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Cryptochrome restores dampened circadian rhythms and promotes healthspan in aging *Drosophila*

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Summary

Circadian clocks generate daily rhythms in molecular, cellular, and physiological functions providing temporal dimension to organismal homeostasis. Recent evidence suggests two-way relationship between circadian clocks and aging. While disruption of the circadian clock leads to premature aging in animals, there is also age-related dampening of output rhythms such as sleep/wake cycles and hormonal fluctuations. Decay in the oscillations of several clock genes was recently reported in aged fruit flies, but mechanisms underlying these age-related changes are not understood. We report that the circadian light–sensitive protein CRYPTOCHROME (CRY) is significantly reduced at both mRNA and protein levels in heads of old *Drosophila melanogaster*. Restoration of CRY using the binary GAL4/UAS system in old flies significantly enhanced the mRNA oscillatory amplitude of several genes involved in the clock mechanism. Flies with CRY overexpressed in all clock cells maintained strong rest/activity rhythms in constant darkness late in life when rhythms were disrupted in most control flies. We also observed a remarkable extension of healthspan in flies with elevated CRY. Conversely, CRY-deficient mutants showed accelerated functional decline and accumulated greater oxidative damage. Interestingly, overexpression of CRY in central clock neurons alone was not sufficient to restore rest/activity rhythms or extend healthspan. Together, these data suggest novel anti-aging functions of CRY and indicate that peripheral clocks play an active role in delaying behavioral and physiological aging.

Key words: *Cryptochrome*; circadian clock; aging; healthspan; *Drosophila*.

Introduction

Circadian clocks are intrinsic mechanisms that generate daily rhythms in behavior, physiology, and cellular processes, ensuring temporal homeostasis coordinated with day/night cycles (Reddy & O'Neill, 2010). Increasing evidence suggests bidirectional relationships between circadian clocks and aging. On the one hand, genetic or environmental disruptions of the clock function accelerate physiological aging, onset of late life diseases, and mortality risk in mammals (Davidson *et al.*, 2006; Kondratov *et al.*, 2006; Antoch *et al.*, 2008; Yu & Weaver, 2011). On the other hand, aging is also known to impair circadian rhythms as evidenced by disruption of sleep/wake cycles, dampening of hormonal rhythms, and weakening of clock gene oscillations (Valentinuzzi *et al.*, 1997; Huang *et al.*, 2002; Hofman & Swaab, 2006; Kondratova & Kondratov, 2012). Age-related decline in temporal coordination of metabolic, physiological, and neurological functions has profound effects on health and disease susceptibility, yet the mechanisms underlying the decay of the circadian system are not understood.

Bidirectional relationships between circadian clocks and aging have also been established in *Drosophila melanogaster*. Disruption of the circadian clock increases the susceptibility of aging fruit flies to oxidative stress and neurodegeneration (Krishnan *et al.*, 2009, 2012). Conversely, aging flies show fragmented sleep/activity patterns and dampened clock gene oscillations (Koh *et al.*, 2006; Luo *et al.*, 2012; Rakshit *et al.*, 2012; Umezaki *et al.*, 2012). In the current study, we used *Drosophila* to address mechanisms underlying the decay of the circadian system and investigate whether this decay could be reversed in aging flies.

The molecular mechanism of the circadian clock is based on transcription–translation feedback loops that are evolutionarily conserved from flies to mammals (Stanewsky, 2003; Yu & Hardin, 2006). In fruit flies, *Clock* (*Clk*) and *cycle* (*cyc*) genes encode transcription factors that form CLK–CYC activator complexes and stimulate the expression of genes *period* (*per*) and *timeless* (*tim*) early at night. Subsequently, PER and TIM proteins form heterodimers and accumulate in the cell nuclei repressing CLK–CYC transcriptional activity and thus suppressing their own transcription (Hardin, 2011). In another negative feedback loop, CLK–CYC complexes induce the expression of transcription factors *Par domain protein 1ε* (*Pdp1ε*) and *vrille* (*vri*) that act as an activator and repressor of *Clk* (Cyran *et al.*, 2003). This basic clock mechanism is cell autonomous and operates in the central and peripheral clock cells. The central clock is formed by a network of ~150 pacemaker neurons in the fly brain, which regulate rest/activity rhythms (Nitabach & Taghert, 2008). Peripheral clocks are found in many cells of the nervous system, such as retinal photoreceptors, glia, sensory neurons, and in non-neural tissues in the head and body (Hardin, 2011; Xu *et al.*, 2011).

High-amplitude oscillations of *per*, *tim*, *Pdp1ε*, and *vri* mRNA as well as PER and TIM proteins observed in young flies are significantly reduced in heads of aging flies (Luo *et al.*, 2012; Rakshit *et al.*, 2012). Oscillations of PER and TIM proteins were also evaluated in central clock neurons (Luo *et al.*, 2012; Umezaki *et al.*, 2012); however, these neurons constitute only a small fraction of clock cells compared with peripheral oscillators, which are therefore responsible for reduced clock gene oscillations observed in the heads. The causes of dampening of clock gene oscillations are not clear. In young flies, the blue light photoreceptive flavin-binding protein CRYPTOCHROME (CRY) is critical for the synchronization of individual oscillator cells (Emery *et al.*, 1998; Stanewsky *et al.*, 1998). CRY targets TIM for degradation after lights on and synchronizes the circadian oscillations with external day/night cycles (Busza *et al.*, 2004). In addition to acting as circadian photoreceptor that mediates light input into the clock, CRY appears to function as the central clockwork component in peripheral clocks. Indeed, in hypomorphic cry^b mutants, rhythmic expression of *per* and *tim* at mRNA

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and protein levels is abolished in the peripheral clock cells but not in the central clock neurons (Stanewsky *et al.*, 1998). Furthermore, CRY is necessary for circadian clock functions in other peripheral oscillators under constant darkness (Ivanchenko *et al.*, 2001; Krishnan *et al.*, 2001), but its role is not yet understood. Mammalian mCry1 and mCry2 are not photoreceptive and act as circadian transcriptional regulators in the clock negative feedback loop (Kume *et al.*, 1999). Interestingly, a recent report shows that human HsCRY-1 protein confers light-independent biological activity in transgenic *Drosophila* (Vieira *et al.*, 2012), suggesting functional similarities between fly and human CRY. More unexpectedly, it was shown that endogenous fly CRY also regulates considerable light-independent transcriptional activity in *Drosophila* (Vieira *et al.*, 2012). An increased repertoire of CRY functions in flies including effects on metabolism (Fogle *et al.*, 2011; Seay & Thummel, 2011; Kumar *et al.*, 2012) suggest that CRY may act via multiple mechanisms that remain to be understood.

As mentioned above, rhythmic expression of *per* and *tim* is abolished in peripheral clock cells such as photoreceptors and glia of hypomorphic *cry*^{b} mutants (Stanewsky *et al.*, 1998). Age-related dampening of *cry* mRNA oscillations was recently reported in heads of flies, along with significantly reduced mRNA oscillations of *per*, *tim*, *Pdp1ε*, and *vri* (Luo *et al.*, 2012; Rakshit *et al.*, 2012). We reasoned that reduced levels of *cry* in heads of old flies may be responsible for diminished cycling of *per* and *tim* mRNA, similar as in young *cry*^{b} mutants (Stanewsky *et al.*, 1998).

In this study, we observed that CRY is reduced with age at both mRNA and protein levels and attempted to replenish CRY in old flies using the binary GAL4/UAS system. We report that overexpression of CRY in all clock-expressing cells improves the mRNA oscillatory amplitude of several genes involved in the clock mechanism and restores strong circadian rest/activity rhythms in old flies. We further show that flies with elevated CRY levels have significantly improved healthspan during aging. Conversely, *cry*-null mutants show accelerated functional decline and accumulate greater oxidative damage, together suggesting novel anti-aging role of CRY. Interestingly, restored rest/activity rhythms and healthspan–benefiting effects are only observed when *cry* is overexpressed in all clock cells, but not when *cry* overexpression is restricted to the central clock neurons, suggesting that peripheral clocks play an active role in maintaining organismal health during aging.

Results

CRY is reduced at both mRNA and protein levels in heads of old flies

We obtained daily mRNA expression profiles of *cry* in the heads and bodies of adult Canton S (CS) males on day 5 (young), 35 (middle aged), and 50 (old). In heads of young flies, *cry* mRNA showed expected daily oscillations, with peak at Zeitgeber time (ZT) 4–8 and trough at ZT 16 (Fig. 1A). The levels and amplitude of *cry* mRNA oscillations were significantly dampened in 35-day-old flies and further reduced on day 50 (Fig. 1A, Table S1), similar to a recent report (Luo *et al.*, 2012). In contrast to heads, there was no statistically significant effect of age on *cry* oscillations in bodies (Table S1).

Given the age-related decline in *cry* mRNA levels, we compared CRY protein profiles in head extracts of 5- and 50-day-old CS flies. In young flies, the highest levels of CRY protein were detected near the end of the dark phase at ZT 0/24. CRY declined during the day to a trough at ZT 12. By comparison, CRY levels were significantly lower in old flies across all time points except ZT 16 (Fig. 1B, Table S1).

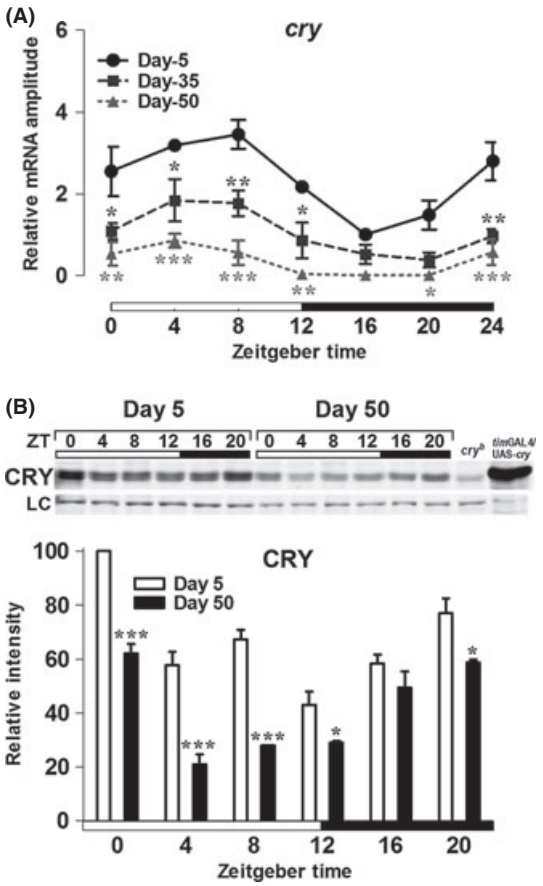

Fig. 1 CRY is reduced with age at both mRNA and protein levels. (A) Daily mRNA profiles of *cry* on days 5, 35, and 50 in heads of CS males, normalized to the trough (ZT 16) values set at 1 for day 5. White and black horizontal bars mark periods of light and dark, respectively. Each data point represents mean ± SEM of three independent RNA samples. Statistical significance between day 5 vs. 35 and day 5 vs. 50 values was determined by two-way ANOVA with Bonferroni's *post hoc* test (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$). (B) Western blot showing CRY protein profile in head extracts of 5- and 50-day-old CS males with *cry*^{b} and *tim*>CRY serving as negative and positive controls. Bar graph (below) indicates the relative band intensity for different ages and time points, with signal intensity at peak (ZT 0) in 5-day-old flies set as 100. Values are mean ± SEM of three independent bioreplicates. Statistical significance between day 5 vs. 50 values was determined using two-way ANOVA with Bonferroni's *post hoc* test and is denoted by ***$P < 0.001$ and *$P < 0.05$ (LC – loading control).

Old flies with elevated CRY have increased oscillatory amplitude of several genes involved in the clock mechanism

We and others recently reported that the oscillatory amplitude of *per*, *tim*, *Pdp1ε*, and *vri* is dampened during aging (Luo *et al.*, 2012; Rakshit *et al.*, 2012); however, the underlying mechanisms are not fully understood. As *per* and *tim* transcription is reduced and nonrhythmic in young *cry*^{b} mutants (Stanewsky *et al.*, 1998), we investigated whether CRY deficiency may contribute to the dampened oscillations of clock genes in old flies. We increased wild-type CRY levels in all central and peripheral clock cells by combining the *tim*GAL4 driver with the UAS-*cry* responder (*tim*>CRY). As a control, *tim*GAL4 flies were crossed to UAS-*cry*^{b} (*tim*>CRY^{B}), which carries a missense mutation at the putative flavin-binding residue, rendering CRY^{B} protein light-insensitive and unstable (Stanewsky *et al.*, 1998; Emery *et al.*, 2000; Busza *et al.*, 2004). As a second control, *tim*GAL4 was crossed to *w*^{1118} (*tim*GAL4/+).

We first determined that CRY protein was significantly elevated in heads of both young and old *tim*>CRY flies, compared with the unstable mutant protein in *tim*>CRY^{B} (Fig. S1). At both ages, CRY levels in heads of *tim*>CRY flies were lower during the day (ZT 4) than at night (ZT 16), suggesting that the ectopic CRY undergoes light-dependent degradation similar as the endogenous CRY. To determine whether higher CRY levels affected the expression of clock genes, we obtained daily expression profiles of *per* and *tim* in heads of old flies. The control *tim*GAL4/+ flies showed considerably dampened daily oscillations of *per* mRNA with trough at ZT 4 and a shallow peak between ZT 12 and 16 on day 50, consistent with recent reports (Luo *et al.*, 2012; Rakshit *et al.*, 2012). In contrast, age-matched *tim*>CRY flies had significantly higher amplitude of *per* mRNA expression with a well-defined peak at ZT 16 (Fig. 2, Table S2), when a peak is usually observed in young flies (Rakshit *et al.*, 2012). Expression of mutant CRY^{B} protein did not increase *per* levels in old *tim*>CRY^{B} flies, and they had a very similar mRNA profile as the *tim*GAL4/+ control. We next measured the expression profile of *tim*, which encodes TIM protein that forms heterodimers with PER. Flies overexpressing CRY had higher amplitude of *tim* cycling due to significantly higher levels at the peak time point (ZT 16), compared with the age-matched controls (Fig. 2, Table S2).

To determine whether other components of the clock mechanism are enhanced by the overexpression of CRY, we examined the mRNA profiles of *Pdp1ε* and *vri* that are also activated by CLK/CYC complexes and oscillate in phase with *per* and *tim*. We observed that mRNA oscillations for the two genes were significantly higher in heads of 50-day-old *tim*>CRY flies than in both controls (Fig. 2, Table S2). Together, these data suggested that flies overexpressing CRY could have increased levels of *Clk* and *cyc* genes leading to increased CLK/CYC-mediated activation of *per*, *tim, Pdp1ε,* and *vri*. To begin addressing this question, we examined the expression levels of *Clk* and *cyc* mRNA in heads of old males. Control flies showed expected *Clk* mRNA oscillations, with peak levels at ZT 4 and trough at ZT 16. There was no significant difference in *Clk* mRNA profiles in heads of *tim*>CRY flies compared with both controls (Fig. 2, Table S2). The expression of *cyc* is nonrhythmic (Bae *et al.*, 2000) and does not significantly change with age in flies (Rakshit *et al.*, 2012). Similar as for *Clk*, there was no difference in *cyc* mRNA levels in heads of *cry*-overexpressing flies and controls (Fig. 2, Table S2).

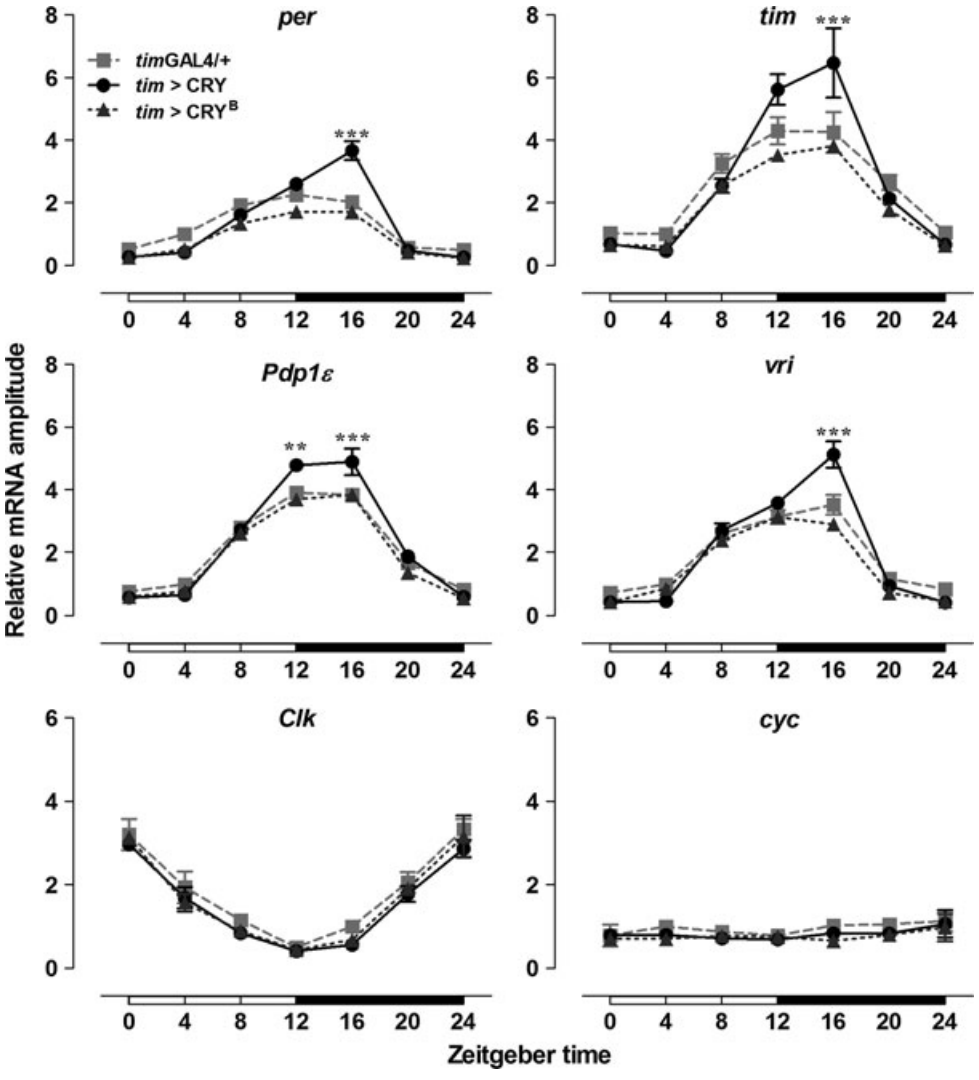

Fig. 2 Old flies with elevated CRY have increased clock gene oscillations. Daily mRNA profiles of clock genes in heads of *tim*GAL4/+, *tim*>CRY, and *tim*>CRY^{B} males on day 50, normalized to the trough (ZT 4/ ZT 16 for *Clk*) values set at 1 for *tim*GAL4/+ control. White and black horizontal bars mark periods of light and dark, respectively. Each data point represents mean ± SEM of three independent RNA samples. Statistical significance between the genotypes was determined by two-way ANOVA with Bonferroni's *post hoc* test and is denoted by ***$P < 0.001$ and **$P < 0.01$.

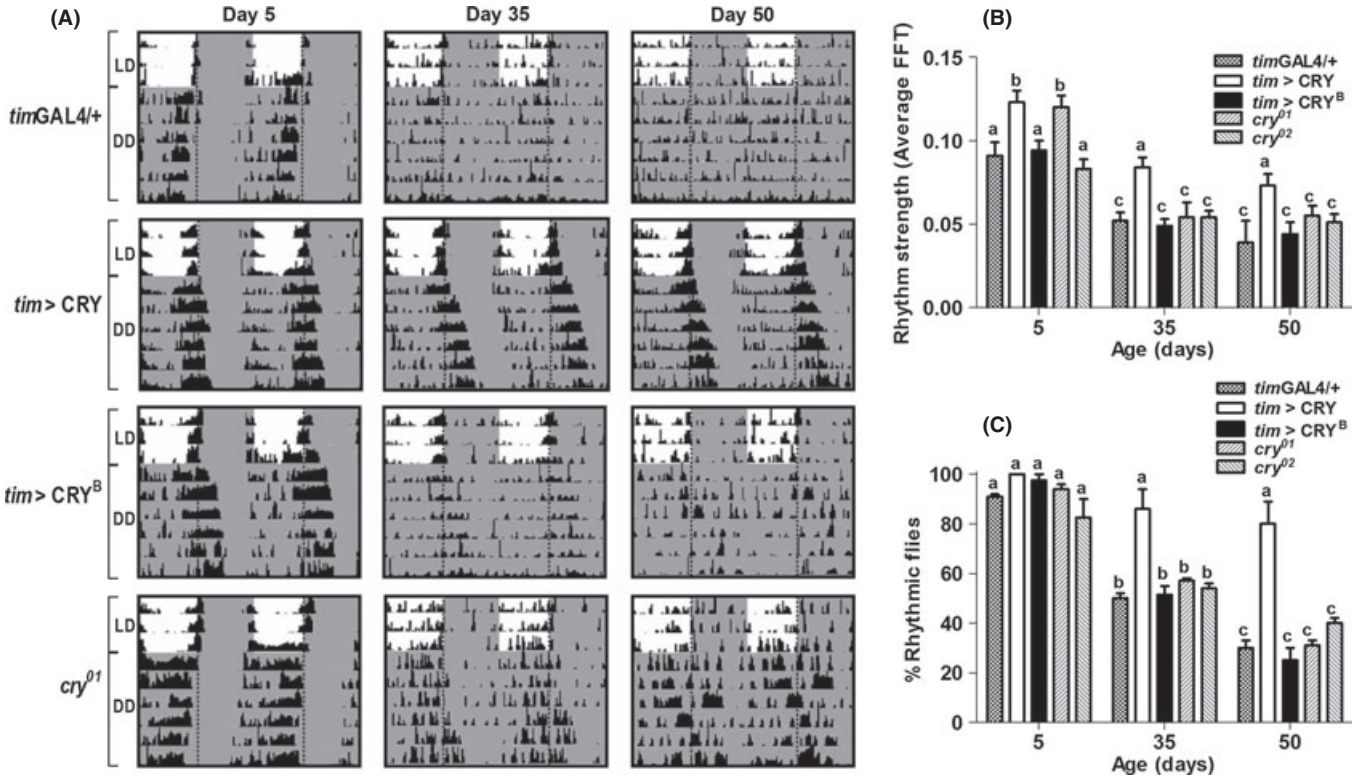

Fig. 3 CRY overexpression prevents disruption of locomotor activity rhythms. (A) Locomotor activity profiles of representative 5-, 35-, and 50-day-old males of the indicated genotypes. Flies of each age were monitored in LD (12:12) for 3-d, followed by 7-d in DD at 25 °C. Shaded areas represent periods of darkness. Vertical dotted lines indicate time of lights-off (ZT/CT 12). (B) Average rhythm strength and (C) percentage of rhythmic flies on days 5, 35, and 50. Flies with FFT values >0.04 were considered rhythmic. Values are mean ± SEM of two independent activity analyses. Total number of flies analyzed for each genotype is indicated in Table S3. Statistical significance was determined using two-way ANOVA with Bonferroni's *post hoc* test, and bars with different letters are significantly different at $P < 0.05$.

CRY overexpression prevents age-related weakening of rest/activity rhythms

Higher oscillations of several clock genes in heads of old CRY-overexpressing flies prompted us to examine the strength of behavioral rhythms in these flies. We recorded locomotor activity rhythms in *tim*>CRY and control flies on days 5–15, 35–45, and 50–60, to assess age-related changes in rhythm strength and other circadian parameters. In addition, we also monitored activity across lifespan in two lines of *cry*-null flies (cry^{01} and cry^{02}) that show normal rest/activity rhythms when young (Dolezelova *et al.*, 2007). Males were monitored for 3 days in LD 12:12 followed by 7 days in DD. Young control flies showed bimodal activity distribution in LD with morning and evening peaks and anticipation of lights-on and off (Fig. S2, Table S3). There was a general age-related reduction in average LD activity counts accompanied by the loss of morning anticipation. However, 50-day-old *tim*>CRY flies showed pronounced morning and evening peaks, while one or both of these peaks were attenuated in control or *cry*-null flies at this age (Fig. S2), suggesting that the well-known role of CRY in circadian photoreception persists in old flies overexpressing CRY.

Our analysis of free-running rhythms in DD revealed that 50- to 60-day-old *tim*>CRY flies maintained remarkably robust rest/activity rhythms, while those rhythms decayed in most control flies (Fig. 3). The average rhythm strength (FFT) was significantly higher in young *tim*>CRY flies than in controls and remained higher across lifespan (Fig. 3B). Young *cry*-null flies had strong activity rhythms as expected (Dolezelova *et al.*, 2007), and interestingly, the average FFT for cry^{01} was significantly higher than for cry^{02} (Fig. 3B, S3A). To better understand senescence in context of rhythm strength, we calculated the time required for this function to decline to 50% and 75% of its peak value as described (Martin *et al.*, 2005). This analysis suggests that aging *tim*>CRY flies have longer FFT decline time than cry^{01}, while both showed similar FFT at young age (Fig. S3A).

Overall calculation of the percentage of rhythmic flies pooled from two independent experiments demonstrated that *tim*>CRY flies were still 82% rhythmic on days 50–60 when the proportion of rhythmic flies decreased to below 40% in control and *cry*-null lines (Fig. 3C, Table S3). Additional analysis of rhythmicity as a function of age for all genotypes confirmed that *tim*>CRY flies had reduced rates of rhythm loss and extended rhythmicity decline times compared with all other genotypes examined (Fig. S3B). Persistence of behavioral rhythms in DD suggests that a durable intrinsic circadian system is maintained in old *tim*>CRY flies.

Replenishment of CRY in all clock-expressing cells improves the healthspan of aging flies

Correlational data suggest links between degradation of circadian rhythms and accelerated aging; however, it is not known whether rejuvenation of the circadian system is associated with extended healthspan. Our finding that replenishment of CRY restores molecular and behavioral rhythms in aging flies provided a unique opportunity to examine the relationship between strength of circadian rhythms and the rate of aging. One of the important biomarkers of aging in *Drosophila* is the decline of climbing ability, which can be measured via the RING assay

that utilizes negative geotaxis to assess vertical mobility (Rhodenizer *et al.*, 2008). We used the RING assay to compare climbing ability in flies with altered CRY levels across lifespan. On day 5, the climbing ability of *tim*>CRY flies was not different from the *tim*GAL4/+ *or* *tim*>CRY^{B} controls; however, both *cry*^{01} and *cry*^{02} mutants had significantly lower climbing ability even at this young age (Fig. 4A, Table S4). The climbing ability declined significantly in both controls and *cry*-null flies on day 35. In contrast, *tim*>CRY flies maintained significantly higher vertical mobility that was not different from day 5 ($P > 0.05$). Flies of all genotypes further lost their climbing ability with progressing age; however, *tim*>CRY flies performed significantly better than controls even on day 50 (Fig. 4A, Table S4). This is confirmed by additional calculations showing that *tim*>CRY flies had the longest decline times for climbing ability (Fig. S3C).

Another biomarker of aging is the accumulation of oxidatively damaged proteins, which can be biochemically measured as levels of protein carbonyls (PC) in the total protein extract (Krishnan *et al.*, 2009). Because alterations in CRY levels affected climbing ability, we tested whether the accumulation of oxidative damage in old age was also affected. We quantified PC in heads of 50-day-old flies at ZT 8, which is the peak of PC accumulation (K. Rakshit, unpublished data). Indeed, *tim*>CRY flies had significantly lower PC accumulation in heads, compared with the controls (Fig. 4B). On the other hand, both *cry*^{01} and *cry*^{02} flies had higher PC levels than the controls, and this difference reached statistical significance in *cry*^{02} flies. Together, these data suggest that CRY may have novel anti-aging functions related to proteostasis.

An important aspect of healthy aging is the ability to withstand homeostatic insults, such as oxidative stress. We reported that flies with disrupted clocks showed significantly increased mortality risk after short-term oxidative challenge (24 h of 100% hyperoxia) in the middle or old age (Krishnan *et al.*, 2009). We used this assay here to test whether flies with elevated CRY levels are capable of buffering short-term oxidative challenge later in life, by exposing 50-day-old flies to 24-h hyperoxia. This treatment significantly shortened the lifespan of *tim*GAL4/+ and *tim*>CRY^{B} flies. In contrast, *tim*>CRY flies were resilient to this stress and had very similar survival curves as their normoxia controls, despite a few initial deaths (Fig. 4C, Table S5). The lifespan of *tim*>CRY flies under normoxia was ~7% longer than control flies, albeit this difference was not statistically significant.

Overexpression of CRY in central pacemaker neurons does not improve rest/activity rhythms during aging

In young flies, locomotor activity rhythms are controlled by ~150 central pacemaker neurons in the brain (Nitabach & Taghert, 2008). The *tim*GAL4 driver increases CRY in both central and peripheral clock cells. Therefore, we next examined whether overexpression of CRY in central pacemaker neurons alone is sufficient to restore locomotor activity rhythms during aging. We initially used the *Pdf*GAL4 driver to overexpress CRY or CRY^{B} in PDF-positive lateral ventral neurons (LN$_v$s), which are crucial for DD activity rhythms in young flies (Nitabach & Taghert, 2008). Also, *Pdf*GAL4-driven UAS-*cry* can rescue behavioral photoresponses in *cry*^{b} mutants (Emery *et al.*, 2000). Surprisingly, we found that *Pdf*-driven expression of CRY in LN$_v$s is not sufficient to prevent age-related decay of behavioral rhythms (activity of *Pdf*GAL4 in PDF-positive clock neurons was confirmed by crossing with UAS-*gfp* – not shown). *Pdf*>CRY flies showed weakening and age-related fragmentation of rest/activity rhythms similar as the *Pdf*>CRY^{B} controls (Fig. 5A). There was no significant difference in the average daily activity in LD between *Pdf*>CRY and *Pdf*>CRY^{B} flies (Fig. S4, Table S3). Analysis of fly activity in DD revealed very similar age-related

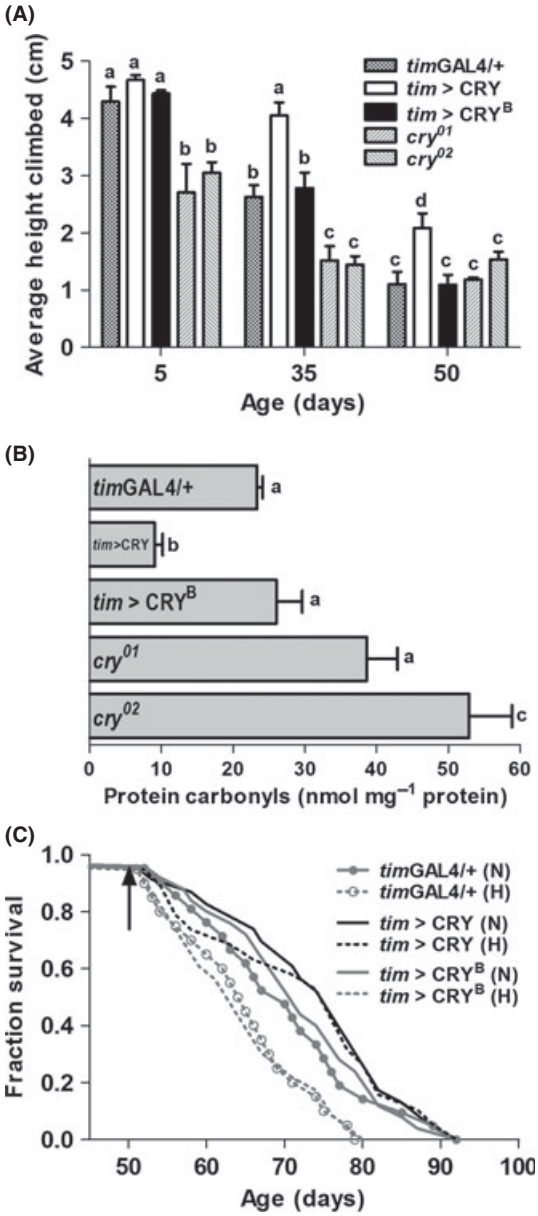

Fig. 4 Flies overexpressing CRY have improved healthspan. (A) Vertical mobility was measured by the RING assay in 5-, 35-, and 50-day-old males of the indicated genotypes. Bars represent mean height climbed (±SEM), based on testing four vials per genotype, each containing 25 flies. Statistical significance was determined using two-way ANOVA with Bonferroni's *post hoc* test, and bars with different letters are significantly different at $P < 0.05$. (B) Oxidative damage in the form of protein carbonyls (PC) was measured in heads of 50-day-old males of the indicated genotypes at ZT 8. *tim*>CRY flies had significantly lower PC compared with the *tim*GAL4/+ and *tim*>CRY^{B} controls, and *cry*^{01}. There was significantly higher PC accumulation in heads of *cry*^{02} flies compared with *tim*GAL4/+ control. Bars represent mean carbonyl levels (±SEM), based on testing 3 independent sets of flies, each containing 75 flies in 3 technical repeats of 25 flies each. Statistical significance was determined using one-way ANOVA with Tukey's *post hoc* test, and bars with different letters are significantly different at $P < 0.05$. (C) Lifespan of *tim*GAL4/+, *tim*>CRY, and *tim*>CRY^{B} flies in normoxia (solid lines) and following 24-h hyperoxia (dotted lines) on day 50 (marked by arrow). In normoxia, there was no significant difference in mean survival curves among the genotypes. Hyperoxia on day 50 significantly shortened the lifespan of *tim*GAL4/+, and *tim*>CRY^{B} controls ($P < 0.05$) but not *tim*>CRY flies.

decline in the rhythm strength (Fig. 5B) and the percentage of rhythmic flies (Fig. 5C) in both *Pdf*>CRY and *Pdf*>CRY^{B} flies, with no statistical difference at any tested age (Table S3). Additional analysis of rhythm strength and % rhythmicity functions with age confirmed similar rates of decline in *Pdf*>CRY and *Pdf*>CRY^{B} flies (Fig. S5A,B).

We then overexpressed CRY or CRY^{B} using another driver line *cry*GAL4-39, which is active in additional groups of central pacemaker neurons regulating rest/activity rhythm (Klarsfeld *et al.*, 2004). We confirmed that the *cry*GAL4-39 driver drives the expression in several groups of pacemaker neurons but not in peripheral clocks by crossing with UAS-*gfp* (not shown). Despite that a larger subset of central pacemaker neurons was targeted, rhythms of locomotor activity decayed at a similar rate in *cry*39>CRY flies as in *cry*39>CRY^{B} controls (Figs 5D–F, S4, S5A–B, Table S3).

Rescue of CRY in central pacemaker neurons alone does not improve healthspan

We tested whether rescue of CRY in the central pacemaker neurons affects biomarkers of aging that were improved by CRY overexpression in both central and peripheral clocks. There was no statistically significant difference in climbing ability of *Pdf*>CRY or *cry*39>CRY flies compared with their respective controls at any physiological age (Fig. 6A, S5C), nor was there any statistically significant difference in PC accumulation in heads of old *Pdf*>CRY or *cry*39>CRY flies compared with their respective *Pdf*>CRY^{B} and *cry*39>CRY^{B} controls (Fig. 6B).

Discussion

The decline of circadian output rhythms is a common signature of aging in animals including humans. It was recently reported in *Drosophila* that aging is associated with dampened molecular oscillations of core clock genes in peripheral but not central oscillators (Luo *et al.*, 2012; Rakshit *et al.*, 2012). Given the persistence of molecular oscillations in the central clock, there is currently no explanation as to why rest/activity rhythms lose their robustness during aging. The results of our study suggest that enhancement of peripheral clocks may prevent degradation of the circadian output. Furthermore, our data suggest a cause and effect relationship between the molecular and behavioral decay of circadian rhythms and fly healthspan.

We observed reduction in *cry* mRNA levels in heads of old flies, consistent with a previous study (Luo *et al.*, 2012). Interestingly, *cry* decline was observed in the heads but not in bodies similar to our results on other clock genes (Rakshit *et al.*, 2012), suggesting that peripheral clocks in the fly head are more susceptible to aging than clocks in the body tissues. We also report that CRY protein is significantly reduced in heads of 50-day-old CS flies at most time points, although it still oscillates in a phase similar to young flies. This suggests that the circadian system is severely attenuated yet functional in old flies.

Our data show that age-related CRY decline is functionally linked to decay of the circadian system, as overexpression of wild-type CRY significantly improved the amplitude of *per*, *tim*, *Pdp1ε*, and *vri* genes in heads of old flies. These results may seem surprising as it was reported that overexpression of CRY together with PER using the eye-specific *gmr*GAL4 driver suppressed *tim* mRNA expression (Collins *et al.*, 2006). However, this study also reported that overexpression of CRY alone significantly increased the amplitude of *tim* and *vri* in young flies (Collins *et al.*, 2006), consistent with our current data in old flies. Further studies are required to determine whether replenishment of CRY in peripheral clock cells may enhance the transcriptional activity of CLK–CYC complexes in the heads. Although we show that neither aging nor CRY overexpression altered *Clk* and *cyc* mRNA levels, downstream post-translational mechanisms or epigenetic modifications could be conceivably affected in old flies and restored by CRY overexpression. In support of this notion, it was reported that CLK protein levels are constitutively low in heads of *cry^{b}* flies (Collins *et al.*, 2006). Further experiments would be required to understand the mode of CRY action in maintaining molecular circadian rhythms. Interestingly, age-related decay of the circadian clock at molecular and behavioral levels was reported in mPer1 and mCry2 knockout mice (Oster *et al.*, 2003).

Our study reveals that restoration of CRY in all clock-expressing cells prevented the decay of locomotor activity rhythms with age. While rhythms in LD were somewhat improved in *tim*>CRY flies consistent with its role as a photoreceptor, remarkable differences were observed in DD where 35- and 50-day-old flies with overexpressed CRY showed almost young-like rest/activity rhythms, suggesting that the free-running circadian system was intact in those flies. We also found that augmentation of CRY in central clock neurons alone was not sufficient to restore rest/activity rhythms. While only central clocks are required for behavioral rhythms in young flies (Hardin, 2011), our data suggest that peripheral clocks may additionally be necessary for maintaining neuronal homeostasis and preventing the degradation of circadian output pathways in aging individuals. A recent study suggests that circadian rhythms may be also strengthened by *Pdf* overexpression (Umezaki *et al.*, 2012), suggesting that circadian rejuvenation may be achieved in more than one way. Interestingly, decay of clock output pathways despite strong mPer2 oscillations in the central clock (SCN) has been recently reported in mouse (Nakamura *et al.*, 2012).

Our data show that CRY-mediated strengthening of circadian rhythms is associated with deceleration of functional decline normally observed in aging flies. Namely, CRY-overexpressing flies maintained significantly higher climbing ability than their controls at every age tested, suggesting that these flies had a slower rate of aging. Furthermore, these flies had significantly lower oxidative damage in old age. Consistent with these findings, they were also able to recover from exposure to short-term hyperoxia. In contrast, *cry*-null flies showed accelerated functional decline and higher accumulation of oxidatively damaged proteins. Together, these results suggest that strong circadian rhythms are important for maintaining the health of an organism during aging and also point toward novel anti-aging functions of CRY. While several studies demonstrated that genetic or environmental disruptions of the clock function accelerate physiological aging and age-related diseases, it is not clear whether rejuvenation of the circadian system can delay aging. Here, we achieved circadian rejuvenation by ectopic increase in age-reduced protein CRY and demonstrate that this treatment extends healthspan in flies. CRY supplementation and, conversely, CRY deficiency at the same chronological age shifted the readouts of aging in opposite directions. Namely, *tim*>CRY flies showed better climbing vigor and reduced oxidative damage than age-matched controls, while climbing was impaired even in the young *cry*-null flies and they accumulated greater oxidative damage during aging. A limitation of our study is that the *tim*GAL4 driver causes supra-physiological increase in the levels of CRY, and the associated phenotype may be a result of excess of CRY instead of a mere replenishment with age. However, *tim*>CRY flies did not show aberrant phenotypes that could suggest off-target effects in molecular or behavioral parameters measured. Moreover, *tim*>CRY^{B} controls show results very similar to wild-type controls despite supra-physiological levels of *cry* mRNA (not shown).

Taken together, our data suggest that CRY-mediated improvement in circadian rhythms may delay functional aging. This could be linked to the

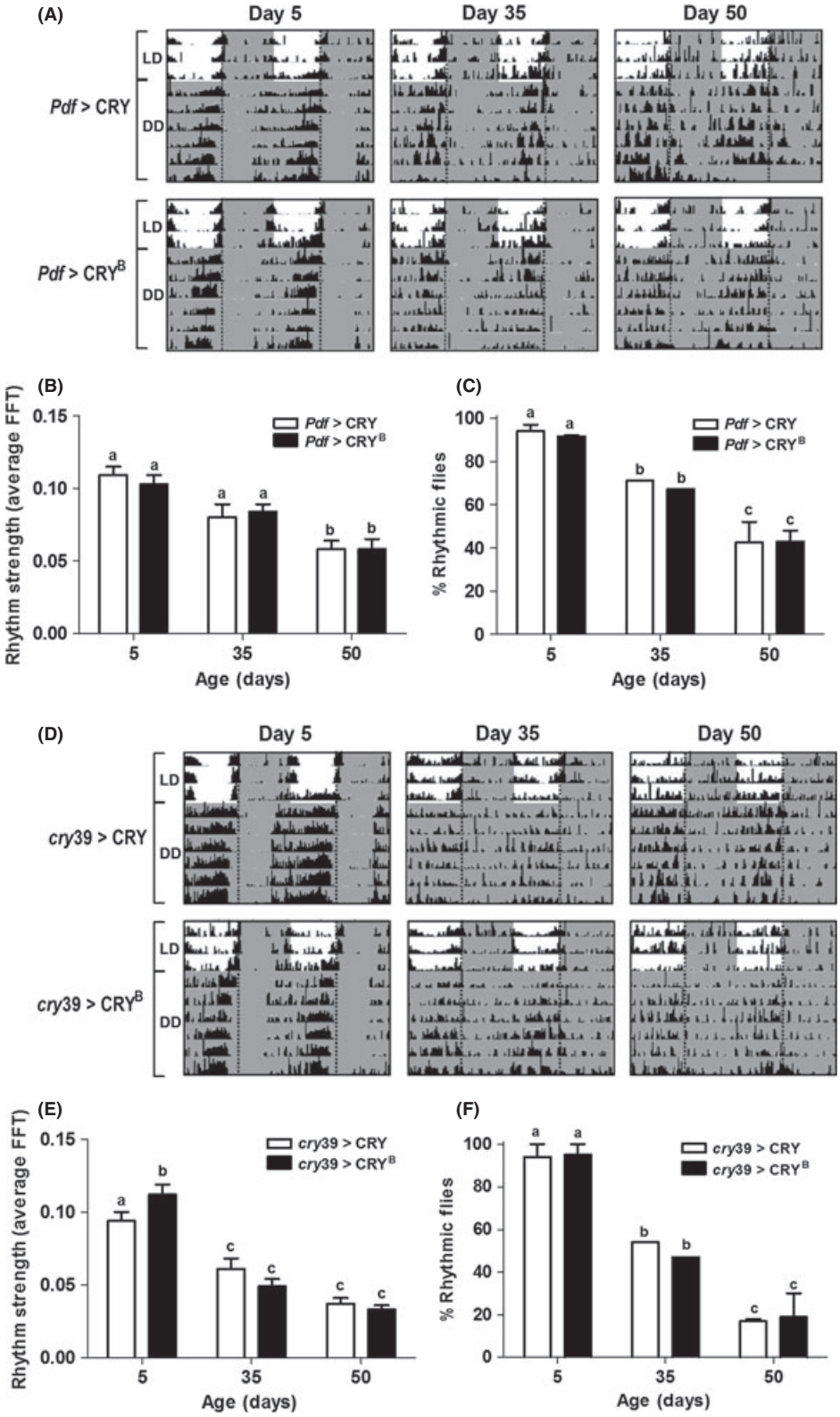

Fig. 5 Rescue of CRY in central pacemaker neurons alone does not improve locomotor activity rhythms. (A) Locomotor activity profiles of representative *Pdf*>CRY and *Pdf*>CRY^B males on days 5, 35, and 50. Flies of each age were monitored in LD (12:12) for 3-d, followed by 7-d in DD at 25 °C. Shaded areas represent periods of darkness. Vertical dotted lines indicate time of lights-off (ZT/CT 12). (B) Average rhythm strength and (C) percentage of rhythmic *Pdf*>CRY and *Pdf*>CRY^B flies on days 5, 35, and 50. (D) Representative locomotor activity profiles, (E) average rhythm strength, and (F) percentage of rhythmic *cry*39>CRY and *cry*39>CRY^B flies on days 5, 35, and 50. (B, C, D, F – flies with FFT values >0.04 were considered rhythmic). Values are mean ± SEM of two independent activity analyses. Total number of flies analyzed for each genotype is indicated in Table S3. Statistical significance was determined using two-way ANOVA with Bonferroni's *post hoc* test, and bars with different letters are significantly different at $P < 0.05$.).

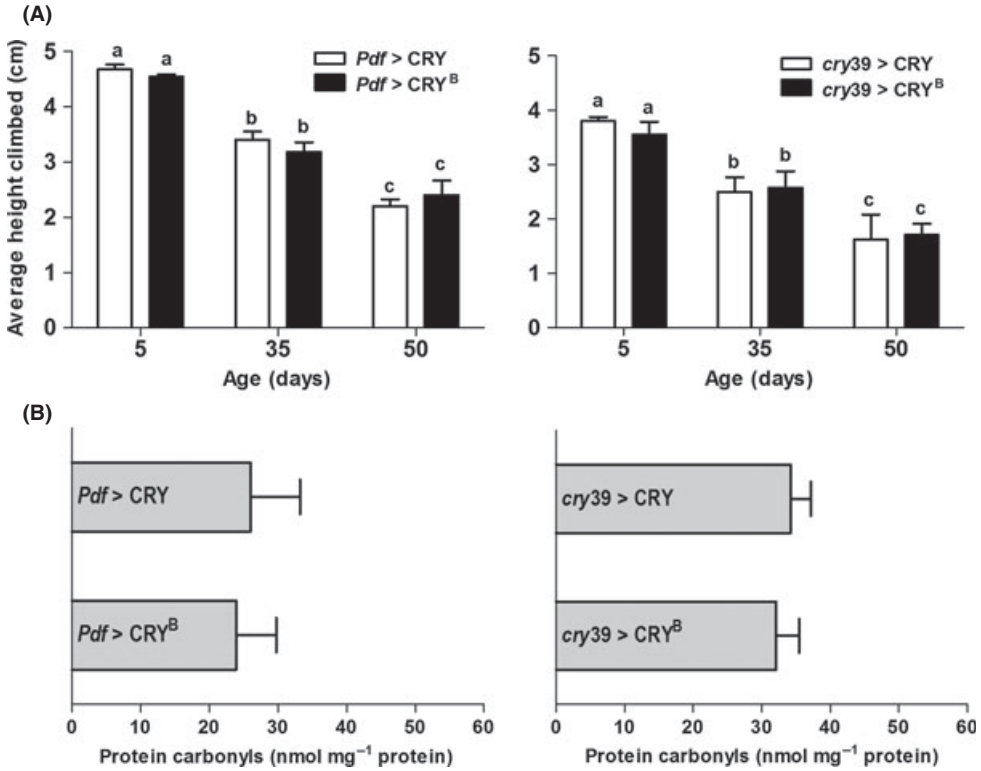

Fig. 6 CRY-restoration in central pacemaker neurons alone does not improve healthspan. (A) Vertical mobility was measured by the RING assay in 5-, 35-, and 50-day-old males of the indicated genotypes. Bars represent mean height climbed (±SEM), based on testing four vials per genotype, each containing 25 flies. Statistical significance was determined using two-way ANOVA with Bonferroni's *post hoc* test, and bars with different letters are significantly different at $P < 0.05$. (B) Oxidative damage in the form of protein carbonyls (PC) was measured in heads of 50-day-old males of the indicated genotypes at ZT 8. Bars represent mean carbonyl levels (±SEM), based on testing 3 independent sets of flies, each containing 75 flies in three technical repeats of 25 flies each. Statistical significance was determined using one-way ANOVA with Tukey's *post hoc* test.

enhanced expression of clock-controlled effector genes that are involved in many pathways maintaining temporal homeostasis (Wijnen & Young, 2006; Krishnan *et al.*, 2008). On the other hand, we cannot exclude that healthspan–benefiting effects of CRY overexpression may be uncoupled from circadian rhythms and instead linked to its pleiotropic effects. Several novel noncircadian functions of CRY have been recently suggested in *Drosophila* (Fogle *et al.*, 2011; Seay & Thummel, 2011; Kumar *et al.*, 2012). Intriguingly, recent *in vivo* analysis comparing the transcriptional activity of human and fly *cryptochromes* in transgenic *Drosophila* suggests that these proteins may share common signaling pathways in DD, regulating genes implicated in stress response (Vieira *et al.*, 2012).

In summary, we demonstrate that age-related dampening of clock gene oscillations and daily activity rhythms can be significantly improved by the genetic manipulation of a protein that is best known for its role in circadian phototransduction. We provide evidence that overexpression of CRY helps slow down the aging process and reverse age-associated phenotypes. Our study suggests novel anti-aging role of the gene *cryptochrome* in *Drosophila,* as it has profound effects on the health and fitness most likely by improving clock-controlled effector genes. However, the mode of CRY action as an anti-aging factor will require future studies.

Experimental procedures

Fly rearing

Drosophila melanogaster were reared on 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25 °C in 12-h light per 12-h dark (LD) cycles (with an average light intensity of ~2000 lux). By convention, lights-off is denoted as Zeitgeber time (ZT) 12. For experiments on aging flies, cohorts of 50–75 mated males were housed in 8-oz round bottom polypropylene bottles (Genesee Scientific, San Diego, CA, USA) inverted over 60-mm Falcon Primaria tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) containing 15 mL of diet. Diet dishes were replaced daily without CO_2, after tapping flies to the bottom of the bottle. For other assays, cohorts of 25 mated males were reared in narrow vials (Genesee Scientific).

Fly stocks

Mated canton S-mated males were used for RNA and protein measurements of *cry* during aging. For the *cry* overexpression studies, w^{1118} served as the wild-type control to which driver and responder lines were backcrossed for 8 generations. Driver lines w^{1118};+;BsR-B (*Pdf*GAL4) (Park *et al.*, 2000), *w*;*cry*Gal4$^{\#39}$;+ (*cry39*GAL4) (Klarsfeld *et al.*, 2004) and *w*;*tim*Gal4(62);+ (*tim*GAL4) (Kaneko & Hall, 2000) were crossed to either responder line w^{1118};UAS-*cry*24;+ (UAS-*cry*) (Emery *et al.*, 1998) or w^{1118};w^{+} UAS*cry*^{b}31;+ (UAS-*cry*^{b}) (Emery *et al.*, 2000) as a control. Additionally, *cry*^{01} and *cry*^{02} mutants (Dolezelova *et al.*, 2007) were backcrossed to w^{1118} and used in some experiments.

Quantitative real-time polymerase chain reaction

Three independent bioreplicates of flies were collected at 4-h intervals around the clock on days 5, 35, and 50. Total RNA was extracted from

fly heads and bodies separately using Tri Reagent (Sigma, St. Louis, MO, USA). The samples were purified and treated with Takara Recombinant DNase I (Clontech Laboratories Inc., Mountain View, CA, USA). Synthesis of cDNA was achieved with the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed on the StepOnePlus Real-Time machine (Applied Biosystems, Carlsbad, CA, USA) under default thermal cycling conditions, with a dissociation curve step. Every reaction contained Power SYBR Green (Applied Biosystems), 10 ng cDNA, and 400 nM primers. Primer sequences are available upon request. Data were analyzed using the $2^{-\Delta\Delta CT}$ method with mRNA levels normalized to the gene *rp49*. Relative mRNA amplitude was calculated with respect to the trough levels on day 5 set as 1 (for aging study) or trough levels of *tim*GAL4/+ (control) set as 1 on day 50 (for *cry* overexpression studies).

Western blotting

Three independent bioreplicates of 5- and 50-day-old males of different genotypes were collected at specific ZT points. About 5–10 fly heads per time point were homogenized on ice in Laemmli buffer, sonicated, boiled at 100 °C for 5 min, and centrifuged at 12000 g at 4 °C. A constant ratio of the buffer (7 μL per head) was used to ensure equal protein loading and separation on 10% acrylamide gel. Proteins were transferred to the 0.45 μm polyvinylidene fluoride (PVDF) Immobilon-FL membrane (Millipore Billerica, MA, USA) and incubated in 1X TBST (10 mM Tris, 0.15 M NaCL, 0.1% Tween-20, pH 7.5) + 5% milk for 2 h, then overnight at 4 °C with 1:2000 anti-CRY (Rush *et al.*, 2006) in blocking buffer. Membranes were treated for 2 h with 1:20 000 goat anti-rabbit IRDye680 (LI-COR Biosciences, Lincoln, NE, USA). Proteins were quantified using the LI-COR Odyssey INFRARED IMAGING SYSTEM software (v. 3.0, LI-COR Biosciences, Lincoln, NE, USA).

Locomotor activity analysis

Flies were entrained in LD 12:12 at 25 °C. Locomotor activity of 5, 35, and 50-day-old males was recorded for 3 day in LD 12:12, followed by 7 day in constant darkness (DD) using the Trikinetics locomotor activity monitor (Waltham, MA, USA). For a quantitative measure of circadian rhythmicity in DD, fast Fourier transform (FFT) analysis was conducted using CLOCKLAB software (Actimetrics; Coulbourn Instruments, Whitehall, PA, USA). Flies with FFT values <0.04 were classified as arrhythmic, ones with values of 0.04–0.08 were classified as weakly rhythmic, whereas flies with FFT values >0.08 were considered strongly rhythmic. Flies with both weak and strong rhythms that showed a single peak in the periodogram were included in the calculation of the free-running period using the CLOCKLAB software (Actimetrics, Wilmette, IL, USA).

Rapid iterative negative geotaxis (RING) assay

Vertical mobility was tested using the RING assay as described (Rhodenizer *et al.*, 2008; Krishnan *et al.*, 2012). Briefly, four groups of 25 mated males of each age and genotype were transferred into empty vials without anesthesia, and the vials were loaded into the RING apparatus. The apparatus was tapped three times in rapid succession to initiate a negative geotaxis response. The flies' movements in tubes were videotaped and digital images captured 4 s after initiating the behavior. The climbed distance was calculated for each fly and expressed as average height climbed in the 4-s interval. The performance of flies in a single vial was calculated as the average of five consecutive trials (interspersed with a 30-s rest).

Protein carbonyl (PC) assay

To assess oxidative damage, protein carbonyls were measured in head homogenates of 50-day-old males of various genotypes at 370 nm after reaction with 2,4-dinitrophenylhydrazine (DNPH), using a Synergy 2 plate reader (BioTek, Winooski, VT, USA), as described previously (Krishnan *et al.*, 2008). Results were expressed as nmol mg^{-1} protein using an extinction coefficient of 22 000 M^{-1} cm^{-1}.

Hyperoxia treatment and lifespan analysis

For hyperoxia exposure, four cohorts of 25 males in narrow vials with diet were placed in a Plexiglass chamber filled with oxygen (100% medical grade) flowing at a constant rate (300 mL min^{-1}) for 24-h, as described (Krishnan *et al.*, 2009). Control flies were transferred to narrow vials with diet and kept under normoxia. After the treatment, flies were transferred from narrow vials to bottles as described in the fly rearing section. Diet was replaced on alternate days without anesthesia, and mortality was recorded daily.

Statistical analysis

Data were statistically analyzed with GRAPHPAD PRISM (v.5.0) and GRAPHPAD INSTAT (v.3.0; San Diego, CA, USA). The qRT-PCR, locomotor activity analysis, and RING data were evaluated by two-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test. Additionally, we plotted FFT, % rhythmic, and RING data as points to better visualize the slope of functional decline with age for each genotype and calculated the time required for each function to decline to 50% and 75% of its peak value, interpolated from second-order polynomial curves as described (Martin *et al.*, 2005). PC data were evaluated by one-way ANOVA with Tukey's *post hoc* test. For Western data, the relative strength of the signals was quantified using LI-COR Image analysis software (v.3.0) and subjected to two-way ANOVA with Bonferroni's *post hoc* test. Lifespan and survival curves were plotted following Kaplan–Meier survival analysis, and statistical significance of curves was assessed using the log-rank (Mantel–Cox) test.

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Author contributions

KR and JMG designed the study. KR conducted all the experiments and wrote the manuscript. JMG provided critical feedback and revised the first draft. The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Western blot showing CRY protein levels in head extracts of 5 and 50 day-old *tim*>CRY flies and controls at ZT 4 and 16. LC represents the loading control. CRY levels were lower in the morning (ZT 4) than at night (ZT 16) in both young and old *tim*>CRY flies, suggesting light sensitivity of ectopic CRY.

Fig. S2 Average daily activity profiles of 5, 35, and 50 day-old males of the indicated genotypes in LD (12:12). Each bar represents daily activity counts in a 15 min bin averaged for 3 days.

Fig. S3 Analysis of the rate of senescence in males of the indicated genotypes for the following parameters A) FFT, B) % Rhythmicity, and C) Vertical mobility. For each parameter, the graph on the left side shows average values obtained on days 5, 35, and 50, while bar diagrams on the right indicate the time required for function to decline to 50% and 75% of its peak value, interpolated from second-order polynomial curves.

Fig. S4 Average daily activity profiles of 5, 35, and 50 day-old males of the indicated genotypes in LD (12:12). Each bar represents daily activity counts in a 15 min bin averaged for 3 days.

Fig. S5 Analysis of the rate of senescence in males of the indicated genotypes for the following parameters A) FFT, B) % Rhythmicity, and C) Vertical

mobility. For each parameter, the graph on the left side shows average values obtained on days 5, 35, and 50, while bar diagrams on the right indicate the time required for function to decline to 50% and 75% of its peak value, interpolated from second-order polynomial curves.

Table S1 Statistical analysis of gene expression (qPCR) and protein (Western Blotting) data by two-way ANOVA with Bonferroni's *post-hoc* test.

Table S2 Statistical analysis of gene expression (qPCR) data in heads of 50 day-old flies by two-way ANOVA with Bonferroni's *post-hoc* test.

Table S3 Locomotor activity analysis of flies overexpressing CRY.

Table S4 Statistical analysis of Rapid Iterative Negative Geotaxis (RING) data by two-way ANOVA with Bonferroni's *post-hoc* test.

Table S5 Median lifespan (days) is shown for indicated genotypes with n = sample size.