Dietary restriction and the transcription factor clock delay eye aging to extend lifespan in *Drosophila Melanogaster*

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Many vital processes in the eye are under circadian regulation, and circadian dysfunction has emerged as a potential driver of eye aging. Dietary restriction is one of the most robust lifespan-extending therapies and amplifies circadian rhythms with age. Herein, we demonstrate that dietary restriction extends lifespan in *Drosophila melanogaster* by promoting circadian homeostatic processes that protect the visual system from age- and light-associated damage. Altering the positive limb core molecular clock transcription factor, CLOCK, or CLOCK-output genes, accelerates visual senescence, induces a systemic immune response, and shortens lifespan. Flies subjected to dietary restriction are protected from the lifespan-shortening effects of photoreceptor activation. Inversely, photoreceptor inactivation, achieved via mutating rhodopsin or housing flies in constant darkness, primarily extends the lifespan of flies reared on a high-nutrient diet. Our findings establish the eye as a diet-sensitive modulator of lifespan and indicates that vision is an antagonistically pleiotropic process that contributes to organismal aging.
Circadian rhythms are ~24 h oscillations in behavior, cellular physiology, and biochemistry, which evolved to anticipate and manage predictable changes associated with the solar day (e.g., predator/prey interactions, nutrient availability, phototoxicity, etc.)\(^1\). Circadian rhythms are generated by endogenous clocks that sense time-cues (e.g., light and food) to govern rhythmic oscillations of gene transcriptional programs, synchronizing cellular physiology with daily environmental stressors\(^2\). In addition to keeping time, the molecular clock regulates the temporal expression of downstream genes, known as Clock-controlled genes, to promote tissue-specific rhythms in physiology\(^3\). The *Drosophila* molecular clock is comprised of transcriptional-translational feedback loops, where the transcription factors Clock (CLK) and Cycle (CYC) rhythmically activate their own repressors, Period (PER) and Timeless (TIM)\(^4\). This feedback loop not only exists in central pacemaker neurons, where it sets rhythms in locomotor activity, but it also functions in peripheral tissues, such as the eye\(^4\).

Aging is associated with a progressive decline in visual function and an increase in the incidence of ocular disease. *Drosophila* photoreceptor cells serve as a powerful model of both visual senescence and retinal degeneration\(^5,6\). *Drosophila* and mammalian photoreceptors possess a cell-intrinsic molecular clock mechanism that temporally regulates many physiological processes, including light sensitivity, metabolism, pigment production, and susceptibility to light-mediated damage\(^7\). Visual senescence is accompanied by a reduced circadian amplitude in core-clock gene expression within the retina\(^8\). This reduction in retinal circadian rhythms may be causal in eye aging, as mice harboring mutations in their core-clock genes, either throughout their entire body or just in their photoreceptor cells, display several early-onset aging phenotypes within the eye. These mice prematurely form cataracts and have reduced photoreceptor cell light sensitivity and viability\(^8\). However, the molecular mechanisms by which the molecular clock influences eye aging are not fully understood.

Dietary restriction (DR), defined as reducing specific nutrients or total calories, is the most robust mechanism for delaying disease and extending lifespan\(^9\). The mechanisms by which DR promotes health and lifespan may be integrally linked with circadian function, as DR enhances the circadian transcriptional output of the molecular clock and preserves circadian function with age\(^10\). Inversely, high-nutrient diets (i.e., excess consumption of protein, fats, or total calories) repress circadian rhythms and accelerate organismal aging\(^11,12\). However, how DR modulates circadian rhythms within the eye, and how these rhythms influence DR-mediated lifespan extension, had yet to be examined.

Herein, we sought to elucidate the circadian processes that are activated upon DR by performing an unbiased, 24 h time-course mRNA expression analysis in whole flies. We found that circadian processes within the eye are highly elevated in expression in flies reared on DR. In particular, DR enhanced the rhythmic expression of genes that encode proteins that are involved in light adaptation (i.e., calcium handling and deactivation of rhodopsin-mediated signaling). Building on this observation, we demonstrate that the majority of these circadian phototransduction genes were transcriptionally regulated by CLK. Eliminating CLK function either pan-neuronally, or just in the photoreceptors, accelerated visual decline with age. Furthermore, disrupting photoreceptor homeostasis increased systemic immune responses and shortened lifespan. Several eye-specific CLK-output genes that were upregulated in expression in response to DR, were also required for DR-to slow visual senescence and extend lifespan.

Results

Dietary restriction amplifies circadian transcriptional output and delays visual senescence in a CLK-dependent manner. To determine how DR changes circadian transcriptional output, we performed a series of microarray experiments over the span of 24 h in *Canton-S* wild-type flies (whole fly) housed in light-dark (LD) 12:12 h and reared on either a high-yeast (5%; ad libitum, AL) diet or a low-yeast (0.5%; DR) diet (Supplementary Fig. 1a), identifying rhythmic transcript expression with the circadian algorithm JTK_CYCLE (Supplementary Data 1). A full description of the fly strains utilized in this study is in Supplementary Data 2. In LD conditions, transcripts displaying 24 h oscillations may be driven by the core-molecular clock (“circadian”) or by rhythmic lighting cues\(^13\). To control for the effect of light on rhythmic gene expression, we also performed time-course microarrays under similar conditions in flies that lack an endogenously generated circadian transcriptional rhythm (tim\(^{01}\) mutants). We reasoned that the oscillatory transcripts identified in tim\(^{01}\) flies are likely driven by light. Therefore, we defined clock-controlled transcripts as those that oscillate only in wild-type flies but not in tim\(^{01}\) mutants (Supplementary Fig. 1a). Wild-type flies maintained on DR displayed nearly twice the number of circadian transcripts compared to flies reared on AL (Fig. 1a, b and Supplementary Fig. 1b), while the circadian mutant tim\(^{01}\) flies failed to show a significant increase in oscillating transcripts on DR (Supplementary Fig. 1c). These data indicate that the DR-mediated increase in circadian transcripts requires functional molecular clocks and is independent of mechanisms influenced by rhythmic lighting cues (i.e., masking). Circadian gene expression in the wild-type flies was also more robust on DR vs AL; DR-specific oscillators were statistically more rhythmic (lower JTK_CYCLE circadian P-values) and displayed larger circadian amplitudes than AL-specific oscillators in the *Canton-S* but not in tim\(^{01}\) mutant flies (Supplementary Fig. 1d, e). Diet also drastically altered the wild-type circadian transcriptional profile, as only 16% of DR oscillators were also oscillating on AL (Supplementary Fig. 1b). Furthermore, the AL and DR circadian transcriptomes were enriched for distinct processes (Supplementary Fig. 1f, g and Supplementary Data 1).

Transcripts that oscillate on both AL and DR diets were highly enriched for genes associated with the canonical phototransduction signaling cascade (Fig. 1c, d), which is the process by which *Drosophila* photoreceptor cells, the primary light-sensitive neurons, transduce light information into a chemical signal\(^14\). Briefly, light-mediated conversion of rhodopsin proteins to their meta-rhodopsin state stimulates heterotrimeric Gq proteins that activate a phospholipase C (NORPA), which produces secondary messengers and promotes the opening of Transient Receptor Potential channels (TRP, TRPL), ultimately allowing Ca\(^{2+}\) and Na\(^{+}\) to depolarize the photoreceptor cell\(^15\). Although the phototransduction transcripts were cyclic on both diets, on DR their expression became more rhythmic (lower JTK_CYCLE P-values and larger circadian amplitudes) and elevated (~2-fold increase in expression across all timepoints) (Fig. 1e and Supplementary Fig. 1j). The phototransduction components were similarly upregulated in expression in the tim\(^{01}\) mutants, but did not display a 24 h rhythmicity (Supplementary Fig. 1k), indicating that their oscillatory expression patterns are driven by the intrinsic molecular clock, as opposed to being primarily light-responsive. Since our time-course analyses were performed in whole-fly, we queried publicly available circadian transcriptomes from wild-type heads to further investigate the rhythmic oscillations of eye-related transcripts\(^16\). The majority of the DR-sensitive phototransduction genes also robustly cycled in wild-type heads (Supplementary Table 1).
In *Drosophila* and mammals, visual function oscillates to align with daily changes in ambient illumination from the sun, which can be 10⁶–10⁸-fold brighter during the day than at night. Photoreceptors are unique in that they have evolved mechanisms responsible for maintaining homeostasis in the presence of light-induced calcium ion gradients that are magnitudes greater than what other neuronal populations experience. Mechanisms of light adaptation within photoreceptors include the rapid (millisecond) closure of TRP channels (facilitated via enzymes scaffolded by INAD), rhodopsin internalization from the rhabdomere membrane (e.g., ARR1, ARR2), and calcium efflux (e.g., CALX). Acrophase analyses (time of peak expression) revealed that circadian transcripts whose gene products encode proteins that promote photoreceptor activation (Ca²⁺ influx) reach peak expression during the dark phase, while genes that encode proteins that terminate the phototransduction response (i.e., deactivation of rhodopsin-mediated signaling) peak in anticipation of the light phase (Supplementary Fig. 1l). These findings provide a potential mechanistic explanation for the rhythmic response pattern in light sensitivity observed in *Drosophila* photoreceptors (See Supplementary Discussion 1 for additional interpretations).

To determine if molecular clocks mediate the enhanced expression of phototransduction genes on DR, we measured the transcriptome of fly heads with pan-neuronal overexpression of a dominant-negative form of the core-clock factor, CLK (Elav-GS-GAL4 > UAS-CLK-Δ1; denoted nCLK-Δ1) (Supplementary Fig. 2a). To avoid potential developmental defects related to altered CLK function, we used the drug-inducible (RU486) GeneSwitch driver to express CLK-Δ in adult flies. Genes downregulated in nCLK-Δ1 heads were enriched for light-response pathways, including "response to light stimulus" and "deactivation of rhodopsin signaling" (Fig. 2a, b and Supplementary Data 3). Additionally, genes that were both circadian in wild-type heads and downregulated in nCLK-Δ1 were highly enriched for homeostatic processes related to eye function, while downregulated genes in nCLK-Δ1 that were non-circadian in wild-type heads displayed no such enrichment (Supplementary Fig. 2c and Supplementary Data 4). Together, this indicates that CLK governs the transcriptional regulation of many eye-related processes in *Drosophila*.

Given DR’s ability to improve homeostasis across an array of tissues, and its ability to enhance the circadian rhythmicity of light-response genes, we examined how diet and CLK influence visual function with age. We longitudinally quantified the positive phototaxis response of wild-type flies (*Canton-S* and *Oregon-R*) reared on either AL or DR diets (experimental setup in Supplementary Fig. 3a). A full description of the positive phototaxis responses and accompanied statistics for all strains used in this study are in Supplementary Data 5. Compared to AL-fed flies, DR slowed the decline in positive phototaxis observed with age (Supplementary Fig. 3b, c). Importantly, this effect...
**Fig. 2** Dietary restriction delays visual senescence in a CLK-dependent manner. **a** GO enrichment scores corresponding to downregulated light-response genes in heads from RNA-Seq of nCLK-Δ1 (Elav-GS-GAL4 > UAS-CLK-Δ1) vs controls. *P*-values were calculated with hypergeometric distribution (findGO.pl, HOMER) with no adjustment for multiple-hypothesis testing. **b** Heatmap of normalized RNA-Seq expression corresponding to the gene-ontology category “Deactivation of rhodopsin-mediated signaling” (GO:0016059) in nCLK-Δ1 and controls at zeitgeber times 0 and 12 (lights on and lights off, respectively). **c** Positive phototaxis responses for nCLK-Δ1 flies. For each timepoint results are represented as mean values of percent positive phototaxis ±SEM (n = 24 biologically independent cohorts of 20 flies examined over three independent experiments, N = 480 flies per condition). *P*-values were determined by two-tailed Student’s *t*-test (unpaired), comparing responses between diet and/or genotype at each timepoint. **d** Boxplots of electroretinogram amplitudes for nCLK-Δ1 flies and controls at day 14 and 21. Data are presented as Tukey multiple comparison of means: The horizontal line within each box is the median, the bottom and top of the box are lower and upper quartiles, and the whiskers are minimum and maximum values. (n = 8 and 12 biologically independent flies measured at day 14 and 21, respectively, examined over 1 independent experiment). *P*-values were determined by two-tailed Student’s *t*-test (unpaired), comparing responses between diet and/or genotype at each timepoint. Source data are provided as a Source Data file.

cannot solely be attributed to diet-dependent changes in locomotor activity, as climbing activity and phototaxis declined at different rates with age (Supplementary Fig. 3d). Compared to wild-type flies, DR minimally protected *clk*<sup>out</sup> (clk-null) flies from age-related declines in phototaxis (Supplementary Fig. 3e). Unlike the *clk*<sup>out</sup> mutant line, DR enhanced positive phototaxis responses in flies with mutations in the gene encoding the blue-light-sensitive Cryptochrome protein (*cry*<sup>Δ1</sup>, *cry*<sup>Δ2</sup>, and *cry*<sup>B</sup> mutants) (Supplementary Fig. 3g–i). This discrepancy between diet-dependent responses to phototaxis in core-molecular clock mutants shows that DR’s ability to improve phototaxis responses is not reliant on CRY’s ability to photo-entrain the molecular clock<sup>24</sup>, while CLK function is required. nCLK-Δ1 and nCLK-Δ2 (an additional dominant-negative CLK mutant, Elav-GS-GAL4 > UAS-CLK-A2) flies displayed accelerated declines in positive phototaxis with age compared to controls (Fig. 2c and Supplementary Fig. 3f).

Since the positive phototaxis assay measures a behavioral response to light, we next evaluated how diet and CLK directly influence photoreceptor function with age by performing extracellular electrophysiological recordings of the eye (electroretinograms, ERG<sup>25</sup>). A full description of the ERG responses and accompanied statistics for the fly strains used in this study are in Supplementary Data 6. We observed larger ERG amplitudes, i.e., the light-induced summation of receptor potentials from the photoreceptors<sup>26</sup>, in control flies reared on DR vs AL on day 14 (Fig. 2d). Furthermore, the DR-mediated enhancements in the ERG amplitudes were significantly reduced in nCLK-Δ1 flies with age (Fig. 2d).

Since the Elav-GS-GAL4 driver is expressed in a pan-neuronal fashion (i.e., photoreceptors and extra-ocular neurons), we sought to examine how manipulating CLK function solely within photoreceptors influences visual function with age. To this end, we crossed UAS-CLK-Δ1 mutant flies and flies that over-express wild-type CLK (UAS-clk, denoted CLK-OE) with a photoreceptor-specific GAL4 driver line under the temporal control of the temperature-sensitive GAL80 protein (Trpl-GAL4; GAL80<sup>ts</sup> > UAS-CLK-Δ1, denoted prCLK-Δ1 and Trpl-GAL4; GAL80<sup>ts</sup> > UAS-CLK-OE, denoted prCLK-OE). To avoid potential alteration of normal CLK function during development, prCLK-Δ1 and prCLK-OE flies were raised at 18 °C (GAL80 active, GAL4 repressed) and then transferred to 30 °C (GAL80 repressed, GAL4 active) following eclosion. When compared to control flies (Trpl-GAL4/++; GAL80<sup>ts</sup>/+), prCLK-Δ1 flies displayed accelerated declines in both positive phototaxis and ERG amplitude with age, while prCLK-OE flies displayed a slower rate of visual senescence (Fig. 3a, b and Supplementary Fig. 4b). By day 14, prCLK-OE flies displayed a clear DR-mediated improvement in positive phototaxis compared to prCLK-Δ1 flies reared on AL (Fig. 3a). Interestingly, we failed to observe significant differences in diet-dependent responses (AL vs. DR) in the ERG’s of prCLK-OE flies at any timepoint (Fig. 3b),

![supplementary figure 3g](https://example.com/supplementary-figure-3g.png)

**Supplementary Figure 3g**

- Detection of visible light
- Phototransduction, visible light
- Response to light stimuli
- Deactivation of rhodopsin-mediated signaling
- Retina homeostasis

**Boxplots of Electroretinogram Amplitudes**

- Time (days)
- Amplitude (mV)

**Fold-change in RNA-Seq expression**

- AL cnt
- DR cnt
- AL nCLK-Δ1
- DR nCLK-Δ1

**Delta Phototaxis Index (%)**

- Time (days)
- Phototaxis index (%)

**Delta Amplitude**

- Time (days)
- Amplitude (mV)
suggesting that the reduced phototaxis responses on AL diets observed in these lines were likely caused by behavioral and/or extra-ocular functional changes with age. We next visualized tangential sections of the eye to determine if the differences in phototaxis and ERG we observed via diet and genotype (prCLK-Δ1 and prCLK-OE) were associated with morphological changes to the ommatidium and photoreceptors. We failed to observe major morphological changes between genotypes on day 2, indicating that these groups developed a normal ommatidial structure, with the rhodobemere of the R1-7 photoreceptors clearly visible with no apparent degeneration (Fig. 3c). In agreement with the positive phototaxis and ERG data, we observed massive photoreceptor degeneration in the prCLK-Δ1 flies, while the control and prCLK-OE flies displayed normal ommatidial structure and rhodobemeres (Fig. 3c). Together, the gene expression, phototaxis, ERG, and morphological observations indicate that DR functions in a CLK-dependent manner to delay photoreceptor aging in the fly.

nCLK-Δ drives a systemic immune response and reduces longevity. Age-related declines in tissue homeostasis are accompanied by elevated immune responses and inflammation.\textsuperscript{27,28} Interestingly, we found that genes upregulated in nCLK-Δ1 fly heads were significantly enriched for immune and antimicrobial humoral responses (Fig. 4a, b). In Drosophila, damage-associated molecular patterns can induce a sterile immune response that is characterized by the expression of antimicrobial peptides (AMPs), similar to the effects of infections by pathogens.\textsuperscript{29} We quantified the mRNA expression of AMPs in the bodies of nCLK-Δ1 and nCLK-Δ2 flies to determine if neuronal damage signals propagate throughout the body to drive systemic immune responses; the Drosophila fat body generates high levels of AMPs in response to intrinsic damage signals.\textsuperscript{29} The RT-PCR primer sequences used in this study are in Supplementary Data 7. AMP expression (atta, diptB, and dro) was reduced in control flies reared on DR compared to AL; however, expression of nCLK-Δ1 and nCLK-Δ2 elevated AMP expression on DR (Fig. 4c and

**Fig. 3 Photoreceptor clocks modulate visual function and degeneration with age.** a Positive phototaxis responses for prCLK-Δ1 flies (Trpl-GAL4; GAL80Δ5 > UAS-CLK-Δ1), prCLK-OE (Trpl-GAL4; GAL80Δ5 > UAS-Clk), and control flies (Trpl-GAL4; GAL80Δ5/+ ) reared at 30 °C. For each timepoint results are represented as mean values of percent positive phototaxis ±SEM (control and prCLK-Δ1: n = 24 biologically independent cohorts of 20 flies examined over three independent experiments, N = 480 flies per condition, prCLK-OE: n = 16 biologically independent cohorts of 20 flies examined over 2 independent experiments, N = 320 flies per condition). P-values were determined by two-tailed Student’s t-test (unpaired), comparing responses between diet and/or genotype at each timepoint. b Boxplots of electroretinogram amplitudes for prCLK-Δ1, prCLK-OE, and control flies reared at 30 °C. The sample sizes (n) corresponding to (b) can be found in the “Statistics and Reproducibility” section within the Methods. Data are presented as Tukey multiple comparison of means: The horizontal line within each box is the median, the bottom and top of the box are lower and upper quartiles, and the whiskers are minimum and maximum values. P-values were determined by two-tailed Student’s t-test (unpaired), comparing responses between diet and/or genotype at each timepoint. c Tangential sections through prCLK-Δ1, prCLK-OE, and control retinas at day 2 and day 10. R1-7 photoreceptors are apparent within each hexagonally shaped ommatidia. White bars in bottom right corners represents 10 microns. The number of biologically independent replicates for each group within this experiment are in the Statistics and Reproducibility section. Additional representative images are included with the source data file. Source data are provided as a Source Data file.
Fig. 4 nCLK-Δ1 flies display elevated immune responses and shortened lifespan. a GO enrichment scores corresponding to upregulated inflammatory genes in heads from RNA-Seq of nCLK-Δ1 vs controls. P-values were calculated with hypergeometric distribution (findGO.pl, HOMER) with no adjustment for multiple-hypothesis testing. b Heatmap of normalized RNA-seq expression corresponding to the gene-ontology category “Antimicrobial humoral responses” (GO:0019730) in nCLK-Δ1 and controls. c Relative expression of AMP genes (attA, diptB, and dro) calculated by RT-qPCR with mRNA isolated from nCLK-Δ1 bodies. Results are plotted as average Log2 fold-change in expression calculated by the ΔΔ-Ct method, normalized to DR vehicle-treated control samples, as well as the housekeeping gene rp49 ± SEM (n = 3 biologically independent cohorts of flies, N = 30 flies per cohort). P-values were determined by two-tailed Student’s t-test (unpaired), comparing Log2 fold-changes in expression. d Relative mRNA expression of immune genes (attA, diptB, and dro) calculated by RT-qPCR with mRNA isolated from bodies of w1118 and rhodopsin mutant flies housed in 12:12 h LD. Results are plotted as average Log2 fold-change in expression calculated by the ΔΔ-Ct method normalized w1118 DR control samples as well as rp49 ± SEM (n = 3 biologically independent cohorts of flies, N = 30 flies per cohort). P-values were determined by two-tailed Student’s t-test (unpaired), comparing Log2 fold-changes in expression. e Kaplan-Meyer survival analysis of nCLK-Δ1 flies (Elav-GS-GAL4 > UAS-CLK-Δ1) reared at 25 °C. Survival data are plotted as an average of three independent lifespan repeats. Control flies (vehicle-treated): AL N = 575, DR N = 526; nCLK-Δ1 flies (RU486 treated): AL N = 570, DR N = 565. f Kaplan-Meyer survival analysis of prCLK-Δ1 flies (Trp1-GAL4; GAL80ts > UAS-CLK-Δ1) and control (Trp1-GAL4/+, GAL80ts/+) flies reared at 30 °C. Survival data are plotted as an average of three independent lifespan repeats. Control flies: AL N = 599, DR N = 501; prCLK-Δ1 flies: AL N = 513, DR N = 564. Source data are provided as a Source Data file.
Supplementary Fig. 5a). To further investigate this systemic inflammatory response, we isolated and quantified hemolymph from nCLK-D1 and control flies. In agreement with the transcriptional activation of AMPs in both the heads and bodies of nCLK-D1 flies, we found the most highly upregulated protein in nCLK-D1 hemolymph to be the antimicrobial peptide, AtTC (Supplementary Fig. 5b). Furthermore, we observed an enrichment for proteins associated with translational activation (e.g., cytoplasmic translation and ribosomal biogenesis) within the upregulated proteins in the nCLK-D1 hemolymph, which may reflect the activation of hemocytes, the immune effector cells in Drosophila (Supplementary Data 8)30. Taken together, these data demonstrate that disrupting neuronal CLK function elevates systemic immune responses. However, as with all tissue-specific driver systems, we cannot rule out the possibility that our ELAV-GS-GAL4 driver expresses in a small population of non-neuronal cell types, which, in theory, could contribute to the elevated systemic inflammatory responses and/or influence lifespan.

To determine if photoreceptor degeneration induces a systemic immune response in Drosophila, we forced photoreceptor degeneration by knocking down AtTPα within the eye (GMR-GAL4 > UAS-AtTPα-RNAi), and quantified the expression of AMPs within the bodies. The AtTPα gene encodes the catalytic alpha subunit of the Na⁺/K⁺-ATPase responsible for reestablishing ion balance in the eye during light responses31,32. Our decision to use AtTPα knockdown as a model of photoreceptor degeneration was motivated by previous reports indicating that its expression is under circadian regulation33 and that its knockdown in the eye results in aberrant ion homeostasis that drives age-dependent, light-independent photoreceptor degeneration34. Ocular knockdown of AtTPα rendered flies blind in both AL and DR conditions compared to controls (Supplementary Fig. 5c). Knocking down AtTPα in the eye also drove the expression of AMPs within the bodies of flies reared on either an AL or DR diet (Supplementary Fig. 5d). Thus, DR fails to suppress immune responses in the context of forced photoreceptor degeneration.

Since we found that photoreceptor degeneration induced systemic immune responses, we postulated that reducing phototransduction should reduce inflammation. To assess how stress from environmental lighting influences immune responses, we analyzed a circadian microarray dataset comparing gene expression changes in wild-type (y,w) heads in flies reared in 12 h light and 12 h darkness (12:12 LD) or constant darkness13. We found immune response genes to be among the most highly enriched processes upregulated in the flies housed in 12:12 LD vs constant darkness (Supplementary Fig. 5e, f and Supplementary Data 9). We quantified AMPs within the bodies of flies harboring rhodopsin-null mutations to evaluate how the different photoreceptor subtypes influence systemic immune responses. The Drosophila ommatidia consist of eight photoreceptors (R1–8) that express different rhodopsins with varying sensitivities to distinct wavelengths of light35. The R1-6 photoreceptors express the major rhodopsin Rh1, encoded by ninaE, while the R7 photoreceptor expresses either Rh3 or Rh4. The R8 photoreceptor expresses either Rh5 or Rh636. The rhodopsin-null mutants [ninaEΔ377, rh3Δ38, rh4Δ39, or rh6Δ40] displayed reductions in immune marker expression in their bodies compared to w1118 outcrossed controls (Fig. 4d). Taken together, these findings indicate that suppression of rhodopsin-mediated signaling is sufficient to suppress systemic immune responses in Drosophila housed in LD conditions.

Given the strong associations between chronic immune activation and accelerated aging, we measured the lifespans of nCLK-D flies27. Both nCLK-D1 and nCLK-D2 flies displayed significantly shortened lifespans, with a proportionally greater loss in median lifespan in flies reared on DR compared to AL (Fig. 4e and Supplementary Fig. 6a–c). Detailed information on the survival analyses performed in this study and accompanied statistics are in Supplementary Data 10. nCLK-D flies have altered CLK function throughout all neurons; however, it is possible that the lifespan-shortening effect observed in these lines was substantially driven by the loss of CLK function within the eye; Others have demonstrated that CLK is highly enriched (>5-fold) within photoreceptors compared to other neuronal cell types in Drosophila (Supplementary Fig. 6d). Furthermore, overexpressing CLK within just photoreceptors (prCLK-D1) also shortened lifespan (Fig. 4f). These findings argue that neuronal CLK function is required for the full lifespan extension mediated by DR and indicate that the function of the transcription factor CLK within photoreceptors is essential for the maintenance of visual function with age as well as organismal survival.

**DR protects against lifespan shortening from photoreceptor cell stress.** Previous reports have demonstrated that exposure to light can decrease lifespan—extending the daily photoperiod, or housing flies in blue light both reduce longevity41,42. Since DR delays visual senescence and promotes the rhythmic expression of genes involved in photoreceptor homeostasis (i.e., light adaptation, calcium handling), we investigated how diet influences survival in the context of light and/or phototransduction. To test the interrelationship between diet, light, and survival, we housed w1118 (white-eyed) in either a 12:12 LD cycle or constant darkness. Housing flies in constant darkness extended the lifespan of flies reared on AL, while the lifespans of flies reared on DR were unaffected (Fig. 5a). Constant darkness failed to extend the lifespan of red-eyed (w+) Canton-S wild-type flies (Supplementary Fig. 7a), suggesting that the ATP-binding cassette transporter encoded by w, and the red-pigment within the cone cells, help to protect against lifespan shortening from diet- and light-mediated stress43. White-eyed, photoreceptor null flies (homozygous for TRP365 mutation44) reared on AL failed to display lifespan extension in constant darkness (Supplementary Fig. 7b), indicating that the lifespan-shortening effects of light exposure are primarily mediated by the photoreceptors.

We performed survival analyses in rhodopsin-null flies to examine how activation of the different photoreceptor subtypes influences lifespan on AL and DR. In agreement with the reduction in systemic immune responses observed in the rhodopsin-null strains, these flies were also longer lived in comparison to w1118 outcrossed controls (Fig. 5b–e). Furthermore, rh6G mutants, which displayed the largest reductions in inflammation, also displayed the greatest extension in lifespan compared to the other rhodopsin-null lines. Gqa4 mutant flies45, which harbor a mutation in the G-protein that mediates activation of TRP channels downstream of rhodopsin, also displayed increased longevity compared to control flies (Fig. 5f). Interestingly, with the exception of rh4L, rhodopsin-null mutations and Gqa4 mutants primarily extended lifespan on AL, indicated by the hazard ratios in Fig. 5g. We next sought to investigate how increases in rhodopsin-mediated signaling influence survival. To this end, we knocked down the major arrestin, encoded by arr1, within the eyes of flies (GMR-GAL4 > UAS-arr1-RNAi). ARR1 is required for light-mediated rhodopsin internalization from the rhabdomere membrane into endocytic vesicles, thus suppressing rhodopsin-mediated signaling and associated Ca2+–mediated phototoxicity/cell death46,47. arr1 mRNA expression is circadian in wild-type heads (Supplementary Fig. 1m) and is also a direct CLK target (CLK ChIP-Seq., Supplementary Table 1). Additionally, arr1 expression was significantly downregulated in heads of nCLK-DN1 flies compared to controls (Supplementary Fig. 7c). In agreement with its
Despite its physiological role in light adaptation, we found that arr1-RNAi knockdown flies were hypersensitized to light (Supplementary Fig. 7d). In contrast to the rhodopsin-null strains, which displayed greater proportional improvements in survival on AL vs DR, arr1-RNAi knockdown flies displayed a significant shortening of lifespan on DR, while the lifespan on AL was indistinguishable from the control (Fig. 5b). Together, these findings suggest that modulation of rhodopsin-mediated signaling is sufficient to regulate lifespan in Drosophila.

Next, we used an optogenetics approach to examine how chronic photoreceptor activation influences survival in flies reared on AL or DR. This approach allowed us to directly compare the survival of isogenic flies with or without photoreceptor activation, while avoiding the potentially confounding effect that extra-ocular light-sensing might have on survival. Namely, we expressed the red-light-sensitive csChrimson cation channel in photoreceptors (Trpl-GAL4 > UAS-csChrimson (red-shifted)) supplemented with retinal or vehicle control and housed in 12:12 h red light:dark. Survival data are plotted as an average of two independent lifespan repeats. j Survival analysis of eye-specific ATPα RNAi knockdown flies vs RNAi control flies. Survival data are plotted as an average of three independent lifespan repeats. The total number of flies (N) corresponding to each lifespan in Fig. 5 can be found in the “Statistics and Reproducibility” section within the Methods. Source data are provided as a Source Data file.

Although DR protected flies from lifespan shortening from the optogenetic activation of photoreceptors, we found that forcing photoreceptor degeneration, by knocking down ATPα in the eye (GMR-GAL4 driver) shortened the lifespan of both AL and DR (Fig. 5j). In addition to knocking down ATPα throughout all eye cell types, we also targeted ATPα expression specifically within the cone cells that surround the photoreceptors (Spa-GAL4 > UAS-ATPα-RNAi). We found that knockdown of ATPα mRNA within cone cells was also sufficient to accelerate visual declines with age and reduce the lifespan in flies reared on AL and DR (Supplementary Fig. 8a, b). Similarly, eye-specific knockdown (achieved via GMR-Gal4) of Nervana 2 (nrv2-RNAi) and Nervana 3 (nrv3-RNAi), which encode the Beta subunit of the Na⁺K +ATPase of the eye34 also reduced phototaxis responses and shortened lifespan (Supplementary Fig. 8c–f). Taken together, these data support a model where DR protects flies from lifespan shortening caused by photoreceptor stress, as chronic photoreceptor activation reduces survival in flies reared on AL while having minimal to no effect on flies reared on DR. Inversely, photoreceptor deactivation primarily improves survival of flies reared on AL.

**Eye-specific, CLK-output genes modulate lifespan.** We next sought to determine if CLK-output genes in the eye influence age-
related visual declines and lifespan. We employed a bioinformatics approach to identify candidate eye-specific circadian genes transcriptionally regulated by CLK (Supplementary Fig. 9a and Supplementary Data 11). First, we compared age-associated changes in photoreceptor-enriched gene expression to genes that were differentially expressed on DR compared to AL. More than half of the photoreceptor-enriched genes that were downregulated with age were also upregulated on DR at ZT 0 and ZT 12 (upper left quadrant of Fig. 6a, b and Supplementary Fig. 9a). We then subset this gene list, selecting just transcripts whose expression was downregulated with age and upregulated on DR, and examined how their expression changed in nCLK-Δ1 fly heads (Supplementary Fig. 9a). From this analysis, we identified G-protein beta-subunit 76 C (Gβ76c), retinin, and tetraspanin 42Ej (sun) as genes that were significantly downregulated in nCLK-Δ1 fly heads at ZT 0 and/or ZT 12 (Fig. 6c, d and Supplementary Fig. 9a, e–g). Gβ76c encodes the eye-specific G beta-subunit that plays an essential role in terminating phototransduction14,50. Retinin encodes(311,127),(360,142), one of the four most highly expressed proteins in the lens of the Drosophila compound eye31. Furthermore, Retinin functions in the formation of corneal nanostructures, knockdown of which results in degraded nanostructures and a reduction in their anti-reflective properties52. Sun encodes for a lysosomal tetraspanin concentrated in the retina that protects against photoreceptor degeneration by degrading rhodopsin in response to light53. We analyzed a published CLK Chromatin Immunoprecipitation (ChIP-chip) dataset in Drosophila compound eye52. We analyzed a published CLK Chromatin Immunoprecipitation (ChIP-chip) dataset in Drosophila compound eye52.
sun in Drosophila eye tissue\textsuperscript{54} (Supplementary Fig. 9h and Supplementary Table 1), which supports our bioinformatics approach and provides further evidence that sun is an eye-specific CLK-output gene. Eye-specific knockdown of Gβ76c (GMR-GAL4 > UAS-Gβ76c-RNAi), retinin (GMR-GAL4 > UAS-retinin-RNAi), and sun (GMR-GAL4 > UAS-sun-RNAi) reduced phototaxis responses (Fig. 6e and Supplementary Fig. 9i), and shortened lifespan in comparison to RNAi control flies (GMR-GAL4 > UAS-mCherry-RNAi) (Fig. 6f, Supplementary Fig. 9j). These findings indicate that DR and CLK function together in the regulation of eye-specific circadian genes involved in the negative regulation of rhodopsin signaling (i.e., phototransduction termination). Furthermore, these observations support previous findings that lifespan extension upon DR requires functional circadian clocks\textsuperscript{10,55}, and establishes circadian CLK-output genes as diet-dependent regulators of eye aging and lifespan in Drosophila.

Discussion

Progressive declines in circadian rhythms are one of the most common hallmarks of aging observed across most lifeforms\textsuperscript{56}. Quantifying the strength, or amplitude, of circadian rhythms is an accurate metric for predicting chronological age\textsuperscript{57}. Many cellular processes involved in aging (e.g., metabolism, cellular proliferation, DNA repair mechanisms, etc.) display robust cyclic activities. Both genetic and environmental disruptions to circadian rhythms are associated with accelerated aging and reduced longevity\textsuperscript{58,59}. These observations suggest that circadian rhythms may not merely be a biomarker of aging; rather, declines in circadian rhythms might play a causal role. The observation that DR and DR-memetics, such as calorie restriction and time-restricted feeding, improve biological rhythms suggests that clocks may play a fundamental role in mediating their lifespan-extending benefits.

Herein, we identified circadian processes that are selectively amplified by DR. Our findings demonstrate that DR amplifies circadian homeostatic processes in the eye, some of which are required for DR to delay visual senescence and improve longevity in Drosophila. We report that disrupting CLK function within photoreceptors accelerates visual declines and shortens lifespan, while overexpressing wild-type CLK protects against age-associated declines in vision and rescues AL-dependent declines in photoreceptor function. Our data also demonstrate that photoreceptor stress has deleterious effects on organismal health; overstimulation of the photoreceptors induced a systemic immune response and reduced longevity.

Among the more interesting and unexpected findings of this study is the observation that the Drosophila eye influences systemic immune responses, as we observed elevated AMP expression in the bodies of flies overexpressing CLK-\(\Delta\) pan-neuronally and in flies with forced photoreceptor degeneration (ATPα-RNAi). It is possible that GAL4 misexpression may promote inflammatory responses in the fly bodies, although we found a reduction in systemic inflammation in the rhodopsin-null lines suggesting that this phenomenon can originate at the photoreceptor. We also found that these systemic immune responses correlate with lifespan changes (increased body AMP expression is associated with declines in longevity and vice versa), similar to what is observed with chronic inflammation or “inflammaging” in other models\textsuperscript{60}. However, we cannot conclude whether neuronal or eye-mediated increases in systemic inflammation are causal to aging in other tissues. Furthermore, the mechanisms by which the Drosophila eye, and, more specifically, the photoreceptor influence systemic immune responses are unclear. We speculate that photoreceptor degeneration may disrupt the retinal-blood barrier such that damage signals from the eye propagate through to the hemolymph and activate AMP expression in distal tissues. Future studies are aimed at elucidating this mechanism, and its effect on longevity.

Our findings establish the eye as a diet-sensitive regulator of lifespan. DR’s neuroprotective role in the photoreceptors appears to be mediated via the transcription factor CLK, which promotes the rhythmic oscillation of genes involved in the suppression of phototoxic cell stress (Fig. 7 and Supplementary Discussion 1). Given that CLK transcriptionally regulates circadian and non-circadian transcripts, future investigations may determine whether the time-of-day regulation of these genes by CLK is germane to promoting eye health with age. These studies may also examine whether the DR-mediated benefits on visual senescence and photoreceptor viability are mediated solely by CLK as a transcription factor (as demonstrated here) or whether circadian clock function (rhythmic output) is required. Our findings also support the notion that age-related declines in the visual system impose a high cost on an organism’s physiology. Perhaps this provides an alternative hypothesis for why several cave-dwelling animals, whose visual systems have undergone regressive evolution (e.g., cave-dwelling fish and naked-mole rats), are especially long-lived\textsuperscript{61}. Failing to develop a visual system may act as a pro-survival mechanism allowing organisms to avoid the damage and inflammation triggered by age-related retinal degeneration. Ultimately, developing a visual system, which is critical for reproduction and survival, may be detrimental to an organism later in life. Thus, vision may be an example of an antagonistically pleiotropic mechanism that shapes lifespan.

Methods

Experimental materials. A full detailed description of the materials, primer sequences, software packages, and commercial assays used in this study are reported in Supplementary Data 7. Data was collected with Microsoft Excel (version 16.38) and figures and statistics were generated in GraphPad Prism software (version 9).

Fly stocks. The genotypes of the Drosophila melanogaster lines used in this study are listed in Supplementary Table 2. The following lines were obtained from the Bloomington Drosophila Stock Center: Oregon R (25125), GMR-GAL4 (1104), Elav-GS-GAL4 (43642), Trpl-GAL4 (52274), clk\textsuperscript{wor} (56754), UAS-csChrimson (55134), UAS-CLK-\(\Delta\) (36318), UAS-CLK-\(\Delta\) (36319), G876-RNAi (28507), tsp42E/sun-RNAi (29392), retinin-RNAi (57398), AP7ε-RNAi (28073), nvr2-RNAi (28666), nvr2-RNAi (60367), and mCherry-RNAi (Bloomington RNAi-Cnt, 35785). The following lines were obtained from the Vienna Drosophila Resource Center: arr1-RNAi (22196), RNAi-cnt (empty vector, 60100). The UAS-CR line
Age of fly strains used in this study. Canton-S and timfl2 mutant flies (11 days old) were used for the time-course microarray experiment. nCLK-Δ1 flies (11 days old) were used for the RNA-Seq experiment. The following strains (6, 10, 14, and 22 days old) were used in photo-stimulation experiments: nCLK-Δ1, prCLK-Δ1, prCLK-Δ1OE, GMR-GAL4 > RNAi-cnt, GMR-GAL4 > Gbeta76c-RNAi, GMR-GAL4 > retininal-RNAi, GMR-GAL4 > mRNA-RNAi, GMR-GAL4 > Gbeta76c-RNAi, GMR-GAL4 > retininal-RNAi, GMR-GAL4 > mRNA-RNAi, Canton-S, Oregon-R, yw60, nCLK-Δ1, cry01, cry02, cryb, GMR-GAL4 > AtPax-RNAi, GMR-GAL4 > arr1-RNAi, GMR-GAL4 > nvr2-RNAi, GMR > G > nvr3-RNAi, Spa-GAL4 > RNAi-cnt, Spa-GAL4 > AtPax-RNAi. The following strains (6, 10, 14, and 25 days old) were used in ERG experiments: nCLK-Δ1, prCLK-Δ1, prCLK-Δ1OE, GMR-GAL4 > R > RNAi-cnt, Spa-GAL4 > mRNA-RNAi. The following strains (6 days old to death) were used in lifespan experiments: nCLK-Δ1, nCLK-Δ1OE, GMR-GAL4 > RNAi-cnt, Spa-GAL4 > mRNA-RNAi, Spa-GAL4 > R > RNAi-cnt, Spa-GAL4 > AtPax-RNAi. nCLK-Δ1 flies at 18 days of age were used in the hemolymph mass-spectrometry experiment.

Fly husbandry and survival analyses. All flies were maintained at 25 ± 1 °C, 60% humidity under a 12 h:12 h LD cycle (~750lux, as measured with a Digital Lux Meter, Dr. Meter Model LX1330B) unless otherwise indicated. Fly stocks and crosses were set in standard vials containing 1.5% yeast extract, 5% sucrose, 0.04% agar, 8.5% cornmeal, and 1% acid mix (a 1:1 mix of 10% propionic acid and 83.6% phosphoric acid). Fly bottles were seeded with live yeast prior to collecting virgins or setting up crosses. Mated adult progeny were then transferred to ad libitum (AL) or dietary restriction (DR) media within three days of eclosion. Adult female flies used in experiments were transferred to fresh media every 48 h at which point deaths were recorded for survival analysis. AL and DR fly media differed only in their percentage of yeast extract, respectively, containing 5% or 0.5% (Yeast Extract, B.D. Bacto, Thermo Scientific). The resulting count data were clustered, and data were scaled by row for normalization across timepoints. We employed the heatmap2 (Galaxy Version 3.0.1) function from R ggplot2 package to visualize bioinformatics data. Heatmap visualizations were generated using the Heatmapper package 3.34.3. Transcript-level expressions from the four independent biological replicates were averaged for each timepoint.

Heatmap visualizations. We employed the heatmap2 (Galaxy Version 3.0.1) function from R ggplot2 package to visualize bioinformatics data. Data were not clustered and data were scaled by row for normalization across timepoints.

Electroretinogram assays. ERGs were performed and analyzed in two independent laboratories. ERGs were recorded for eight nCLK-Δ1 adult female flies reared on AL or DR diets in a microelectrode chamber implemented with a Keithley 4280 at 4 °C at the Baylor College of Medicine (BCM), and at day 21 (25 days of age) at the University of California, Santa Barbara (UCSB). ERGs were recorded for prCLK-Δ1 and prCLK-Δ1OE female flies at UCSB reared on AL or DR and maintained at either 18 °C or 30 °C at 8 days old, 6, 10, 14, and 18 days old. BCM: ERG recordings were performed as in ref 68. Two glass electrodes were filled with Ringer’s solution and electrode cream was applied to immobilized flies. A reference electrode was placed on the thorax, while the seq, experimental design, RNA extraction: Frozen flies were vortexed to remove heads and mRNA from each biological replicate of pooled heads was isolated with the Quick-RNA Mini Prep Kit (Zymo Research #11-328), per manufacturers’ instructions. Fragment library preparation and deep sequencing: Library preparation was performed by the Functional Genomics Laboratory at the Berkeley Core Research Facility at University of California, Berkeley. cDNA libraries were produced from the low-input RNA using the Takara SMART-Seq v4 ULTRA-low input RNA kit. An S220 Focused-Ultrasonicator (Covaris) was used to fragment the DNA, and library preparation was performed using the KAPA hyper prep kit for DNA (KK8504). Truncated universal adapter stubs were used for ligation, and indexed primers were used during PCR amplification to complete the adapters and to enrich the libraries for adapter-ligated fragments. Samples were checked for quality on an AATI (now Agilent) Fragment Analyzer. Samples were then transferred to the Vincent J. Coates Genomics Sequencing Laboratory (GSL), another QB3-Berkeley Core Research Facility at UC Berkeley, where Illumina sequencing library molarity was measured with quantitative PCR with the Kapa Biosystems Illumina Quant qPCR Kits on a BioRad FXC Connect thermal cycler. Libraries were then pooled evenly by molarity and sequenced on an Illumina NovaSeq6000 150PE S4 flowcell, generating 25 M read pairs per sample. Raw sequencing data were converted into fastq format, sample-specific files using the Illumina FastqTools v2.20 (v2.20.10.40) software on the UCSB supercomputer server. Read alignment and differential expression analyses: Raw fastq reads were filtered by the Trimmomatic software44 (Trimmomatic-0.36) to remove Illumina-specific adapter sequences and the minimal length was set to 36 (MINLEN) for trimming sequences. The paired-end filtered reads were then aligned to D. melanogaster reference genome was utilized as the gene annotation file with specific strand information set to “stranded (Reverse)”. The resulting count files (tabular format) were then analyzed with DESeq2 (Galaxy Version 2.11.40.7 + galaxy7) with fit-type set to “local”, and P-values of less than 0.05 (adjusted for multiple testing) were considered differentially expressed between factor levels. Normalized count reads were outputted for visualization of expression (heatmaps), and Supplementary Data 3 contains normalized read counts across all experimental samples. UCSC genome browser visualization: The makeUCSCfile software package from HOMER (v4.11) was utilized to generate bedGraph files for visualizing changes in tag density at exon 2 of crk comparing nCLK-Δ1 and control samples (Supplementary Fig. 2b).

Garcian time-course expression analysis. Mated Canton-S and timfl2 female flies were reared on AL or DR diets for seven days at 25 ± 1 °C, under a 12:12 h light-dark (LD) regime. Beginning on the seventh day, four independent biological replicates (per diet/timepoint) of approximately 35 female flies (approximately 11 days old) were collected on dry ice every 4 h for 20 h starting at ZT 0 (six total timepoints, 48 total samples). RNA extraction, DNA amplification/labeling, and gene expression arrays were performed following the same protocols as in ref 53. In summary, RNA was isolated from whole-fly lysates with Qiagen’s RNeasy Tissue Mini Kit (74804), and RNA quantity and quality were accessed with a Nanodrop and Agilent’s bioanalyzer (RNA 600 Nano Kit (5067-15811)). DNA amplification from total RNA was performed using Sigma’s TransPlex Complete Whole Blood Hybridization Kit (WTA) and purified with a QIAquick PCR Purification Kit (28104). Gene expression labeling was performed with NimbleGen One-Color DNA Labeling Kit (0522355001) and hybridized to NimbleGen 12-Plex gene expression arrays. Arrays were quantitated with NimbleGen’s NimbleScan2 software (version 2.6), and downstream expression analyses were conducted using 3.2.1.4 (https://www.nature.com/naturecommunications) package 3.34.3. Transcript-level expressions from the four independent biological replicates were averaged for each timepoint.

ncrL-Δ1 RNA-seq analyses. nCLK-Δ1 (Elav-GAL4 > UAS-nCLK-Δ1) adult female flies were developed on standard stock food (1.5% yeast-extract) for four generations. At 18 days old, flies were then transferred on DR diets or DR diets treated with RU486 or vehicle control at 25 ± 1 °C, under a 12:12 h LD regime. Diets were changed approximately every 48 h, until the seventh day at which point flies were flash-frozen (~11 days old) on dry ice at ZT 0 and ZT 12 (lights-on and -off, respectively). See Supplementary Fig. 2a for RNA-seq library description. Sequencing libraries were produced by the Functional Genomics Laboratory at the Berkeley Core Research Facility at University of California, Berkeley. cDNA libraries were produced from the low-input RNA using the Takara SMART-Seq v4 Ultra-low input RNA kit. An S220 Focused-Ultrasonicator (Covaris) was used to fragment the DNA, and library preparation was performed using the KAPA hyper prep kit for DNA (KK8504). Truncated universal adapter stubs were used for ligation, and indexed primers were used during PCR amplification to complete the adapters and to enrich the libraries for adapter-ligated fragments. Samples were checked for quality on an AATI (now Agilent) Fragment Analyzer. Samples were then transferred to the Vincent J. Coates Genomics Sequencing Laboratory (GSL), another QB3-Berkeley Core Research Facility at UC Berkeley, where Illumina sequencing library molarity was measured with quantitative PCR with the Kapa Biosystems Illumina Quant qPCR Kits on a BioRad FXC Connect thermal cycler. Libraries were then pooled evenly by molarity and sequenced on an Illumina NovaSeq6000 150PE S4 flowcell, generating 25 M read pairs per sample. Raw sequencing data were converted into fastq format, sample-specific files using the Illumina FastqTools v2.20 (v2.20.10.40) software on the UCSB supercomputer server. Read alignment and differential expression analyses: Raw fastq reads were filtered by the Trimmomatic software44 (Trimmomatic-0.36) to remove Illumina-specific adapter sequences and the minimal length was set to 36 (MINLEN) for trimming sequences. The paired-end filtered reads were then aligned to D. melanogaster reference genome was utilized as the gene annotation file with specific strand information set to “stranded (Reverse)”. The resulting count files (tabular format) were then analyzed with DESeq2 (Galaxy Version 2.11.40.7 + galaxy7) with fit-type set to “local”, and P-values of less than 0.05 (adjusted for multiple testing) were considered differentially expressed between factor levels. Normalized count reads were outputted for visualization of expression (heatmaps), and Supplementary Data 3 contains normalized read counts across all experimental samples. UCSC genome browser visualization: The makeUCSCfile software package from HOMER (v4.11) was utilized to generate bedGraph files for visualizing changes in tag density at exon 2 of crk comparing nCLK-Δ1 and control samples (Supplementary Fig. 2b).

Heatmap visualizations. We employed the heatmap2 (Galaxy Version 3.0.1) function from R ggplot2 package to visualize bioinformatics data. Data were not clustered and data were scaled by row for normalization across timepoints.
food (with or without 200 μM RU486 when indicated) at a density of 10–25 flies per tube prior to and after phototaxis measurements. Approximately 160–480 flies were used per experiment. Flies eluted free of phototaxis were from the S-Traps collected following the following morning in the same elution tube as follows: 80 μl of 50 nM TEAB was spun through for 1 min at 1000 g. Eighty microliters of 0.5% formic acid was spun through for next 1 min at 1000 g. Finally, 80 μl of 50% acetonitrile in 0.5% formic acid was spun through the S-Trap column for 1 min at 4000 g. These pooled elution solutions were dried in a speed vac and then resuspended in 0.2% formic acid. Desalting: The redissolved peptide samples were desalted with stage tips containing a C18 disk, concentrated, and resuspended in aqueous 0.2% formic acid containing “Hyper Reaction Monitoring” indexed retention time peptide standards (rRT, Bioynios). Mass spectrometry: The proteomic sample preparation: nCLK–Δ1, nCLK–Δ2, GMR–GAL4 > mCherry—RNAi (cnt), GMR–GAL4 > arl1–RNAi, GMR–GAL4 > Atp8–RNAi, GMR–GAL4 > retinin–RNAi, GMR–GAL4 > sun–RNAi, GMR–GAL4 > ninaE–RNAi, GMR–GAL4 > rh32–RNAi, prCLK–Δ1, prCLK–OE, Spa–RNAi–GAL4 and Spa–RNAi–cnt. RNA extraction and cDNA preparation. Adult female flies (strains nCLK–Δ1, nCLK–Δ2, w1118, mnl27, rht3, rdh4, rdh7, GMR–GAL4 > Atp8–RNAi, GMR–GAL4 > mCherry–RNAi (cnt), GMR–GAL4 > arl1–RNAi, GMR–GAL4 > Atp8–RNAi, GMR–GAL4 > retinin–RNAi, GMR–GAL4 > sun–RNAi, GMR–GAL4 > ninaE–RNAi, GMR–GAL4 > rh32–RNAi, prCLK–Δ1, prCLK–OE, Spa–RNAi–GAL4 and Spa–RNAi–cnt.) were collected, homogenized, and RNA was isolated using the Quick-RNA MiniPrep Kit (Zymo Research, 11-328). RNA was collected in 30 μl DNase/RNase-free water and quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). For each experiment, 120–180 age-, genotype-, and diet-matched flies were collected, and three independent RNA extractions were performed. To extract RNA from heads, 40–60 flies were used; to extract RNA from bodies, 20–30 flies were used. cDNA preparation: The iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, 1708841) was used to generate cDNA from RNA extracted from heads and bodies. For each group, 1 μg of total RNA was used in a volume of 4 μl Script master mix, then brought to 20 μl with 18 μl DNase/RNase-free water. A T1000 thermocycler (BioRad) was used for first-strand RT-PCR, with 25 cycles of denaturing (95 °C for 2 min), reverse PCR (30 cycles at 52 °C), and inactivation of the reaction (5 min at 85 °C).

Real-time quantitative PCR. Reactions were performed in a 384-well plate. Each reaction contained 2 μl of 1:20 diluted cDNA, 1 μl of primers (forward and reverse at 10 μM), 5 μl SensiFast SYBR Green No-ROX Kit (BIOLINE, BIO-98020), and 2 μl of DNase/RNase-free water. The qPCR reactions were performed with a Light Cycler 480 System (Roche Applied Science) with the following run protocol: pre-incubation (95 °C for 2 min), forty PCR cycles of denaturing (95 °C for 5 s, ramp rate 4.8 °C/s), and annealing and extension (60 °C for 20 s, ramp rate 2.5 °C). The PCR primer sequences (forward and reverse) are in Supplementary Data 7.

Hemolymph mass spectrometry. Proteomic sample preparation: nCLK–Δ1 female flies (Elav-GeneSwitch–GAL4 > UAS-nCLK–Δ1) were reared on AL diet plus RU486 or vehicle control (N = 300 flies per biological replicate, n = 3 biological replicates). On day 14 (18 days old), flies were snap-frozen on dry ice and transferred to prechilled vials. The vials were vortexed for 5–10 s to remove heads and the frozen bodies were transferred to room temperature vials fitted with 40 μm filters. Headless bodies were thawed at room temperature for 5 min and spun at 2000 x for 2 min. Following the spin, hemolymph collected homogeneously from each vial, and the bodies remained within the filters. Digestion: A Bicinchoninic Acid protein assay (BCA) was performed for each of the hemolymph samples and a 100 μg aliquot was used for trypptic digestion for each of the six samples. Protein samples were added to a lysis buffer containing a final concentration of 5% SDS and 50 mM triethylammonium bicarbonate (TEAB), pH 7.2–5.5. The samples were reduced to 20 μM dithiothreitol (DTT) for 10 min at 50 °C, subsequently cooled at room temperature for 10 min, and then alkylated with 40 mM iodoacetamide (IAA) for 30 min at room temperature in the dark. Samples were acidified with a final concentration of 1.2% phosphoric acid, resulting in a visible protein precipitate. 90% methanol in 100 mM TEAB was added at a volume of seven times the acidified lysate volume. Samples were vortexed until the protein coagulated was thoroughly dissolved in the 90% methanol. The entire volume of the samples was spun through the micro S-Traps (Prolifit) in a flow-through Eppendorf tube. Samples were spun through in 200 μl aliquots for 20 s at 4000 x g. Subsequently, the S-Traps columns were washed with 200 μl of 90% methanol in 100 mM TEAB (pH 7.1), twice for 20 s each at 4000 x g. S-Traps columns were placed in a clean elution tube and incubated for 1 h at 47 °C with 125 μl of trypsin digestion buffer (50 mM TEAB, pH –8) at a 1:25 ratio (protease/protien, wt/wt). The same mixture of trypsin digestion buffer was added again for overnight incubation at 37 °C. The protein hydrolysates were then washed with fresh TEAB (pH 7.1) 2 times, vortexed, and placed on an Eppendorf for 5 min to remove any remaining precipitate. The supernatant was transferred to an Eppendorf tube for further analysis. The pooled elution solutions were dried in a speed vac and then resuspended in water for further analysis.

Identification of photoreceptor-enriched CLK-out genes. Diagram of bioinformatics results reported in Supplementary Fig. 5A. Gene lists are reported in Supplementary Data 11. We identified the top 1000 photoreceptor-enriched genes from ref. 74. We then filtered this list for genes that oscillate in a circadian fashion, and that are downregulated with age from ref. 16 (GSE81100). Approximately 1/3 of the photoreceptor-enriched genes (366 genes) were expressed in a circadian fashion in young wild-type heads and approximately one-half of these (172 genes) displayed a significant loss in expression with age (5- vs 55-day old heads). We further analyzed the remaining gene lists to identify those that are significantly upregulated on DR compared to AL at either ZT 0 or ZT 12 from control (vehicle-treated) samples from our nCLK–Δ1 RNA-Seq analyses. Transcripts with a DESeq2 P < 0.05 (non-adjusted) were considered differentially expressed. To increase the chance of including false negatives we utilized the raw P-value of the adjusted P-values (multiple testing correction) and performed additional experiments to validate these downstream targets. For the final validation, we analyzed the genes that were significantly downregulated in nCLK–Δ1 on DR (RU486 vs vehicle-treated controls), resulting in the identification of G76c, retinin, and sun.

Tangential retinal sectioning and imaging. Control, prCLK–OE, and prCLK–Δ1 female flies were reared on AL or DR food for 2 or 10 days. Flies were then decapitated (6 and 14 days old) and whole-fly heads were fixed in 2.5% glutar-aldehyde overnight and then transferred into 2.0% osmium tetroxide for approximately 4 h. The heads were then dehydrated in 100% ethanol and embedded in Epon. Tangential retinal sections (~0.5 micron slices) were stained with 0.1% Toluidine Blue. Image capture was performed with a Nikon Ni-E upright microscope with a motorized stage, MQA18000 DS-FI3 Microscope Camera controlled by the NIS Elements 5.2 software. The microscope was set to ‘brightfield’, auto-exposure was set to ‘continuous’, power at 100%, and auto-white was selected for each image taken with either a ×20 and ×40 objective.

Identification of differential gene expression in flies reared in LD vs DD. Gene expression changes in heads of y.w. flies housed in 12:12 LD vs constant darkness (DD) were accessed from ref. (GSE8342)13. Fold-changes in response to light were calculated by averaging the changes in expression at each timepoint from a circadian microarray and comparing expression between flies housed in LD vs. DD. Individual genes were scored as significantly differentially expressed by performing a Student’s t-test (paired, two-tailed) with P ≤ 0.05.
Statistics and reproducibility. The individual biological replicates "n" and the number of individual flies "N" is denoted in each figure legend along with the particular statistical test utilized. The P-value statistics are included in each figure. All error bars are represented as the standard error of the mean (SEM), and all graphs were generated in PRISM 9 (GraphPad). The experiments in this manuscript were performed with populations of female flies (i.e., typically greater than 20 flies by biological replicate).

Figure 3b: The number of biologically independent flies used are as follows (Day 2, 6, 10, 14): AL cnt 10, 14, 14, 10 flies, AL prCLK-Δ1, 4, 5, 4, 0 flies, AL prCLK-OE 10, 10, 9, 10 flies, DR cnt 10, 10, 10, 10 flies, DR prCLK-Δ1 5, 6, 7, 0 flies, DR prCLK-OE 10, 10, 12, 10 flies. The ERG amplitudes for the prCLK-Δ1 flies on day 14 were non-responsive (flat) so we did not include those data in the graph. The ERG amplitudes were collected from flies examined over two independent experiments.

Figure 3c: The number of biologically independent flies with similar observable phenotypes to the main figure are as follows (Day 2, 10): AL cnt 4, 2 flies, AL prCLK-Δ1 2, 3 flies, AL prCLK-OE 2, 2 flies, DR cnt 3, 6 flies, DR prCLK-Δ1 3, 1 flies, DR prCLK-OE 4, 3 flies.

Figure 5: The number of flies used for lifespan analyses are as follows: (a) LD housed flies: AL N = 560, DR N = 584; DD housed flies: AL N = 460, DR N = 462. (b-g) w^{1118}, ninaE^{17} flies: AL N = 314, DR N = 511; w^{1118}, rh^{55} flies: AL N = 543, DR N = 597; w^{1118}, rh^{56} flies: AL N = 550, DR N = 593; w^{1118}, rh^{65} flies: AL N = 533, DR N = 563; w^{1118}, Gαq flies: AL N = 403, DR N = 400. (h) RNAi control flies: AL N = 365, DR N = 351; arrl-1 RNAi flies: AL N = 333, DR N = 322. (i) Retinal-treated flies: AL N = 289, DR N = 236; Vehicle-treated flies: AL N = 256, DR N = 126. (j) RNAi control flies: AL N = 493, DR N = 490; ATPla RNAi flies: AL N = 510, DR N = 535. Survival data is plotted as an average of three independent lifespan repeats.

Time-course microarray analyses. Four independent biological replicates (per diet/timepoint) of approximately 35 Canton-S female flies were collected on dry ice every 4 h for 20 h starting at ZT 0 (six total timepoints, 48 total samples). Differential expression was determined by two-tailed Student’s t-test (paired) comparing the averaged transcript-level expression values between AL and DR samples across all timepoints, and P-values less than 0.05 were considered significant. The JTK_CYCLE algorithm (v3.0) was utilized to identify circadian transcripts from the AL and DR time-course expression arrays. Transcript-level expression values for each of the four biological replicates (per timepoint/diet) were used as input for JTK_CYCLE, and the period length was set to 24 h. We defined circadian transcripts as those displaying a JTK_CYCLE P-value of less than 0.05 (non-adjusted) in wild-type flies (Canton-S) while displaying a JTK_CYCLE P-value of less than 0.05 (non-adjusted) in circadian mutant flies (time^{2}). Subsequent analyses compared diet-dependent changes in JTK_CYCLE outputs (phase and amplitude).

nCLK-Δ1 RNA-Seq. Three independent biological replicates of 100 mated female flies were utilized per genotype/diet/timepoint. DESeq2 software was utilized and P-values of less than 0.05 (adjusted for multiple testing) were considered differentially expressed between factor levels for gene-ontology enrichment analyses.

ERG responses. For ERG experiments we quantified responses from 6 to 15 individual flies per standard in the field. Statistical significance was determined by two-tailed Student’s t-test (unpaired), comparing ERG responses between diet and genotypes. Full ERG statistics are reported in Supplementary Data 6.

Survival analyses. The Log-rank (Mantel-Cox) test was used to determine statistical significance by comparing average lifespan curves from a minimum of two independent lifespan replicates. Hazard Ratios (log-rank) were also utilized to determine the probability of death across genotypes, lighting conditions, and diet. Detailed Log-rank and hazard ratios for each lifespan are reported in Supplementary Data 10.

Positive phototaxis assay. Statistical significance for the phototaxis index at each timepoint was calculated with the Student’s t-test (two-tailed, unpaired). Two-way ANOVA or mixed-effects models were performed to determine statistical significance between diet, genotype, or time interactions. Full statistical output (two-way ANOVA and t-test) for all phototaxis experiments are reported in Supplementary Data 5.

Real-time quantitative PCR. Fold change in gene expression was calculated using the ΔΔCt method and the values were normalized using p49 as an internal control. P-values were calculated with the pairwise Student’s t-test comparing Log2 fold-changes in expression.

Mass-spectrometric data processing, quantification, and bioinformatics. Mass-spectrometric data-dependent acquisitions were analyzed using the database search engine ProteinPilot (SCIEX 5.0 revision 4769) using the Paragon algorithm (5.0.0.0,4767). Using these database search engine results a MS/MS spectral library was generated in Spectrumator 14.2.200619.47784 (Biognosy). The DIA/SWATH data was processed for relative quantification by comparing peptide intensity from various different timepoints during the cell cycle. For the DIA/ SWATH MS2 data sets quantification was based on XICs of 6-10 MS/MS fragment ions, typically y- and b-ions, matching to specific peptides present in the spectral libraries. Differential protein expression analysis was performed using a paired t-test and P-values were adjusted for multiple testing. Peptides were identified at Q ≤ 0.01%, and significantly changed proteins were accepted at a 5% FDR (Q ≤ 0.01).

Gene-ontology enrichment analysis. To identify enriched gene-ontology (i.e., bioprocess) categories with the resultant lists from bioinformatics approaches, we utilized the “findGO.4p” package from HOMER (v4.11). Full gene-ontology lists including enrichment statistics (calculated assuming hypergeometric distribution, no adjustment for multiple-hypothesis testing) and associated gene lists are reported in supplementary data files. A maximal limit of 200 gene identifiers per GO category was implemented to reduce the occurrence of large, over-represented terms that lack specificity (i.e., metabolism).

Data availability

The time-course microarray data and accompanied JTK_CYCLE statistics that support the findings in this study have been deposited in the Gene Expression Omnibus with the GSE158286 accession code. The RNA-seq data and accompanied differential expression analyses that support the findings in this study have been deposited to GEO with the GSE158905 accession code. The mass-spectrometric raw data generated in this study have been deposited at the MassIVE ID MSV000806781; it is also available at ProteomeXchange with the ID PXD023896. Additional mass-spectrometric details from DIA and DDA acquisitions, such as protein identification and quantitation details are available at the repositories (including all generated Spectrumator and Protein Pilot search engine files). The wild-type circadian gene expression data analyzed in this study are available in the GEO database under accession code GSE811005. The results of DD expression data analyzed in this study are available in the GEO database under accession code GSE937825. All other data supporting the findings of this study are included within the manuscript and supplementary information/data files. Source data are provided with this paper.

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Competing interests
The authors declare no competing interests.

Additional information
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