactivation of rhodopsin signaling (Fig. 3H, S3D-E Fig).

Because light entrains the circadian clock to activate Clock-mediated transcription, we next performed RNA-seq in photoreceptors from young Rh1>GFP^{KASH} flies (D10) reared in constant darkness (DD) and compared this with flies of the same age raised under standard light:dark (LD). Under these conditions, we observed differential expression of genes associated with several biological processes, including phototransduction and circadian rhythm in dark raised flies relative to LD (S3F-G Fig). Because these processes were also enriched in the genes with differential expression upon disruption of CLK:CYC activity, our data suggest that these biological processes are normally regulated by the circadian clock in photoreceptors in response to the light/dark cycle.

Taken together, these data show that the CLK:CYC complex is a major transcriptional regulator of the photoreceptor transcriptome, including the phototransduction pathway. Additionally, our

data suggest that Clock activity regulates gene regulatory networks by regulating expression of TFs that in turn direct expression of a large proportion of the transcriptome in photoreceptors.

Clock activity promotes changes in TF activity and maintains global levels of accessibility in photoreceptors

To investigate the indirect effects of Clock activity on the photoreceptor transcriptome, we further investigated the TFs that showed disrupted expression upon loss of Clock activity. Gene Concept Network (cnet) plots demonstrated significant enrichment of 62 TFs amongst the upregulated genes in flies expressing Clk^{DN} (Fig. 4A). Based on these changes in TF expression, we wondered if disrupting Clock activity would also lead to significant changes in TF activity relative to Rh1>Ctrl flies. To test this, we profiled chromatin accessibility in Rh1>CLK^{DN} versus control, and integrated the RNA-seq and ATAC-seq data using *diffTF* as in Fig. 2. PCA analysis of the accessible chromatin landscape revealed that 77% of the variation was explained by expression of CLK^{DN} (Fig. 4B). In addition, CLK:CYC targets such as *tim*, *per*, *vri* and *Pdp1* exhibited significant decreases in accessibility through-out the gene body upon loss of CLK:CYC activity (Fig. 4C). Importantly, genomic annotation of accessible peaks revealed a stable distribution of peaks in all samples (S4A Fig), suggesting that disruption of CLK:CYC activity does not lead to overall changes in the genome-wide distribution of accessible peaks. Rather, these data show that CLK:CYC activity increases chromatin accessibility at target genes, consistent with the well characterized role of Clk and cyc in transcription activation (51).

Using *diffTF*, we identified 37 TFs with differential activity upon disruption of CLK:CYC. Whereas 15 TFs had decreased activity, 22 showed increased activity in Rh1>CLK^{DN} relative to Rh1>Ctrl (Fig. 4D). Interestingly, several of the genes encoding these Clock-regulated TFs are also directly bound by Clk and cyc at their promoters (see Figure 3), including the eye-specific TFs *oc/Otd* and *ey*, as well as *Xrp1*, *onecut*, *crp* and *Trl*. We also identified increased TF activity of the circadian regulator *vri* (*vri*), which binds and represses Clock expression at the

transcript level (52). Consistent with the reported role of *vri* as a repressor, we observed decreased levels of wild-type *Clk* transcript in Rh1>CLK^{DN} flies using qPCR with primers that differentiate between the wild-type and dominant negative version (see S2B Fig). Thus, these data show that Clock activity contribute to the maintenance of gene regulatory networks in photoreceptors by regulating transcript levels and/or activity of many transcription factors.

Visual inspection of chromatin accessibility tracks showed that the Rh1>CLK^{DN} flies had an overall decrease in chromatin accessibility around promoters and gene bodies. To further evaluate this, we obtained gene metaplots averaging the ATAC-seq signal around promoters for all actively expressed genes in Rh1>CLK^{DN} and Rh1>Ctrl flies. Strikingly, we observed that disruption of Clock activity led to a widespread decrease in chromatin accessibility, and this decrease in global accessibility was exacerbated at D10 relative to D1 (Fig. 4E). These gene-based observations were corroborated by peak-based quantification (S4B Fig). Thus, our data suggest that disruption of Clock activity results in a global decrease in chromatin accessibility in photoreceptors, even though *Clk* and *cyc* only have 475 direct gene targets in this cell type (Fig. 3E). To further gain insight if these changes in accessibility were progressive between D1 and D10 for a given gene, we obtained heatmaps of ATAC-seq fold change signal for actively expressed genes at either D1 or D10 in which both heatmaps were sorted identically (Fig. 4F). These heatmaps revealed that genes that had decreased accessibility at D1 also showed sustained decreases in accessibility at D10, suggesting that Clock has a global role in increasing chromatin accessibility in photoreceptors at a large fraction of actively expressed genes.

Taken together, our data shows that Clock activity contributes to the regulation of TF levels and/or activity, which potentially lead to modulation of gene regulatory networks associated with these TFs. In addition, disruption of Clock activity leads to a widespread decrease in chromatin accessibility that is independent from changes in gene expression, suggesting that Clock activity

contributes to the global maintenance of chromatin remodeling of actively expressed genes in *Drosophila* photoreceptors.

Disruption of Clock activity leads to light-dependent retinal degeneration and light-independent accumulation of oxidative stress

Disruption of the phototransduction machinery by mutations in phototransduction genes that leads to loss of function or decreased expression is often associated with retinal degeneration (53). Consistent with the GSEA analysis (see Fig. 3H), cnet plots evaluating RNA-seq fold change for genes associated with phototransduction revealed significant decreases in transcript levels of 22 genes in flies expressing *Clk*^{DN} (Fig. 5A-S5 Fig). These genes included many phototransduction components whose loss leads to light-dependent retinal degeneration, such as *Cds*, *Arr2*, *rdgB*, *trp*, *rdgC*, and *ninaC* (marked with a red asterisk, Fig. 5A) (53). Thus, we hypothesized that disruption of CLK:CYC activity in *Drosophila* photoreceptors would lead to retinal degeneration. To test this, we examined photoreceptor integrity using optical neutralization in Rh1>CLK^{DN} and Rh1>Ctrl flies that were maintained in either standard 12:12 hour light:dark (LD) or free-running, or constant darkness (DD) conditions. We found that Rh1>CLK^{DN} flies showed significant retinal degeneration by D10 when raised under standard light:dark (LD) conditions relative to Rh1>Ctrl flies (Fig. 5B-C). Retinal degeneration was observed in an additional UAS-CLK^{DN} line (not shown), and was progressive with no phenotype observed at D1 and intermediate levels of degeneration at D5, although the CLK^{DN} transcript was detected already by D1 (see S3B Fig). Strikingly, rearing Rh1>CLK^{DN} flies in constant darkness (DD) prevented the onset of retinal degeneration, demonstrating that CLK:CYC activity is required to prevent light-dependent retinal degeneration in adult *Drosophila* photoreceptors.

Clock activity has also been shown to be necessary to protect against oxidative stress. For example, flies carrying the *Clk*^{Drk} allele, which produces a mis-spliced *Clk* transcript and leads to

decreased clock activity (54), show increased levels of reactive oxygen species (ROS) in aging brains (55). Since light is a major source of oxidative stress in the eye (56) and Rh1>CLK^{DN} showed light-dependent retinal degeneration, we hypothesized that Rh1>CLK^{DN} flies exposed to light had increased oxidative stress levels relative to Rh1>Ctrl flies. To test this, we performed a targeted metabolite assay to measure the ratio of reduced and oxidized glutathione (GSH:GSSG) in Rh1>CLK^{DN} and Rh1>Ctrl flies reared in LD or DD conditions at D1 and D10. In this assay, a lower GSH:GSSG ratio is indicative of increased oxidative stress levels (57). We note that given the technical limitations for isolation of intact photoreceptors, we performed these targeted metabolite assays using dissected eyes. Using this approach, we found that oxidative stress levels did not show significant changes at D1 in any condition (Fig. 5D-left). Unexpectedly, we observed a significant decrease in the GSH:GSSG ratio in Rh1>CLK^{DN} flies relative to Rh1>Ctrl flies raised in either LD and DD conditions at D10 (Fig. 5D-right). Thus, our data shows that Clock activity protects the *Drosophila* eye against oxidative stress. However, since expression of CLK^{DN} only caused retinal degeneration in flies reared in LD, it is unlikely that the increased ROS levels are responsible for the retinal degeneration observed in CLK^{DN} flies at D10.

Together, these data suggest that the disruption in expression of the phototransduction machinery in photoreceptors that lack Clock activity is likely responsible for the light-dependent retinal degeneration that we observed. When we performed GO term analysis of genes that were differentially expressed in Rh1>CLK^{DN} at both D10 and D1, observed significant upregulation of genes associated with response to unfolded protein and response to topologically incorrect protein, including many heat shock proteins and chaperones (Fig. 5E-5F). When the light-sensing Rhodopsin 1 (Rh1) is not properly inactivated, this can lead to retinal degeneration in a light-dependent manner (53). Importantly, the onset of light-dependent retinal degeneration associated with failures in Rh1 inactivation can be caused by several factors,

including excessive endocytosis of Rh1 and increased Ca^{2+} influx (53,58–60). We propose that Clock activity is protective in the retina because it promotes expression of genes that contribute to proper recycling of Rh1 upon light-exposure. However, our data also uncover an additional neuroprotective role of Clock by contributing to the response to oxidative stress, which might be important for additional tissues that do not contain the phototransduction machinery. This secondary role of Clock in the cellular response to oxidative stress might become increasingly important in advanced age, providing another potential function for the increased CLK:CYC activity that we observe in aging photoreceptors.

Discussion

The circadian clock maintains daily biological rhythms by controlling the expression of target output genes, and is highly conserved between *Drosophila* and humans (39). Although the eye is not required for circadian behavior in flies (61,62), many genes are rhythmically expressed in the retina including most of the phototransduction machinery (49,63,64). Here, we report that retina-specific disruption of the circadian clock in *Drosophila* leads to progressive light-dependent retinal degeneration. This role for the circadian clock in maintaining visual health is likely conserved in mammals because retina-specific disruption of BMAL1 (*chx10^{Cre};Bmal1^{fl/fl}*) accelerates the loss of cone viability and function in aging mice, which otherwise show normal lifespan (65). The circadian clock also plays a broader role in maintaining health during aging because mice deficient in BMAL1 in all tissues have reduced lifespan and several symptoms of premature aging including cataracts and neurodegeneration (66–68). Moreover, homozygous *Per^{luc}* mice exhibit age-dependent photoreceptor degeneration and premature aging of the retinal pigment epithelium (69). Since the PER::LUCIFERASE fusion protein is wild type and is used as a model for studying circadian rhythms (70), this observation suggests that even very mild changes in the expression or function of core circadian clock regulators can negatively impact the health of the aging eye. Notably, disruption of circadian rhythms in the human eye

contributes to glaucoma and is also implicated in development of myopia (71,72). We note that while preparing this manuscript, a preprint showed that expression of CLK^{DN} under the photoreceptor-specific trpl-Gal4 driver caused decreased phototactic behavior in young flies relative to an age-matched control (73), which is consistent with the rhabdomere loss observed in our study. However, rhabdomere integrity was not tested by the authors of this preprint. We conclude that the circadian clock plays a key role in regulating visual health and photoreceptor survival during aging that is likely conserved across the animal kingdom.

How does disruption of the circadian clock in photoreceptors lead to light-dependent retinal degeneration? Our data suggest that although Clk-dependent transcription is necessary to prevent high levels of oxidative stress in the eye, this is not the proximal cause of retinal degeneration in young flies. Instead, we favor a model in which CLK:CYC directly bind and activate expression of genes encoding the phototransduction machinery in photoreceptors, maintaining the continued expression of these proteins that have an integral role in photoreceptor structure and function. Numerous studies have demonstrated that complete loss of function of individual phototransduction genes results in retinal degeneration, often dependent on light exposure (53). Our data show a significant and substantial decrease in transcript levels of multiple phototransduction genes, and we propose that the cumulative decrease in expression of their corresponding proteins causes the light-dependent retinal degeneration in flies expressing CLK^{DN}. However, because disrupting CLK:CYC activity in photoreceptors had widespread effects on gene expression and chromatin accessibility, we cannot exclude the possibility that other pathways such as autophagy (74), Ca²⁺ signaling (75) and phosphoinositide metabolism (76) also contribute to the onset of light-dependent retinal degeneration.

The circadian clock has been implicated in the oxidative stress response in *Drosophila* (55,77) and in mammals (78). Since the onset of many age-related eye diseases is particularly sensitive

to disruptions in oxidative stress response (79), an increase in ROS levels in Rh1>CLK^{DN} eyes suggests an overall neuroprotective role for the circadian clock regulators in the retina, one which might become increasingly important with advanced age. Under standard laboratory conditions, wild-type flies with pigmented eyes only begin to exhibit the first signs of retinal degeneration after D50 (80). Thus, we hypothesize that the increase in CLK:CYC activity in aging photoreceptors protects against retinal degeneration in part, by promoting expression of genes that combat oxidative stress. Supporting this hypothesis, several stress response genes exhibit cyclic expression patterns in the head only in older flies (D55), and this cyclic expression is dependent on both Clk and oxidative stress (63). However, because these studies were performed in female white-eyed *w¹¹¹⁸* flies (63), which already show substantial retinal degeneration by D15 to D30 (81), it remains to be elucidated if these age-associated changes in cyclic transcription also occur in flies with pigmented eyes.

We identified an age-dependent increase in Clock activity in photoreceptors via an unbiased integrative ATAC-seq and RNA-seq approach, which focused on identifying changes in TF activity during aging. Thus, our initial approach was not focused on circadian biology and the data was obtained from single time-point comparisons; samples were collected at ZT6 +/- 1 for aging experiments or at ZT9 +/- 1 for the CLK^{DN} experiments. Because of this, the increase in Clock activity in aging could reflect a change in the phase of Clock activity during the day, as shown for Monarch butterfly brains (82), rather than increase in amplitude. However, other studies have observed a decrease in protein levels of the Clock repressor PER with age in *Drosophila* photoreceptors but not in pacemaker neurons (83,84), suggesting that the increased Clock activity observed in our study could in fact reflect an increase in the amplitude of CLK:CYC activity in old flies.

Overall, our work identifies a central role for the circadian clock in regulating the photoreceptor transcriptome. We observed that Clock contributes to the expression of many thousands of

430 genes in adult photoreceptors, consistent with reports from mammalian cells showing that Clock
 431 activity regulates the expression of 15% of expressed genes (51). Clock and cycle have been
 432 proposed to act at the top of a regulatory hierarchy to control widespread cyclic transcription in
 433 many cell types by regulating the expression of TFs (21,22). Our data from photoreceptors are
 434 consistent with Clock regulating expression of several important eye-specific TFs in *Drosophila*
 435 photoreceptors, suggesting a mechanism through which Clock and cyc control expression of
 436 many genes indirectly. This is likely a widespread phenomenon across *Drosophila* tissues
 437 because gene regulatory network analysis of cyclic transcripts in brain, gut, Malpighian tubules,
 438 and fat bodies also identified many Clock-regulated TFs, including *h*, *hth*, *Mitf*, and *GATAd* (85).
 439 Interestingly, *period* (*per*⁰¹) and *timeless* (*tim*⁰¹) male mutants have extended lifespan, mediated
 440 by a loss of PER in intestine cells (86), suggesting that increased Clock activity in a particular
 441 tissue can lead to positive outcomes associated with health- and lifespan in a tissue- and sex-
 442 specific manner. Thus, identifying the molecular mechanism that underlie the age-associated
 443 changes in Clock activity in aging photoreceptors and their impact on cellular homeostasis,
 444 which may be specific to the retina or other peripheral clocks in flies, will be an important area of
 445 research for future studies.

Materials and Methods

Fly collection and maintenance

Rh1-Gal4>UAS-GFP^{KASH} ($w^{1118};; P\{w^{+mC}=[UAS-GFP-Msp300KASH]attP2, P\{ry^{+t7.2}=rh1-GAL4\}3, ry^{506}$) (3)flies were maintained on standard fly food as previously described. For aging experiments, flies were collected in a 3 day window after eclosion and transferred to population cages. For CLK^{DN} experiments, flies were collected in a 24 hour period and transferred to population cages. D1 corresponds to flies that were collected the first day immediately after eclosion. Flies for both aging and CLK^{DN} experiments were maintained in a 25°C incubator with a 12:12 hour light:dark cycle, where relative Zeitgeber Time (ZT) 0 corresponds to when the light cycle begins. For aging experiments, male flies were collected and flash-frozen at ZT6 (-/+) 1 hour. For CLK^{DN} experiments, male flies were collected and flash-frozen at ZT9 (-/+) 1 hour. We note that UAS-GFP^{KASH} and additional tagged KASH or QUAS flies (22) are available at Bloomington Drosophila Stock Center (BDSC) for nuclei immunoprecipitation in different tissues (#92580 for the UAS-GFP^{KASH} flies used in this study). Rh1-Gal4 (BDSC #8691), UAS-CLK^{DN} [#1] (BDSC #36319), UAS-CLK^{DN} [#2] (BDSC #36318) and UAS-LacZ (BDSC #8529) fly lines were obtained from BDSC.

Chromatin accessibility and transcriptome analysis of photoreceptors

Nuclei immuno-enrichment (NIE), Omni-ATAC, quantitative PCR (qPCR), and RNA-seq were performed on male *Rh1-Gal4>UAS-GFP^{KASH}* flies at the indicated ages as previously described (22). Three biological replicates were analyzed for all RNA-seq and -ATAC-seq samples. RNA-seq and ATAC-seq data analysis were performed as described in (22,80); details specific to this study are described below.

Primers for quantitative PCR

Primer name	Sequence (5' – 3')
ClkDN_Fwd	CGACAAGGATGATACAGATCAG
ClkWT_Fwd	GCGAGAAGAAGCGACGAGAT
Clk_Rev	ATTGCTGAGGAACGCAGGCT
eIF1a_Fwd	GCTGGGCAACGGTCGTCTGGAGGC
eIF1a_Rev	CGTCTTCAGGTTCTGGCCTCGTCCGG
Rpl32_Fwd	GCTAAGCTGTCGCACAAATG
Rpl32_Rev	CGTTGTGCACCAGGAACTT

469

470 Note: We used the same Clk_Rev (reverse) primer to detect either WT or DN transcript by

471 combining with corresponding forward primer (Fwd).

472 RNA-seq fold change heatmap analysis: Heatmaps were obtained using log₂ transformed fold

473 change values obtained from pair-wise comparisons using DESeq2 (87). For aging heatmaps,

474 DEGs obtained from each comparison with D10 were pooled and de-duplicated. Plots were

475 obtained using R (v4.0.3) package *pheatmap* (v1.0.12).

476 Differential TF activity using diffTF: DiffTF (27) analysis was performed using the default

477 parameters: analytical approach (*nPermutations=0*), integration mode using raw counts

478 obtained from STAR (*--quantMode GeneCounts*) (88), narrow peaks obtained using MACS2

479 (89). TFBS bed files were obtained from PWMScan (90) using the Aug 2014 BDGP Rel6 +

480 ISO1 MT/dm6 target genome with the 353 available motifs from the CIS-BP library (28) with

481 default parameters (*p-value<0.00001*, *Bg base composition 0.29,0.21,0.21,0.29*). Identified TFs

482 were classified as significant if they had an FDR lower than 0.001 in at least *one* of the pair-wise

483 aging comparisons with D10, or in either D1 or D10 analysis for the CLK^{DN} comparisons.

Clk ChIP-seq peak and photoreceptor ATAC-seq overlap: Previously published high-confidence peaks obtained from Clk and cyc ChIP-seq data (49) were downloaded, and genomic coordinates were transformed from the *dm3* to *dm6* genome using the *LiftOver* tool from the UCSC Genome Browser website (91). Genomic overlap was calculated using R (v4.0.3) packages *ChIPpeakAnno* (v3.24.2) and *GenomicRanges* (v.1.42.0).

Transcript per million (TPM) scatter plots: TPM values for each sample and replicate were obtained using TPMCalculator (92). TPMs were averaged between biological replicates and used for scatter plot generation using R package *ggplot2* (v3.3.3). DEGs obtained with DESeq2 were colored on the TPM scatter plots.

Optic neutralization

Optic neutralization and retinal degeneration quantification were performed as previously described (17,93). Five biological replicates were analyzed for each sample and retinal degeneration scores were assessed blindly.

Targeted GSH:GSSG metabolomic assay

25 eyes per sample were collected from male flies of the indicated age, condition, and genotype ($n = 3$). Eyes were placed in a Covaris MicroTube with 110 μ L of blocking solution (62.5 mg NEM, 3 mg EDTA, 5 mg NaHO₃ dissolved in 2 mL of 3:2 parts Water/MeOH (v/v)). Once all eyes were added to the blocking solution 10 μ L of 100ng/ μ L of each internal standard was added ¹³C₂-¹⁵N-GSH-NEM and ¹³C₄-¹⁵N₂-GSSG. Samples were homogenized using a Covaris Ultra Sonicator with the following settings: peak power: 200; duty factor: 10%; cycles per burst: 200; time: 300 seconds. Samples were then processed using an Agilent 1260 Rapid Resolution liquid chromatography (LC) system coupled to an Agilent 6470 series QQQ mass spectrometer (MS/MS) to analyze glutathione (94). (Agilent Technologies, Santa Clara, CA). A Waters T3 2.1 mm x 100 mm, 1.7 μ m column was used for LC separation (Water Corp, Milford, MA). The

508 buffers were A) water + 0.1% formic acid and B) acetonitrile + 0.1% formic acid. The linear LC
 509 gradient was as follows: time 0 minutes, 0 % B; time 2 minutes, 0 % B; time 8 minutes, 30 % B;
 510 time 9 minutes, 95 % B; time 9.1 minutes, 0 % B; time 15 minutes, 0 % B. The flow rate was 0.3
 511 mL/min. Multiple reaction monitoring was used for MS analysis. Data were acquired in positive
 512 electrospray ionization (ESI) mode. The jet stream ESI interface had a gas temperature of
 513 350°C, gas flow rate of 9 L/minute, nebulizer pressure of 35 psi, sheath gas temperature of
 514 300°C, sheath gas flow rate of 9 L/minute, capillary voltage of 4000 V in positive mode, and
 515 nozzle voltage of 1000 V. The Δ EMV voltage was 450 V. Agilent Masshunter Quantitative
 516 analysis software was used for data analysis (version 8.0).

517

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Figure 1. Tissue-specific profiling of the photoreceptor nuclear transcriptome reveals significant changes in early and late aging

A. Schematic of the *Drosophila* compound eye, composed of ommatidia that contain six outer photoreceptors that express Rhodopsin 1 (Rh1). Outer photoreceptor nuclei are labeled with GFP^{KASH}. B. Experimental design for the aging RNA-seq time course. C. Principal Component Analysis (PCA) of aging RNA-seq samples based on gene counts. D. Hierarchically clustered heatmap of aging RNA-seq samples. Only genes that were identified as being differentially expressed in any condition relative to D10 are shown. Z-scores are calculated based on normalized counts obtained using DESeq2, and the heatmap is divided into five clusters based on the dendrogram. E. Gene Ontology (GO) term analysis of significantly enriched genes in each cluster.

Figure 2. Photoreceptors undergo substantial changes in TF activity during aging

A. Heatmap of RNA-seq fold change during aging for significantly differentially expressed genes (DEGs; p-adj<0.05, |FC|>1.5 relative to D10) associated with the GO term “RNA polymerase II-specific DNA-binding transcription factor binding” (GO:0061629). Genes are hierarchically clustered by log₂ fold change values. B. PCA of aging ATAC-seq samples based on the read distribution over binned genome. C. Schematic of differential transcription factor (TF) analysis approach using *diffTF*. D. Bar plot indicating the number of significant age-regulated TFs at each age relative to D10 (p-adj<0.001). E. Hierarchical clustered heatmap of age-regulated TFs with significant changes in activity between any age and D10 (asterisk, p-adj<0.01). Scale represents the relative change in TF activity with red showing higher TF activity in old samples relative to D10, and blue indicating an age-associated decrease in activity. TFs that bind a common motif and cluster together are indicated by a vertical line.

Figure 3. Clock regulates the transcriptional output of phototransduction genes in photoreceptors

A. Schematic of CLK:CYC activity. Expression of CLK^{DN} disrupts Clock-dependent transcription because it binds CYC but does not contain the DNA binding domain. B. Schematic for the RNA-seq experiments. Flies that express the GFP^{KASH} tag and either CLK^{DN} or LacZ (control) in photoreceptors were aged to D1 or D10 prior to NIE. Flies were harvested at the indicated ZT. C. Scatter plot of mean expression (TPM, n=3) for the indicated pair-wise comparisons. Up- and down-regulated DEGs (p-adj<0.05, |FC|>1.5) are colored in red and blue, respectively. Core clock genes *Clk*, *cyc*, *vri*, *per*, *Pdp1* and *tim* are highlighted. D. Genomic overlap of accessible peaks in photoreceptors and CLK:CYC binding sites identified using ChIP-seq in (49). E. Venn diagram comparing overlap of Clock-bound genes (CBGs-green) with genes that were differentially expressed (adj p <0.05, |FC|>1.5) in Rh1>CLK^{DN} relative to control. F. GO term analysis of Clock direct and indirect targets. G. Table showing selected genes identified as Clock direct target genes that were associated with the indicated biological processes. H. Ridge plot of selected GO terms in flies expressing Clk^{DN} versus control at D1 identified using Gene Set Enrichment Analysis.

Figure 4. Clock activity promotes changes in TF activity and maintains global levels of chromatin accessibility in photoreceptors

A. Cnet plot of genes associated with the GO term “DNA-binding transcription factor activity”. Color represents change in expression in CLK^{DN} relative to control at D1. B. PCA of CLK^{DN} and control ATAC-seq samples based on the read distribution over a 10-kb binned genome. C. Genome browser inspection of CPM-normalized ATAC-seq signal for selected core Clock genes (*per*, *tim*, *vri* and *Pdp1*). Scale is normalized to the same height in each comparison. ATAC-seq from Ctrl flies is labeled in black, and CLK^{DN} in yellow. D. Hierarchical clustered heatmap of significant Clock-regulated transcription factors identified between Rh1>CLK^{DN} and Rh1>Ctrl at

either D1 or D10. Scale represents the relative change in TF activity. E. CPM-normalized gene metaplots of ATAC-seq signal centered around Transcription Start Sites (TSS). F. ATAC-seq fold change heatmaps of signal centered around TSS for all actively expressed genes in photoreceptors. Fold change is obtained by dividing signal from Rh1>CLK^{DN} relative to Rh1>Ctrl, and log² transformed to center changes around zero (no change).

Figure 5. Disruption of Clock activity leads to light-dependent retinal degeneration and light-independent accumulation of oxidative stress

A. Cnet plot of DEGs (p-adj<0.05,|FC|>1.5) in Rh1>CLK^{DN} relative to Rh1>Ctrl at D1, associated with phototransduction-related GO terms. Genes associated with light-dependent and -independent retinal degeneration based on published literature are indicated by accent or asterisks. B. Representative images of eyes from flies expressing CLK^{DN} versus control at the indicated age reared in light:dark (LD) or constant dark (DD) conditions. C. Scatter plot showing quantification of retinal degeneration in panel B. Individual eyes are shown as circles with means shown in black (n=5). The distribution for each LD reared sample was compared with the DD control for the same age using ANOVA followed by Tukey-HSD comparing ages, samples, and condition (L:D vs D:D). “n.s.” = not significant, “*” = FDR<0.05, “***” = FDR<0.005). D. Reduced(GSH):Oxidized(GSSG) Glutathione ratios in dissected eyes from flies of the indicated genotype, age, and condition. Statistics were performed as in panel C. E. Bar plots representing enriched GO terms amongst the genes that were upregulated in Rh1>CLK^{DN} at D10 relative to D1. F. Cnet plots of genes identified in panel E.

Supplemental Figure 1.

Pearson correlation heatmap of gene expression profiles from nuclear RNA-seq of aging samples (n = 3). Scores between 0 and 1 shown in each box correspond to Pearson’s r score.

Supplemental Figure 2.

A. Bar plot showing the genomic distribution of accessible peaks identified in ATAC-seq data in Rh1>GFP^{KASH} during aging. Promoter annotation was based on +/- 2 kb around transcription start sites. B. DNA binding motif for CLK and CYC derived from CIS-BP database.

Supplemental Figure 3.

A. Bar plot showing log₂-transformed transcript per million (TPM) values for core Clock genes comparing Rh1>CLK^{DN} and Rh1>Ctrl at either D1 (left) or D10 (right) (mean +/- SD, n=3, P-value obtained using t-test). B. Bar plot showing Clk^{WT} and Clk^{DN} transcript levels measured in heads from male flies of the indicated genotype. Transcript levels are normalized to the geometric mean of housekeeping genes *eIF-1α* and *Rpl32* (mean ± SD; n = 3, P-value obtained using t-test). “ND” = not detected, “*” = p-value<0.05, “***” = p-value<0.005. C. Box plots showing the fold change values for genes identified as direct or indirect targets. Fold change is obtained using DESeq2. P-adjusted > 0.05, |FC|>1.5. P-value is obtained using Wilcoxon test. D,E. Complete ridge plot obtained from Gene Set Enrichment Analysis comparing differentially expressed genes in Rh1>CLK relative to Rh1>Ctrl at D1 (D) or D10 (E). F. Volcano plot showing differentially expressed genes from photoreceptors when flies are reared in free-running conditions (dark:dark) versus light:dark cycle. Significance is equivalent to -log₁₀(adjusted p-value). Genes are colored based on changes in gene expression in D:D relative to L:D. Red is upregulated, and blue is downregulated. DEGs have p-adj < 0.05. G. Gene Ontology (GO) term analysis of genes that were significantly up-regulated (top) or down-regulated (bottom) in D:D relative to L:D.

Supplemental Figure 4.

A. Bar plot showing the genomic distribution of accessible peaks identified in ATAC-seq data from the indicated genotypes and ages. Promoter annotation was based on +/- 2 kb around transcription start sites. B. Volcano plots representing the differentially accessible peaks in

886 Rh1>CLK^{DN} relative to Rh1>Ctrl. Differentially accessible peaks are defined as having a False
887 Discovery Rate (FDR) < 0.05, and absolute fold change (|FC|) > 1.5.

888 **Supplemental Figure 5.**

889 Heatmap showing RNA-seq fold change values for genes associated with phototransduction in
890 flies expressing Rh1>CLK^{DN} relative to Rh1>Ctrl at D1 or D10. Fold change values are
891 represented inside each square, and color-labelled based on fold change score.

892 **S1 Table**

893 Differentially expressed genes comparing each aging time point relative to D10 (n=3).

894 **S2 Table**

895 Differentially expressed genes comparing Rh1>Clk^{DN} relative to Control (n=3) at D1 or D10.

896

Competing interests

Authors declare no competing interests.

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Data availability

All the high-throughput data is available at the Gene Expression Omnibus (GEO) repository under the specific accession codes outlined below:

Data	Accession code	Status
D10 ATAC-seq/RNA-seq	GSE169328	Public
Aging ATAC-seq	GSE184069	Private.
Aging/dark:dark RNA-seq	GSE174515	Private.
Clk ^{DN} /Ctrl ATAC-seq	GSE183746	Private.
Clk ^{DN} /Ctrl RNA-seq		Private.

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913 Funding acquisition and Supervision: Vikki M. Weake

A **B** **C** **D** **E**

A **B** **D** **E** **G****Phototransduction**

ninaE, trp, linc, inaF-B, rdgB, Arr2, rdgC, PIP82, Arr1, trpl, ninaC, dnc, Calx, slgA, ltpr, Gbeta76C

Circadian rhythm

cwo, vri, Moe, tim, per, Clk, homer, nvy, Fbx14, lh

Transcription factors

psq, Lim1, Dif, oc, ey, Xrp1, ttk, shn, ab, vnd, Glu4EF, dwg, Mnt, CrebA, fuss

C **F** **H**

A **B** **C** **D** **E** **F**

A

A

Age (days)
10 20 30 40 50 60

Feature

- Promoter (<=1kb)
- Promoter (1-2kb)
- 5' UTR
- 3' UTR
- 1st Exon
- Other Exon
- 1st Intron
- Other Intron
- Downstream (<=300)
- Distal Intergenic

B

Clk/cyc binding motif

A **B** **C** **D** **E** **F** **G**

A **B**

A

D1 D10

log₂ RNA-seq fold change
relative to Rh1>Ctrl