Dietary Restriction Impacts Peripheral Circadian Clock Output Important for Longevity in *Drosophila*

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Abstract

Circadian clocks may mediate lifespan extension by caloric or dietary restriction (DR). We find that the core clock transcription factor *Clock* is crucial for a robust longevity and fecundity response to DR in *Drosophila*. To identify clock-controlled mediators, we performed RNA-sequencing from abdominal fat bodies across the 24 h day after just 5 days under control or DR diets. In contrast to more chronic DR regimens, we did not detect significant changes in the rhythmic expression of core clock genes. Yet we discovered that DR induced de novo rhythmicity or increased expression of rhythmic clock output genes. Network analysis revealed that DR increased network connectivity in one module comprised of genes encoding proteasome subunits. Adult, fat body specific RNAi knockdown demonstrated that proteasome subunits contribute to DR-mediated lifespan extension. Thus, clock control of output links DR-mediated changes in rhythmic transcription to lifespan extension.

Introduction

Circadian (~24 h) clocks regulate a wide range of rhythmic metabolic, physiological and behavioral parameters to acclimate to environmental changes in light, temperature, and food availability (Patke et al., 2020). Circadian clock disruption has been implicated in advanced aging and the longevity response to caloric or dietary restriction (CR or DR) (Froy, 2018; Galikova and Flatt, 2010; Manoogian and Panda, 2017; Nakahata and Fukada, 2022; Zhu et al., 2022). DR, reduction in food intake without causing malnutrition, robustly extends longevity in various animal models including yeast, worms, flies, and monkeys (Green et al., 2022; Mc Auley, 2022). Yet, the molecular mechanisms by which DR delays aging are not fully understood. Understanding how the clock impacts aging and DR sensitivity may provide novel avenues to understanding aging.

The circadian clock consists of a widely conserved transcriptional feedback loop that drives 24 h molecular oscillations. In flies, the heterodimer transcription factor CLK/CYC forms the positive arm of the loop and activates their repressors, PER and TIM. The PER-TIM complex functions as the negative arm of the loop and inhibits CLK-CYC activity (Allada and Chung, 2010). This feedback loop drives core clock gene rhythms and controls rhythmic physiological, metabolic, and behavioral parameters via clock control of output genes (Patke et al., 2020). Genetically hybrid mice with a deviation of the circadian period from 24 h by over seven minutes showed a higher mortality rate than the mice with less deviated periods (Libert et al., 2012). However, whether the altered circadian period is correlated with or causes the increased mortality is not clear. Genetic inactivation of CYC ortholog *Bmal1* as well as other circadian clock mutants also significantly reduced lifespan in mice (Dubrovsky et al., 2010; Fu et al., 2002; Kondratov et al., 2006; Lee et al., 2010). Yet when *Bmal1* knockout was restricted to adulthood, lifespan was normal (Yang et

al., 2016). While a lifelong DR did not significantly extend lifespan of *Bmal1* knockout mice (Patel et al., 2016a), chronic (~2 mo) DR exposure increased core clock amplitude in mice (Patel et al., 2016b; Sato et al., 2017). This suggests that the circadian clock may be among the molecular mechanisms of DR. However, mice under DR restrict their feeding behavior to a narrow temporal window (Acosta-Rodriguez et al., 2017). Thus, DR induced changes in core clocks may instead be due to the well-known effects of time-restricted feeding (Hatori et al., 2012). Indeed, core clock genes are important for age-dependent cardiac function and lifespan extending effects of time-restricted feeding (Gill et al., 2015; Ulgherait et al., 2021). A recent study revealed that a basal level lifespan extension by DR is further increased when DR is temporally aligned with mice's natural meal timing (i.e., during the night) {Acosta-Rodriguez, 2022 #117}. Thus, it remains unclear whether disruption of the circadian clock itself or other factors, such as a defect during development, results in lifespan reduction and is responsible for the lack of DR response.

Circadian clocks have also been implicated in aging and the DR longevity response in flies as well. DR mortality effects are rapid, fully evident within just 2 ~ 4 days of a diet shift in flies (Mair et al., 2003; McCracken et al., 2020), making them an attractive model organism for DR studies. Loss-of-function mutants in the activator and repressor complexes that "fix" the clock at different points in the cycle have tested the functional significance of the clock in aging and DR. Inhibition of neuronal *Clk* appears to reduce the lifespan extending DR effects, where flies were tested for DR effects with two (ad libitum and DR) diets (Hodge et al., 2022). However, this observation is inconclusive to the role of *Clk* for DR effects as inhibition of neuronal *Clk* decreases food intake {Xu, 2008 #103}. Reduction of food intake can decrease lifespan under DR while increasing lifespan under ad libitum, masking the true DR response {Flatt, 2014 #74}. per^{01} and tim^{01} mutant

flies exhibit inconsistent DR longevity responses perhaps due to differences in microbial content (Katewa et al., 2016; Ulgherait et al., 2020; Ulgherait et al., 2016; Ulgherait et al., 2021). Thus, the role of core clock genes in mediating DR effects could be further clarified. Notably, the rhythmic amplitude of core clock genes of flies is enhanced after chronic (>10 days) DR (Katewa et al., 2016). Knockdown of modestly CLK- and DR- regulated genes in the eye modulate lifespan without apparent effects on DR-dependent longevity (Hodge et al., 2022). *tim* overexpression increased the amplitude of core clock oscillations and extended lifespan under control but not DR diets (Katewa et al., 2016). While clock oscillation amplitudes between *tim* overexpression on a control diet and wild-type flies under DR are comparable, their lifespan remain quite different, suggesting that core clock effects may not be required for lifespan extension (Katewa et al., 2016). Thus, it remains unclear if lifespan extension functions via the circadian clock or instead through clock output genes and what the role of specific clock output genes is in DR-dependent longevity. Using multiple diets, we demonstrate that *Clk* mutants suppress DR longevity and fecundity responses, providing more definitive demonstration of the role of the core clock. Nonetheless, using a shorter-term DR strategy, we reveal that primary DR effects on the circadian transcriptome spare core clock genes, suggesting a primary effect on circadian clock output. Network analysis suggests that a diet-dependent effect on a gene module containing proteasome subunit genes. Moreover, suppression of proteasome subunit expression, predominantly in the abdominal fat body, limits lifespan extension by DR. These results provide crucial genetic evidence that circadian clock output pathways, specifically those involving the proteasome, link DR-mediated changes in rhythmic transcription to lifespan extension. These studies raise the possibility of using chronotherapy to combat aging and age-related diseases.

Results

The Effects of Dietary Restriction on Lifespan and Fecundity Is Dramatically Suppressed in Mutants of the Core Clock Transcription Factor *Clk*

To tease apart the role of the circadian clock in the DR longevity response, we first evaluated the roles of the positive and negative arm of the feedback loop by testing per^{01} as in prior reports (Katewa et al., 2016; Ulgherait et al., 2020; Ulgherait et al., 2016), and Clk^{Jrk}, a dominant negative allele of *Clk* (Allada et al., 1998), which had not been previously examined for DR studies. We applied a common DR regimen where the concentration of both yeast and sucrose are diluted (whole food dilution: Control: 15% [w/v] Sucrose and Yeast, 15SY; DR: 5% [w/v] Sucrose and Yeast, 5SY) in 12hr light: 12hr dark (LD) cycles (Bass et al., 2007; Kabil et al., 2011). We used female flies where DR responses are more robust (Magwere et al., 2004). We observed robust lifespan extension by DR in wild-type *iso31* (w^{1118}, 29%) and comparable extension in per^{01} flies (25%) (Fig. S1; diet*genotype interaction $p > 0.05$ between *iso31* and per^{01}). These results are consistent with one report which showed that per^{01} flies display normal DR extension effects (Ulgherait et al., 2016). However, only ~ 10% lifespan extension by DR was observed in Clk^{Jrk} mutants (Fig. S1; diet*genotype interaction $p < 0.0001$ between *iso*31 and Clk^{Jrk}). Interestingly, the DR responses in per^{01} and Clk^{Jrk} flies were significantly different from each other (Fig. S1; diet*genotype interaction $p < 0.0001$ between per^{01} and Clk^{Jrk}). We hypothesize that Clk^{Jrk} and per^{01} arrest the clock at opposite points in the cycle, only one of which impacts the DR response. Although the DR response is typically tested by comparing lifespan with just two diets (Solovev et al., 2019), it can potentially mis-assign the effects of DR or diet (Flatt, 2014; Tatar, 2007). For example, *chico* mutants show little effect of DR looking at just two diet concentrations but showed an almost identical response if their lifespan was measured across seven diet concentrations

(Clancy et al., 2002). To distinguish between this possibility and a "true" DR response, we performed a reaction norm analysis by comparing the mean lifespan of *Clk*^{Jrk} flies to that of wild-type over serially diluted diets (1, 5, 10, 15, and 20SY) (Bass et al., 2007; Flatt, 2014; Tatar, 2007). As expected in wild-type flies, we observed a reduction of lifespan as food concentration increased from 5SY to 20SY. A reduction of lifespan was also observed when going from 5SY to 1SY, presumably due to malnutrition (Fig. 1A & C). However, mean lifespan of *Clk*^{Jrk} mutant flies showed a more flattened reaction curve to diets (Fig. 1C, diet*genotype interaction $p < 0.0001$ between wild-type *iso31* and *Clk*^{Jrk}), suggesting that *Clk* is a "true" DR gene. For example, *Clk*^{Jrk} flies only show a 14% increase in lifespan between 5SY and 15SY while *iso31* flies show a 34% increase. In addition, while *Clk*^{Jrk} flies are short-lived relative to wild-type at 5SY, they significantly outlived wild-type flies in 1SY (Fig. 1 & S2, $p < 0.0005$ by log-rank test). Similar results were obtained in an independent trial (Fig. S2). It has been suggested that a whole food dilution may cause dehydration effects, especially on high concentrations of yeast (Ja et al., 2009) and sucrose (van Dam et al., 2020), which may lead to a false conclusion on lifespan response to diet. To eliminate this possibility, we additionally tested the DR response of *Clk*^{Jrk} mutant flies using the yeast-restriction strategy, where varying yeast concentration with a fixed sucrose concentration(Bass et al., 2007; Ja et al., 2009; McCracken et al., 2020). We first tested the DR response of *Clk*^{Jrk} mutant flies using the same experimental diets used for Fig.1 and Fig. S3 except with a fixed sucrose concentration at 5%. Although the reaction pattern of *Clk*^{Jrk} flies in this yeast-restriction DR regimen was qualitatively somewhat different from that of whole food dilution, we confirmed that the DR response of the mutants was strongly suppressed as in whole food dilution protocols (Fig. 1 and Fig. S2). We then further confirmed this observation using a different yeast-restriction protocol where purified yeast extract is used (Katewa et al., 2016; Ulgherait et al., 2016)

instead of whole-cell lysates of yeast (McCracken et al., 2020; Min et al., 2007). Although overall mean lifespan and lifespan response to the yeast extract diets (Fig. S4, Table S1) was qualitatively different from those of whole food restriction (Fig. 1 and S2) and whole cell lysates yeast restriction (Fig. S3), DR response of Clk^{Jrk} mutant flies in yeast extract diets was significantly impaired compared to wild-type *iso31* control flies. Thus, across several diet conditions, these data indicate that Clk^{Jrk} robustly suppresses the DR longevity response. While increasing diet concentration has a negative impact on lifespan, it has a strong positive correlation with female fecundity (e.g., egg laying) primarily due to increased protein sources in yeast (Bass et al., 2007; Skorupa et al., 2008). To determine if Clk^{Jrk} also impacted this diet response, we measured egg laying in both *iso31* and Clk^{Jrk} mutant flies over 7 days. As expected, *iso31* flies increased egg production with increased diet concentrations (Fig. 2). However, egg production was strongly decreased in Clk^{Jrk} mutant flies (Fig. 2, $p < 0.0001$ by regression analysis). More importantly, diet-dependent increases in egg laying were significantly suppressed in Clk^{Jrk} mutants (Fig. 2, $p < 0.01$ for pair-wise comparison in each diet by t-test). A similar trend was observed in an independent trial (Fig. S5). Taken together, these data indicate that Clk^{Jrk} strongly disrupts how flies respond to DR at both levels of longevity and fecundity.

Shorter Term Dietary Restriction Selectively Reprograms Circadian Output Genes in the Abdominal Fat Body

Given the role of Clk^{Jrk} in mediating responses to DR, we then asked how the circadian transcriptome responds to DR. A recent RNA-Seq analysis using whole-fly lysates of female flies after seven days of DR or *ad libitum* diets (a yeast extract restriction protocol) suggested that DR alters the circadian transcriptome in whole flies (Hodge et al., 2022). We focused our studies on

the fat body, an analog of the mammalian liver and adipose tissue, given its critical role in mediating the effect of DR (Bai et al., 2012; Banerjee et al., 2012; Dobson et al., 2018; Katewa et al., 2016). Importantly, the fat body also has its own core clock system, including *Clk*, regulating energy metabolism, feeding, and egg-laying (Xu et al., 2011; Xu et al., 2008). To assess DR-dependent circadian rhythms, we performed a fine scale (every 2 h over 24 h) RNA-Seq analysis from dissected abdominal fat body tissues (DiAngelo and Birnbaum, 2009; Xu et al., 2011; Xu et al., 2008) of young (8 days old) *iso31* females. To prepare dissected fat body samples for RNA-Seq analysis, we entrained flies for ~ 5 days under 12h:12h light-dark (12LD) cycles on either control or DR diets. Previous studies showed that ~ 3 days in LD cycles are sufficient to entrain the fat body clock in *Drosophila* (Erion et al., 2016; Xu et al., 2011; Xu et al., 2008). Moreover, DR reshapes mortality of flies within 2 ~ 4 days of diet shift in flies (Mair et al., 2003; McCracken et al., 2020). We also observed that 5 days on DR diet was sufficient to affect metabolism and physiology evidenced by changes in fecundity (Fig. 2 & S3). Thus, our environmental settings in light schedule and diet are sufficient to capture significant diet- and circadian-dependent transcriptional changes important for lifespan extension by DR. We then analyzed the samples from control and DR diets separately to examine if and how DR changes the circadian gene expression pattern in the fat body. For this diet-dependent analysis, we used BooteJTK (Hutchison et al., 2018) to identify rhythmic genes, using a fold-change threshold ≥ 1.5 at an FDR < 0.25. This cutoff corresponds to p values of 0.011 and 0.014 for control and DR, respectively, making the p value cutoff for this study similar to or more stringent than several recent studies (Abruzzi et al., 2017; Eckel-Mahan et al., 2013; Kuintzle et al., 2017; Sato et al., 2017). We found a significant reorganization of the circadian transcriptome by DR (Fig. 3B-C, Fig. 3E-G). Collectively, we identified 623 oscillating in either one or both conditions, of which 136 cycle in both conditions,

188 cycle only in the control diet, and 299 cycle in the DR diet. Thus, there is a net increase of 50% in cycling genes under DR (Fig. 3B-C). Remarkably, core clock genes were not significantly impacted by DR in both phase and peak expression (using thresholds FDR < 0.1 and log2 (fold change) > 0.5 from differential analysis, see methods) (Fig. 3D). This indicates that rhythmicity of core clock genes is largely resistant to short term (~5 days) diet changes. Although peak phases of common rhythmic genes remained largely unaffected (Fig. 3D), DR significantly, albeit modestly, increased overall expression (10-70% in TPM) of many of these common cyclers, although notably none of the core clock genes were increased (Fig. 3D, F & G). From an averaged expression comparison across all time points between control and DR in the 136 common rhythmic genes, 54 genes were significantly increased (t-test, FDR < 0.05) while only one was downregulated (Fig. 3E-G). It shows that short-term DR (5 days) increases expression of robustly rhythmic genes without affecting expression of the core clock genes, arguing that DR impacts rhythmic output while sparing core clocks.

Weighted Gene Coexpression Network Analysis Identifies a DR-Specific Cycling Proteasome Module

In order to identify novel genes and pathways that are associated with or even causal to the lifespan extension under DR and also to understand circadian transcriptomic organization in the fat body under DR, we took a network approach (Zhang et al., 2013). First, we performed Weighted Gene Coexpression Network Analysis (WGCNA) to reconstruct gene coexpression networks from our time-series RNA-Seq data collected under DR diet (See Methods). We identified 41 network modules of co-regulated genes (Table S2). Notably, 12 modules (29%) were “cycling modules”, i.e., those enriched with rhythmic genes (FDR < 0.05). Genes in each of these “cycling modules”

exhibited highly similar phases and waveforms, revealing coordinated circadian gene expression. In order to further examine how the transcriptomic organization is altered by DR, we computed the modular differential connectivity (MDC) (Zhang et al., 2013), which was expressed as a ratio to reflect the difference in gene co-expression strength (i.e., network connectivity) of a module between DR and control diets (see Methods and Supplementary Information). At FDR < 0.05, we identified 14 network modules among the 41 that gained connectivity (MDC > 1) and one network module that lost connectivity (MDC < 1) under DR diet compared to control diet (Table S2). Importantly, three of the differentially connected modules were also cycling modules identified from WGCNA (Fig. S6 A-B), exhibiting higher network connectivity under DR. This suggests that DR may increase the circadian coordination of gene expression in these modules. One of these modules, which we term the "proteasome module", was of special interest, as the protein products from many of the genes in this module are the core and auxiliary components of the proteasome complex (Fig. 4A-C), showing a dramatically higher pathway enrichment scores than the other two modules (Fig. S6C). This observation also agrees with the primary analysis (Fig. 3B, 3C) that, out of the 33 subunits of the proteasome complex in *Drosophila* (Belote and Zhong, 2009), one and 12 proteasome subunits were defined as cycling (fold-change in TPM ≥ 1.5 at an FDR < 0.25 BooteJTK analysis) on control and DR diets, respectively. The proteasome complex functions as one of the major proteolytic degradation machines (von Mikecz et al., 2008). Remarkably, 25 of the 33 subunit genes that comprise the proteasome complex (Belote and Zhong, 2009) were found in this cycling module which gained network connectivity under DR, suggesting a DR-specific circadian coordination in the gene expression. In addition to differential connectivity, DR mildly (~20%) but significantly elevated the expression of 21 genes (FDR < 0.05) in the proteasome

module (Fig. 4D). Thus, this observation suggests that circadian clocks modulate daily proteasome subunit gene expression in a nutrition-dependent manner.

Knockdown of proteasome subunit genes in fat body limited normal lifespan and the effects of dietary restriction

Our finding of DR-induced changes in proteasome gene expression and cycling suggests a potential mechanism by which the clock could mediate DR effects. Loss of protein homeostasis (proteostasis) is a hallmark of aging and can determine lifespan in both humans and model organisms, including flies (Kaushik and Cuervo, 2015; Koyuncu et al., 2021; Meller and Shalgi, 2021; Santra et al., 2019; Yang et al., 2019; Yu and Hyun, 2021). The proteasome plays a central role in proteostasis by clearing, recycling, and breaking down up to ~ 90% of cellular proteins (Jang, 2018; von Mikecz et al., 2008). In multiple animal models, activation of the proteasome system extends lifespan and healthspan (Anderson et al., 2022; Chondrogianni et al., 2015; Kruegel et al., 2011; Munkacsy et al., 2019; Tonoki et al., 2009; Vilchez et al., 2012). For example, in *Drosophila*, global overexpression of *rpn11*, a regulatory subunit of the proteasome complex, extends lifespan while knock-down of *rpn11* decreases lifespan (Tonoki et al., 2009). Similarly, adult-specific ubiquitous overexpression of *prosβ5*, a catalytic subunit of the proteasome, extends lifespan in flies (Nguyen et al., 2019). Moreover, pharmacological inhibition of the proteasome reduces lifespan in a dosage-dependent manner (Tsakiri et al., 2013). Intriguingly, a recent study in worms shows that DR extends lifespan through promoting proteostasis, suggesting a link between DR and the proteasome (Matai et al., 2019). However, in flies, whether fat body proteasome function is linked to aging and/or the DR response has not been reported. To gain insight into the role of the fat body proteasome, we used the mifepristone (RU486)-inducible

GAL4/UAS Gene-Switch (GS) system (Osterwalder et al., 2001; Roman et al., 2001) to knockdown the expression of 11 proteasome subunits predominantly in the adult abdominal fat body using the S106-GS-Gal4 driver (Bai et al., 2012; Jin et al., 2020; Poirier et al., 2008; Roman et al., 2001; Taylor et al., 2022) and assess its effects on lifespan (Fig. S7). Among those genes that displayed the most robust (>25% change) effects on lifespan, we focused on two genes, *prosβ3* and *rpn7*. While we observed some variability among independent trials (Fig. S8), which has previously been observed in longevity assays in *Drosophila* (Bai et al., 2015; Katewa et al., 2016), we found significant reductions in lifespan as well as suppressions of DR-mediated lifespan extension for knockdown of both of these subunits ($p < 0.0001$ and $p = 0.0222$ for *prosβ3* and *rpn7*, respectively for the gene*diet interaction effect from pooled data)(Fig. 5 & S8, Table S1). In the case of *prosβ3*, three out of the four trials demonstrated statistically significant gene*diet interactions (Table S1). Although the S106-GS-Gal4 is the most widely used inducible fat body driver, it also mis-expresses in the digestive system (Poirier et al., 2008). To test whether the reduced DR response by *prosβ3* and *rpn7* knockdown with S106-GS-Gal4 is solely from the abdominal fat body or in the intestine or both, we performed two rounds (trial 1 and 2) of adult- and tissue-specific knockdown experiments using the gut-specific inducible TIGS-2 (TIGS-Gal4) (Poirier et al., 2008; Ulgherait et al., 2020). Although the DR effect was weaker in TIGS-Gal4 control flies (without RU486) compared to other wild-type control flies tested in this study (Fig.1, S2, S3, S7, S8), presumably due to genetic background effects (Jin et al., 2020; Liao et al., 2013; Wilson et al., 2020), knockdown of *prosβ3* and *rpn7* in the gut consistently displayed a stronger lifespan extension by DR (Fig. S9 and Table S1) despite some variation in the lifespan pattern between the two trials. Notably, compared to S106-GS-Gal4 experiment (Fig. 5 and S8), flies with either of the subunits knocked down in the gut suffered much stronger reductions in lifespan

298 regardless of diet types (Fig. S9), implying that the S106-GS-Gal4 results are not significantly
299 affected by leaky misexpression in the gut. Thus, our data indicate that proteasome function in the
300 adult abdominal fat body is important for DR-mediated lifespan extension.
301
302 **Discussion**
303 While circadian rhythms dampen during aging, environmental and genetic perturbations leading
304 to high-amplitude circadian rhythms correlate with many health benefits (Froy, 2018; He et al.,
305 2016). Here we demonstrate that the master circadian clock transcription factor *Clk* is important
306 for DR effects on lifespan and fecundity. We also discovered that DR acutely (~ 5 days) alters the
307 circadian transcriptome in the fat body while sparing the core clock suggesting that the primary
308 effects of DR are on circadian output genes. Using adult- and tissue-specific RNAi, we show that
309 diet sensitive clock-controlled proteasome subunit genes in the abdominal fat body are important
310 for the lifespan extending effects of DR. Our data suggest a role of the clock in DR effects on
311 lifespan and reveal a molecular pathway, the proteasome, through which the clock may exert some
312 of these effects.
313
314 Our data demonstrate a profound role for the transcription factor *Clk* in mediating the effects of DR
315 on two independent phenotypes: lifespan and fecundity. The effects of Clk^{Jrk} on DR-mediated
316 lifespan expansion were robust, replicable, and, importantly, exhibited across a wide range of diets.
317 Testing DR effects by using just two diets can mask a shift in the diet-dependent lifespan curve
318 (Clancy et al., 2002; Flatt, 2014), for example, due to changes in feeding. The fact that we observe
319 a suppressed response to DR across a wide range of diets suggests a strong DR phenotype. In fact,
320 *Clk* is one of just a handful of fly genes (Banerjee et al., 2012; Katewa et al., 2016; Ulgherait et

al., 2016; Wang et al., 2009; Zid et al., 2009) for which this more rigorous standard has been achieved, suggesting a unique and central role of *Clk* in DR. Clk^{Jrk} mutants are also not simply adversely affected by restricted nutrient intake, as may be the case for Bmal1 mutant mice (Kondratov et al., 2006), as Clk^{Jrk} mutants are much more long-lived than *iso31* flies under the 1SY and 1Y malnutrition diet (Fig. 1, S2, S3). Female fecundity in *Drosophila* is strongly correlated with diet concentration (Bass et al., 2007). Clk^{Jrk} flies also exhibited a suppressed diet-dependent fecundity response especially at higher diet concentrations (Fig. 2, S5), resulting in a reduced fecundity response to a range of diets. These data suggest a more general role for *Clk* in dietary sensitivity beyond lifespan. Reduction of fecundity in female Clk^{Jrk} mutants is consistent with previous observations under standard diets (Beaver et al., 2002). While we observed reduced DR effects in Clk^{Jrk} mutants, we observed a relatively robust DR lifespan response in arrhythmic per^{01} mutants. Notably, our results reproduced one of the prior reports (Ulgherait et al., 2016) with per^{01}. Loss of the major activator (*Clk*) and repressor (*per*) stop the clock at different stages of the cycle (Claridge-Chang et al., 2001; Emery et al., 1998; Glossop et al., 1999). As a result, *per* and *Clk* mutants can often yield distinct, even opposing, phenotypes (Keene et al., 2010). While we cannot exclude the possibility that Clk^{Jrk} may not act via control of oscillatory gene expression (see (McDonald and Rosbash, 2001)), we favor the idea that the clock drives daily oscillations between DR-sensitive (low PER) and DR-insensitive (low CLK) states which may be adapted to daily feeding (Xu et al., 2008) and/or the sleep/wake rhythm.

In addition to demonstrating a critical role for *Clk* in mediating the effects of DR on lifespan, we also find that DR reprograms the circadian transcriptome, not by changing core clocks, but rather by inducing or altering rhythmicity of a key set of clock-controlled output genes. In accordance

with the recent guidelines (Hughes et al., 2017), we collected samples every 2 h from two independent cycles of 24 h (every 2h for 48 h), increasing the statistical power for rhythm detection, and computed the false discovery rate. To determine how the circadian clock may mediate DR effects we assessed the circadian transcriptome under DR conditions in the fat body, a tissue important for metabolism, longevity, and DR. Importantly we assessed the transcriptome after just 5 days of DR sufficient time for DR to induce changes in mortality rate (Mair et al., 2003; McCracken et al., 2020). We found that this short-term DR was sufficient to produce ~ 35% more rhythmic genes in total compared to control diet. DR also increases overall expression of the rhythmic genes in the control diet that remain rhythmic under DR (common rhythmic genes) (Fig. 3B-F). This is consistent with the observation in mouse liver that DR increases the number of rhythmic genes as well as their amplitude (Sato et al., 2017), indicating this feature of circadian DR sensitivity is widely conserved. Strikingly, although we discovered that DR for 5 days is sufficient to initiate reprogramming of the circadian transcriptome, core clocks remained virtually unaffected (Fig. 3D), a time at which DR mortality effects are observed (Whitaker et al., 2014). In contrast, increased amplitudes of core clocks in mice liver is seen after 2~ 6 months of DR (Patel et al., 2016b; Sato et al., 2017) and in flies after 10 days (Katewa et al., 2016).

Genetic induction of rhythmic amplitude of the core clock gene *timeless* altered lifespan in a DR-sensitive manner (Katewa et al., 2016). Yet, this *tim* induction was not accompanied by downstream changes in other components of the feedback loop, suggesting the core clock per se was not involved. Thus, we hypothesize that on the time scale of DR-induced changes in mortality rate, core clocks are not affected and that later changes in core clocks reflect an indirect and delayed effect of DR. It will be of interest to determine if short-term DR in mammals also spares

core clock genes. Together, we postulate that core clocks are resistant to amplitude changes by short-term DR regimens while a longer term, depending on species, gradually increases their amplitude, which can further reprogram diet-dependent CCGs. This also implies that the length of DR shapes the pattern of circadian transcriptome reprogramming. Our data suggest a model by which the circadian clock gates the response to dietary restriction to control the complement and amplitude of clock regulated gene expression (Fig. 6). First, the circadian clock rhythmically controls the daily expression of multiple components of the proteasome. Second, DR increases the expression levels and enhances coordinated rhythmic expression of proteasome subunit genes. WGCNA followed by MDC analysis discovered that the transcriptomic organization in the proteasome module was altered by DR. This change is due to enhanced and coordinated rhythmic expression of proteasome subunits by DR (Fig. 4A-C & S6). In addition, DR mildly but significantly increased average expression (t-test with the time-averaged expression analysis, FDR < 0.05) of many of the proteasome subunits (Fig. 4D). In line with the observation by Katewa et al that amplitude changes by DR in core clocks is gradual and requires a minimum of 6 ~ 10 days in a yeast restriction DR (Katewa et al., 2016), we speculate that acute DR-responsive genes/pathways can enhance CLK driven rhythmic processes. Importantly, we provide in vivo evidence that the diet and clock sensitive subunits of the proteasome in the fat body are important for diet sensitive effects on lifespan, providing a pathway of linking clocks, DR, and aging. Although it is well-established in multiple species that proteasome activity decreases during aging and is generally positively correlated with lifespan (Anderson et al., 2022; Chondrogianni et al., 2015; Huang et al., 2019; Kruegel et al., 2011; Nguyen et al., 2019; Pickering et al., 2015; Tonoki et al., 2009; Vilchez et al., 2012), little is known about its tissue-specific contribution to aging and the link to DR. We found that the knock-down of several cycling proteasome subunits

predominantly in the adult fat body, using the S106-GS gal4 (Poirier et al., 2008), significantly reduces lifespan, consistent with whole organism manipulations (Fig. S7). Moreover, further testing for potential DR effects with two selected subunits (*prosβ3* and *rpn7*) revealed that knock-down of these subunits reduced DR effects, providing evidence that suppression of proteasome function in the fat body limits DR-mediated lifespan extension (Fig. 5). While some variability was observed, perhaps due to inconsistent delivery of RU486 to flies (Yamada et al., 2017), in the case of *prosβ3*, significant effects on DR response were observed in three out of four trials. Moreover, combined data from independent trials confirmed knockdown of these subunits reduces the DR effect. We favor the idea that impaired proteasome function contributes to amino acids imbalance, which is known to be critical for DR-mediated lifespan extension in flies (Grandison et al., 2009), leading to reduced DR response (Fig. 6). As DR is suggested to be the most promising intervention to delay aging, the work presented here has important implications for integrating timing into anti-aging therapies. In conjunction with our observations, a recent study also demonstrated that the beneficial effects of time-restricted feeding on longevity is strongly abolished in core clock mutant flies including Clk^{Jrk} flies (Ulgherait et al., 2021), emphasizing the roles of circadian clocks in dietary interventions for health and longevity. We propose that time-of-day activation of proteasome may, at least partially, mediate the beneficial effect of DR. Thus, the daily timing of anti-aging therapies may be crucial for lifespan and healthspan extension.

Materials and Methods

Fly Rearing and Media

All the flies used for experiments were raised on a standard yeast-cornmeal-molasses based diet under a light-dark (LD) 12:12h cycle. The following flies were used in this study. Clk^{Jrk} and per^{01}

413 flies were backcrossed to the wild type (w^{1118}) iso31 line (Bloomington stock number: 5905) 6
414 times. S106-GeneSwitch (S106-GS) (Bloomington stock number: 8151) and RNAi lines were
415 obtained from Bloomington stock center (See Table S1).
416
417 ***Lifespan and Fecundity Assay***
418 For the lifespan assay, young adult female flies (~ 48 hours cohorts) were separated under light
419 CO_2 anesthesia after mating with males for ~ 2 days in food bottles. Separated female flies were
420 kept in groups of 20~25 flies in plastic vials on the Sucrose-Yeast (SY) diet and transferred to
421 fresh food vials every 2~3 days. For the lifespan experiment with Mifepristone (RU486)-inducible
422 GeneSwitch system, flies were kept in the vials containing either vehicle (1% EtOH) or RU486
423 (200 uM). Dead flies were recorded at each transfer. For the fecundity assay, single females (~ 3
424 days old) were placed with two males of similar age in vials containing different SY diets. Vials
425 were changed daily at ZT0 (8 AM) for 7 days and stored at 4°C until the eggs were counted. Vials
426 with dead females or sterile females (no eggs laid) during the assay were removed from the analysis.
427 For both lifespan and fecundity assays, flies were kept at 25°C, 12hr light : 12 dark (12L:12D) and
428 60% relative humidity.
429
430 ***Survival Statistics***
431 Survival analysis, log-rank test to evaluate statistical differences between survival curves, and Cox
432 proportional hazards analysis to evaluate ability of tested genes to modify lifespan in the specified
433 range of diets were performed with JMP® statistical package (version 14, SAS Institute Inc.) with
434 data from replicate vials combined. P values from the Cox proportional hazards analysis represent

435 the probability of main (Gene (G) as a nominal variable and Diet (D) as a continuous variable) and
436 interaction effects (G x D) by the likelihood ratio chi-square test**.**
437
438 ***Fat Body Dissection***
439 Young mated female flies (~ 3 day old) were entrained under either DR diet (5SY) or control diet
440 (15SY) for 5 days in 12L:12D cycles at 25°C. At every 2 hours, flies were directly dissected
441 without dry ice to harvest fat tissues in the abdomen. Pinned flies were cut to remove organs in the
442 abdomen (intestine, ovaries, malpighian tubules, etc). Fat tissues attached to the epidermis were
443 collected (DiAngelo and Birnbaum, 2009; Katewa et al., 2016; Xu et al., 2011; Xu et al., 2008).
444 Fat body from ~ 10 flies were harvested within 10 minutes for each time point of RNA-Seq analysis.
445
446 ***RNA-Seq***
447 Dissected fat body tissues from ~8 days old mated female flies were homogenized in pH 7.4 PBS
448 for 2 min using a Kontes motor and pestle, and were incubated TRIzol LS Reagent (Thermo Fisher,
449 Waltham, MA) for 15 min. RNA was extracted according to the manufacturer's instructions and
450 residual DNA in the homogenized samples was removed by RNAse free DNase I (Thermo Fisher
451 Scientific). Quality of RNA samples were checked with Agilent 2100 Bioanalyzer. cDNA library
452 was constructed with poly(A) selected mRNA using Truseq RNA library preparation kit and then
453 sequenced at the Genomics Core Facility at the University of Chicago on Illumina HiSeq 2000
454 System.
455
456 ***Quantification of Transcript, Normalization, and Batch Correction***

RNA-seq data were quantified at transcript level using Kallisto (Bray et al., 2016), reference transcriptome used FlyBase_r6.14 (Gramates et al., 2017). Quantified transcripts were summed up to the gene level using tximport library (Soneson et al., 2015). The resulting gene set was initially filtered based on the TPM level; genes with less than 1 TPM across more than 40% of time points per condition (including replicates), were removed from the further analysis. Quantified and filtered samples were normalized within condition with RUVSeq library (Risso et al., 2014), under the RUVg protocol using a set of "in-silico empirical" negative controls (the least significantly DE genes based on a first-pass DE analysis performed prior to RUVg normalization). Technical batch correction between conditions was performed with EDASeq protocol, using upper-quartile (UQ) normalization (Risso et al., 2011).

Rhythmicity Detection

Rhythm detection was performed using BooteJTK (Hutchison et al., 2018) on filtered, TPM level data, with parameters set to: period detection for 24 hr, sampling interval 2hr, model function cosine, phases 00-22hr by 2 hr and asymmetries 02-22hr by 2 hr. Genes with FDR corrected p-value < 0.25 and 1.5 > fold change in raw TPM were assumed to be cycling. Rhythm detection was performed on the commonly expressed genes between analyzed conditions (conditions pooled, 7772 IDs) for detection of the most robust cycling in fat body, additionally condition specific detection was performed (2 replicates per conditions, 7937 in control diet, 7865 in DR diet). Differential gene expression analysis was performed using DESeq2 (Love et al., 2014).

WGCNA and MDC

We reconstructed gene coexpression networks under the DR condition using the WGCNA/r package (Langfelder and Horvath, 2008; Zhang and Horvath, 2005). Briefly, we first computed the network adjacency matrix as kij = [0.5 * (1 – rij)] ^ β. kij is the network connectivity and rij is the Pearson correlation coefficient between a pair of genes i and j. Soft power threshold β was chosen so that the topology of the network was scale-free. A topological overlap matrix (TOM) was then computed to evaluate the neighborhood similarities between genes and to classify network genes into modules using hierarchical clustering and dynamic tree cut. We used DAVID (v6.8) to functionally annotate the identified network modules. To identify network modules that were organized by circadian rhythmicity, we tested the enrichment of rhythmically expressed genes (FDR < 0.1 identified using concatenated data from both DR and control diets) in network modules using fisher's exact test. Since we defined the network as a "signed" network (i.e., using kij = [0.5 * (1 – rij)] ^ β, instead of the default choice of kij = |rij| ^ β), genes in the cycling network modules shared highly similar circadian phases (as opposed to also including genes with the exact opposite phases) as well as cycling waveforms. To evaluate changes in network connectivity between DR and control diets, we implemented in R a method to compute modular differential connectivity (MDC) described by Zhang et al., 2013 (Zhang et al., 2013). MDC is defined as the ratio of the summed pairwise connectivity among genes in a module under DR conditions and that of the same genes under control conditions (i.e., MDC = $\sum i \sum j$ kijDR / $\sum i \sum j$ kijcontrol). Statistical significance was determined using a permutation-based FDR approach. Two types of FDR estimates were computed, one based on randomly permuted samples to generate networks with nonrandom nodes but random connections, and the other based on randomly permuted gene labels to generate networks with random nodes but nonrandom connections. The final FDR was determined as the larger of the two estimates. 1000 permutations were computed for each type of

FDR estimates, and FDRs for modules that gained connectivity (MDC > 1) or lost connectivity (MDC < 1) under DR conditions were estimated separately as described by Zhang et.al., 2013 (Zhang et al., 2013).

Differential Expression Analysis

Differential gene expression analysis was performed using DESeq2 library (Love et al., 2014). We used est. count data generated by kallisto, and a union of pre-filtered gene lists for both conditions (gene lists were in agreement with the TPM level pre-filtering, as described above) resulting in 8030 gene IDs. The full model included time, in a form of 3rd degree polynomial, and diet type as factors. We assumed genes with FDR < 0.2 and absolute log2 fold change > 0.5 as differentially expressed between the two dietary conditions, DR (5SY) and control (15SY) diets. Gene functional classification of differentially expressed genes was performed using DAVID (v6.8) (Huang da et al., 2009)**.**

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Author Contributions

RA and DSH conceived and designed the experiments. DSH performed experiments and analyses. JK prepared samples for RNA-seq analysis. ALH, MI, ARD, RIB performed RNA-seq analyses. PJ performed WGCNA and MDC analysis. LA, NW, SA performed lifespan assays. DSH, MI, PJ, WK, RIB and RA wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

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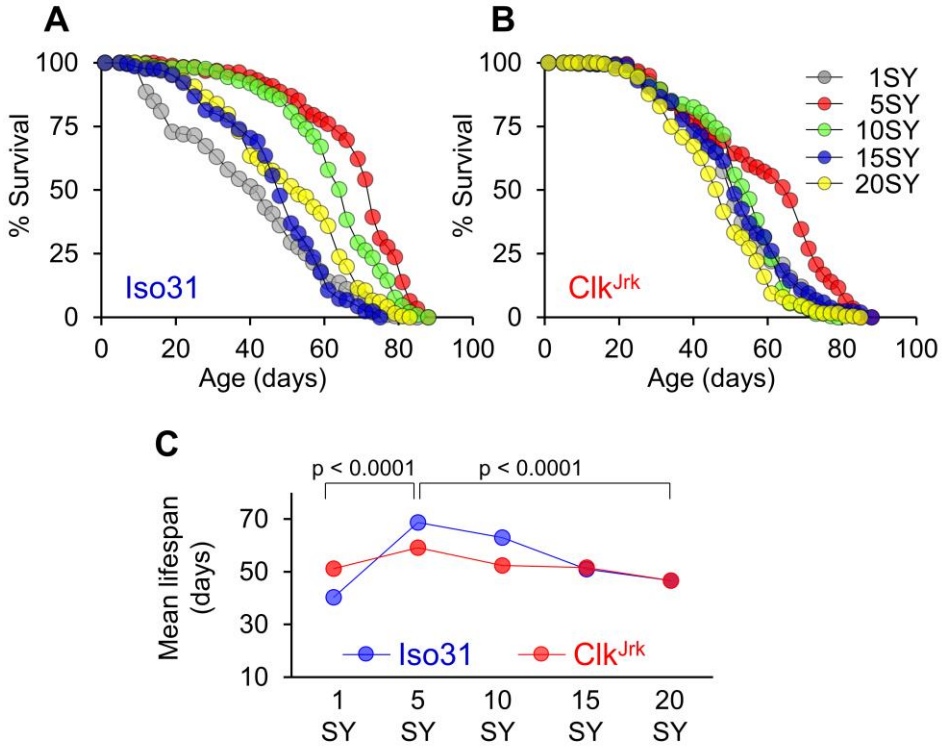


Fig. 1. Reduced longevity response to DR in *Clk^{Jrk}* mutant flies

(A-B) Survival curves of wild-type control flies (*iso31*) and *Clk^{Jrk}* homozygous mutant flies in 1%, 5%, 10%, 15%, and 20% Sucrose-Yeast (SY) diets (total dilution). (C) Mean lifespan plots of *iso31* and *Clk^{Jrk}* flies across different concentrations of SY diets. Basic survival parameters from the Kaplan-Meier method for each diet and genotype are in Table S1. P values represent diet*genotype interaction effects from Cox proportional hazards analysis. Independent replication of the experiment is presented in figure S2 and Table S1.

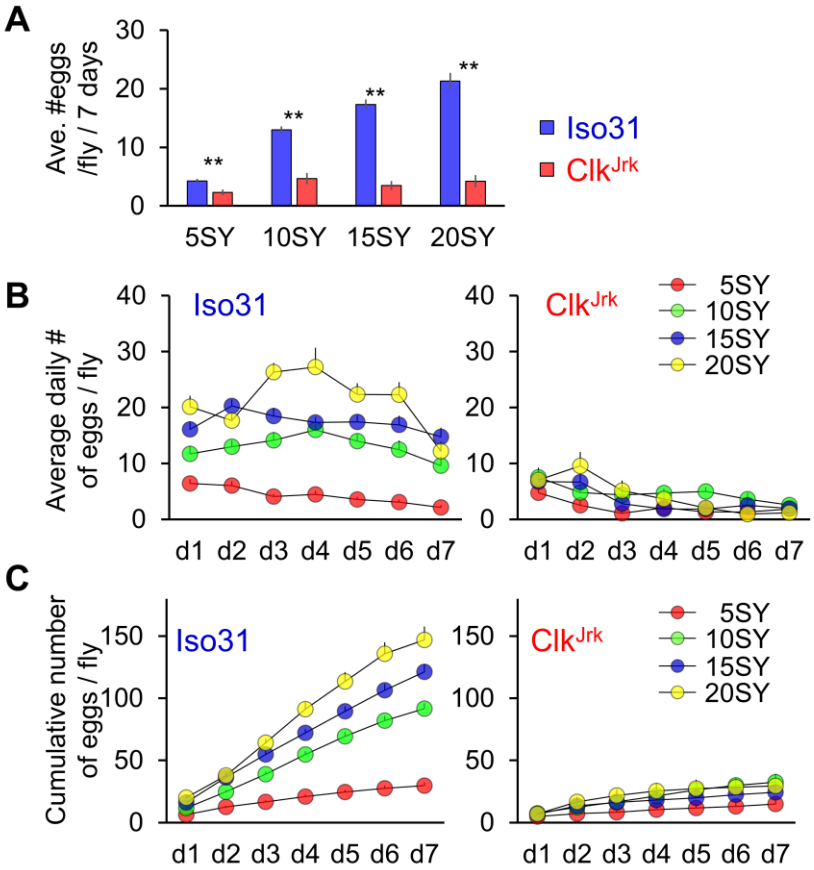


Fig. 2. Reduced fecundity response to diets in *Clk^{Jrk}* mutant flies

(A & B) Cumulative and daily average number of eggs produced per fly for 7 days in wild-type control flies (*iso31*) and *Clk^{Jrk}* homozygous mutant flies in 5%, 10%, 15%, and 20% Sucrose-Yeast (SY) diets. (C) Cumulative number of eggs produced per fly over 7 days. ** $p < 0.01$ by t-test for specified pair-wise comparison. All error bars represent SEM.

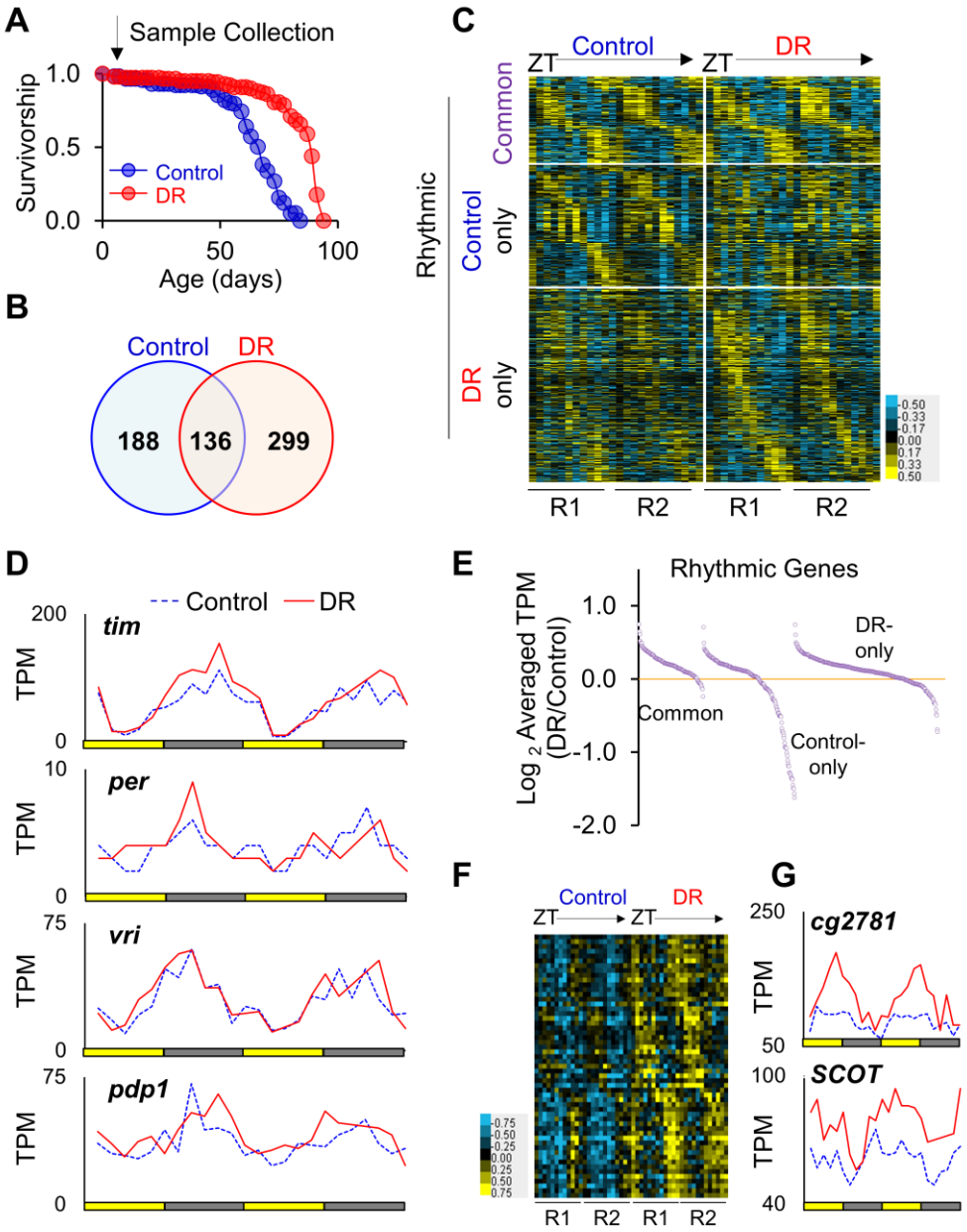
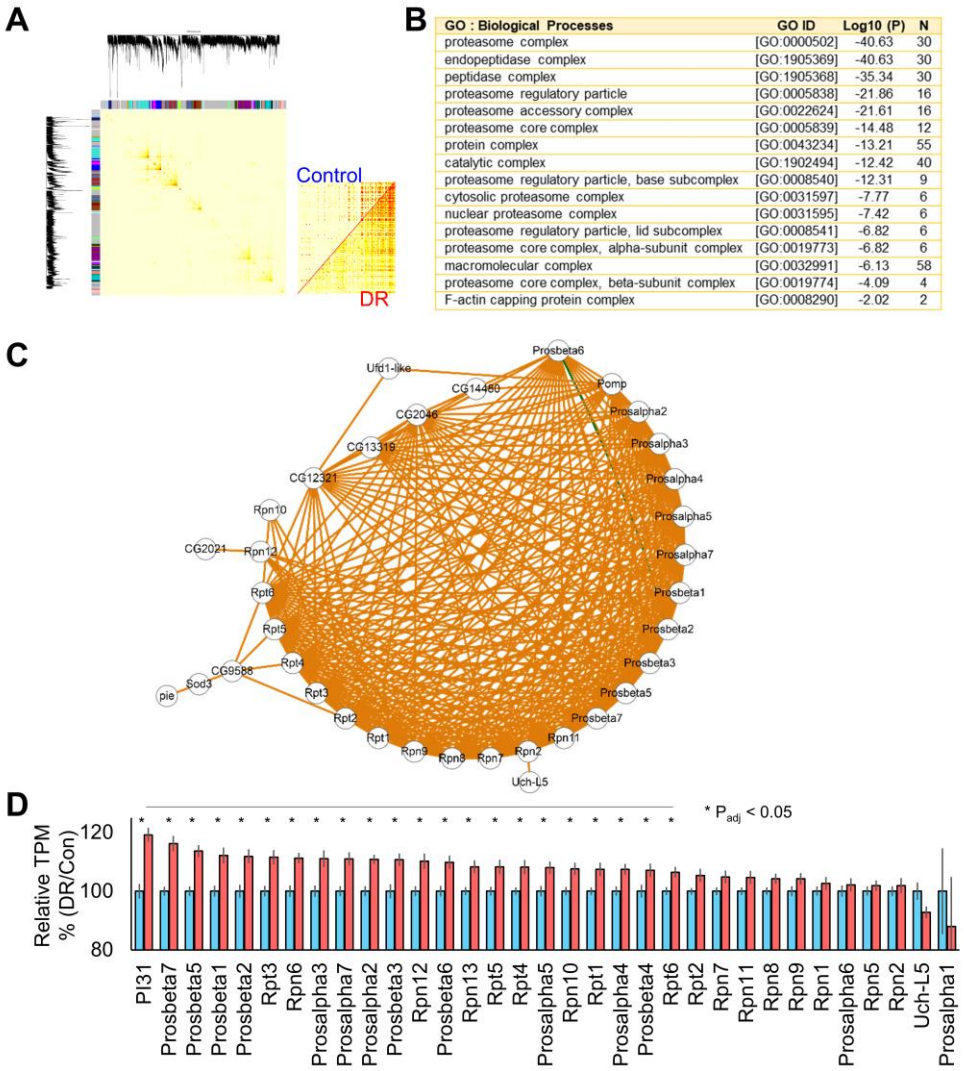


Fig. 3. Effects of DR on circadian transcriptome in the abdominal fat body

(A) Lifespan extension by DR. Samples for RNA-Seq analysis were collected after ~5 days under either control or DR diets. (B) Number of rhythmic genes (0.25 < FDR from Boot eJTK analysis and fold change in TPM ≥ 1.5). (C) Re-organization of circadian transcriptome by DR. Heatmap represents relative expression (Z-score, yellow=high, blue=low expression) of rhythmic genes in each group across 48 h at 2 h intervals (2 replicates of 12 samples for 24 h). Genes in the top panels are rhythmic in both control and DR diets (common); those in the middle panels are rhythmic in control diet (left) but arrhythmic in DR diet (right); those on the bottom panels

are rhythmic in DR diet (right) but arrhythmic in control diet (left). (D) DR failed to affect the expression pattern of core clock genes. (E) Effect of DR in overall time-averaged expression of rhythmic genes in each group. Time-averaged expression in control and DR was compared by t-test with Benjamini-Hochberg correction (Padj ≤ 0.05). TPMs of rhythmic genes in each group were averaged across all time points and were normalized to those in control diet. (F) Increased overall expression in the common rhythmic genes by DR. Heat map represents relative expression (Z-score) of common rhythmic genes across all time points from both control and DR diets. (G) Examples of common rhythmic genes with increased expression by DR.



GO : Biological Processes	GO ID	Log10 (P)	N
proteasome complex	[GO:0000502]	-40.63	30
endopeptidase complex	[GO:1905369]	-40.63	30
peptidase complex	[GO:1905368]	-35.34	30
proteasome regulatory particle	[GO:0005838]	-21.86	16
proteasome accessory complex	[GO:0022624]	-21.61	16
proteasome core complex	[GO:0005839]	-14.48	12
protein complex	[GO:0043234]	-13.21	55
catalytic complex	[GO:1902494]	-12.42	40
proteasome regulatory particle, base subcomplex	[GO:0008540]	-12.31	9
cytosolic proteasome complex	[GO:0031597]	-7.77	6
nuclear proteasome complex	[GO:0031595]	-7.42	6
proteasome regulatory particle, lid subcomplex	[GO:0008541]	-6.82	6
proteasome core complex, alpha-subunit complex	[GO:0019773]	-6.82	6
macromolecular complex	[GO:0032991]	-6.13	58
proteasome core complex, beta-subunit complex	[GO:0019774]	-4.09	4
F-actin capping protein complex	[GO:0008290]	-2.02	2

Fig. 4. Oscillation of proteasome subunits by DR

(A) Identification of the co-expression module enriched with proteasome subunit genes by DR. The "proteasome module" is enlarged at the bottom right of the dendrogram. Gene co-expression networks under the DR diet were built using the WGCNA/r package. A topological overlap matrix (TOM) was then computed to evaluate the neighborhood similarities between genes and to classify network genes into modules using hierarchical clustering and dynamic tree cut (methods). (B) Gene ontology (GO) analysis for the genes in the proteasome module. Enrichment scores in P value were corrected with Benjamini-Hochberg approach with 0.01 as threshold. (C) Physical interaction map among the genes in the proteasome module. Physical interaction was analyzed in the esyN network builder (www.esyN.org) and visualized using the Cytoscape program. (D) Increased overall expression of proteasome subunits by DR. Time-averaged expression in control and DR was compared by t-test with Benjamini-Hochberg correction (Padj ≤ 0.05). All error bars represent SEM.

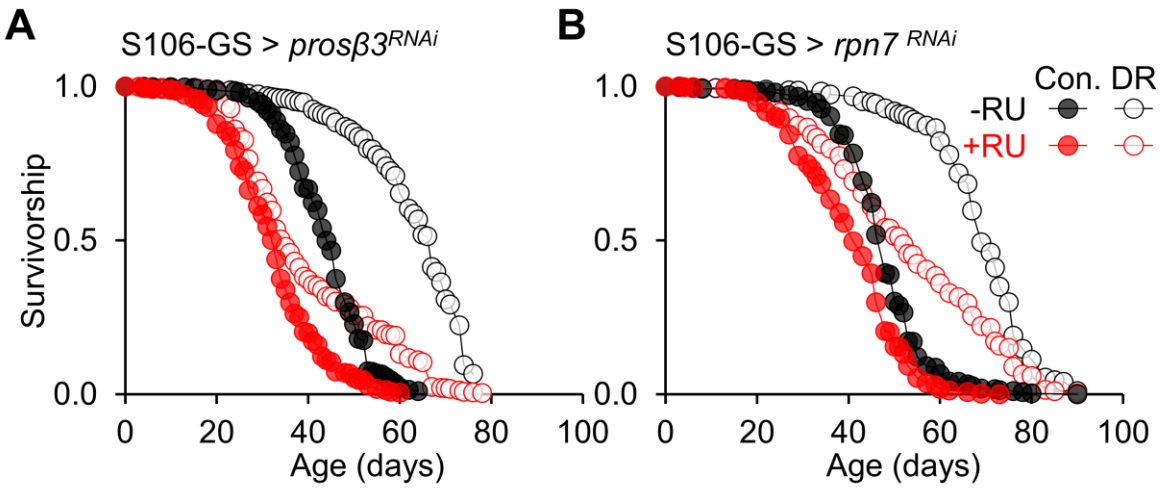
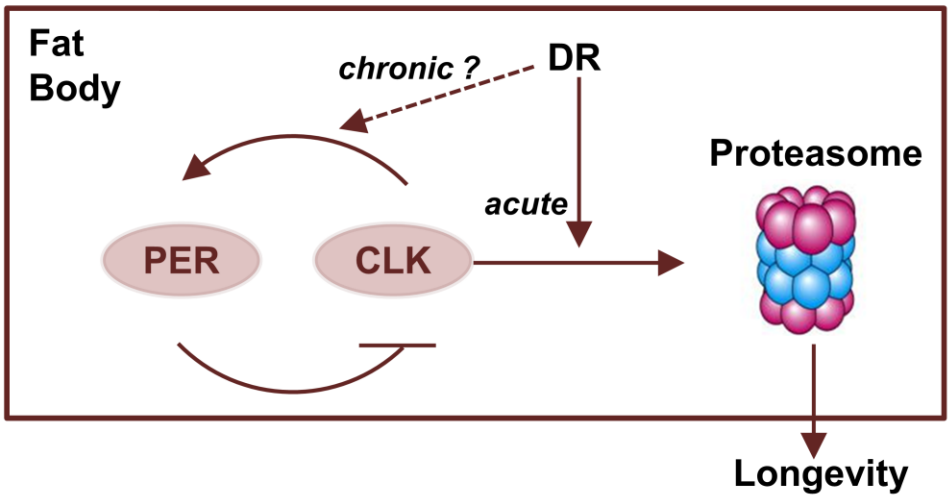


Fig. 5. Reduced longevity response to DR by *prosβ3* and *rpn7* knockdown in the abdominal fat body.

(A-B) Survival curves of the flies with *prosβ3* and *rpn7* knockdown (+RU) in the adult abdominal fat body (S106-GeneSwitch (GS) driver) and their controls (- RU) in control and DR diets. Survival curves were pooled from 3~4 independent trials (See also Fig. S6 and Table S1 for survival curves and detailed statistical analysis for the independent trials). $p < 0.0001$ and $p = 0.0169$ for *prosβ3* and *rpn7*, respectively for the gene*diet interaction effects from Cox proportional hazards analysis.



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922 **Fig. 6. Model for how clock and diet impact proteasome expression to regulate lifespan**