Deviation of innate circadian period from 24 h reduces longevity in mice

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Summary

The variation of individual life spans, even in highly inbred cohorts of animals and under strictly controlled environmental conditions, is substantial and not well understood. This variation in part could be due to epigenetic variation, which later affects the animal's physiology and ultimately longevity. Identification of the physiological properties that impact health and life span is crucial for longevity research and the development of anti-aging therapies. Here, we measured individual circadian and metabolic characteristics in a cohort of inbred F1 hybrid mice and correlated these parameters to their life spans. We found that mice with innate circadian periods close to 24 h (revealed during 30 days of housing in total darkness) enjoyed nearly 20% longer life spans than their littermates, which had shorter or longer innate circadian periods. These findings show that maintenance of a 24-h intrinsic circadian period is a positive predictor of longevity. Our data suggest that circadian period may be used to predict individual longevity and that processes that control innate circadian period affect aging.
Key words: ageing; circadian rhythm; clock; longevity; mice; physical activity; sleep pattern.

Introduction

Longevity is genetically limited. Soon after reaching sexual maturity, the organism starts aging, which manifests itself in fitness decline, increased susceptibility to numerous diseases, and ultimately death. However, some individuals age at different rates compared to others. Even in cohorts of highly inbred animals, which are housed in identical and tightly controlled environments, the variability of life spans can be on the order of 250% (Sanchez-Blanco & Kim, 2011). One of the explanations for such variability could be an innate instability of the epigenetic information set during the development of the organism (Skora & Spradling, 2010). Epigenetic variation that occurs early in development might manifest itself as differences in the specific characteristics of individuals, which would influence the rate of aging, and life span. Other possibilities also exist these include *de novo* mutations, or intrinsic stochasticity of the developmental processes.

A number of aging theories exist that link the rate of organismal aging to the damage caused by reactive oxygen species, instability of genetic programs after the development is completed, or general genome instability (both nuclear and mitochondrial) (Prinzinger, 2005). The circadian clock plays an important role in the above processes. The circadian clock mechanism is well studied (Bass & Takahashi, 2010), and it coordinates behavioral and biochemical processes with day/night cycles. Almost all cells of the organism sustain their own innate clock systems, which interact with environmental cues to optimize metabolism temporally.

Deviation of the innate circadian period from that of the environment can lead to a decrease in fitness in diverse organisms, ranging from cyanobacteria (Ouyang *et al.*, 1998) to mice (Wyse & Coogan, 2010). While investigating free-running period in green alga *Euglena gracilis* and fruit flies *Drosophila melanogaster* (Bruce & Pittendrigh, 1956; Pittendrigh *et al.*, 1958) it was suggested that a match of the innate circadian period to the environmental photoperiod might be beneficial. Indeed, artificial alteration of light cycles shortens the life span of *Drosophila melanogaster*, and 24-h cycle housing conditions yield the longest life span (Pittendrigh & Minis, 1972). Additionally, genetic mutations that disrupt the circadian clock tend to shorten longevity and decrease fitness of fruit flies (Klarsfeld & Rouyer, 1998). In mammals, genetic disruption of the innate clock genes, such as CLOCK or BMAL1, has been associated with numerous diseases of aging such as cataracts (Dubrovsky *et al.*, 2010), cancer (Zhu *et al.*, 2009), psychiatric disorders (Rosenwasser, 2010), and metabolic syndrome (Sadacca *et al.*, 2011).

Here, we use an unbiased approach to investigate the impact of innate circadian rhythm on longevity in a homogeneous population of mice. Our findings suggest that strategies to match innate circadian rhythms to the diurnal cycle may provide benefits for humans, including extended longevity.

Results

To investigate the impact of the individual metabolic characteristics on longevity, we chose to use F1 hybrid mice (CB6F1, purchased from NIA, NIH at 1 year of age), which are derived by crossing two inbred mouse strains BALB/cBy and C57BL/6. Even though these F1 hybrid mice are genetically identical, hybrid vigor alleviates strain-specific diseases, which are often observed in inbred strains (Turturro *et al.*, 1999). Moreover, these F1 hybrid mice are known to exhibit a range of innate circadian periods (Siepka & Takahashi, 2005), possibly due to epigenetic variation.

All animals were singly housed in a climate-controlled environment for the duration of their life span. To measure the activity and circadian rhythm of the mice, we equipped each cage with a running wheel that contained an electronic transmitter to record revolutions. After habituation, all of the animals established a typical circadian activity pattern, where they mostly rested during the 12-h light period and were active during the 12 dark hours. Typical actograms of their running-wheel activity plotted vs. time are presented on Fig. 1A–F (each panel represents one typical animal). After 20 days of entraining, the light cycle was changed to 24-h darkness for 30 days. During this testing phase, animals
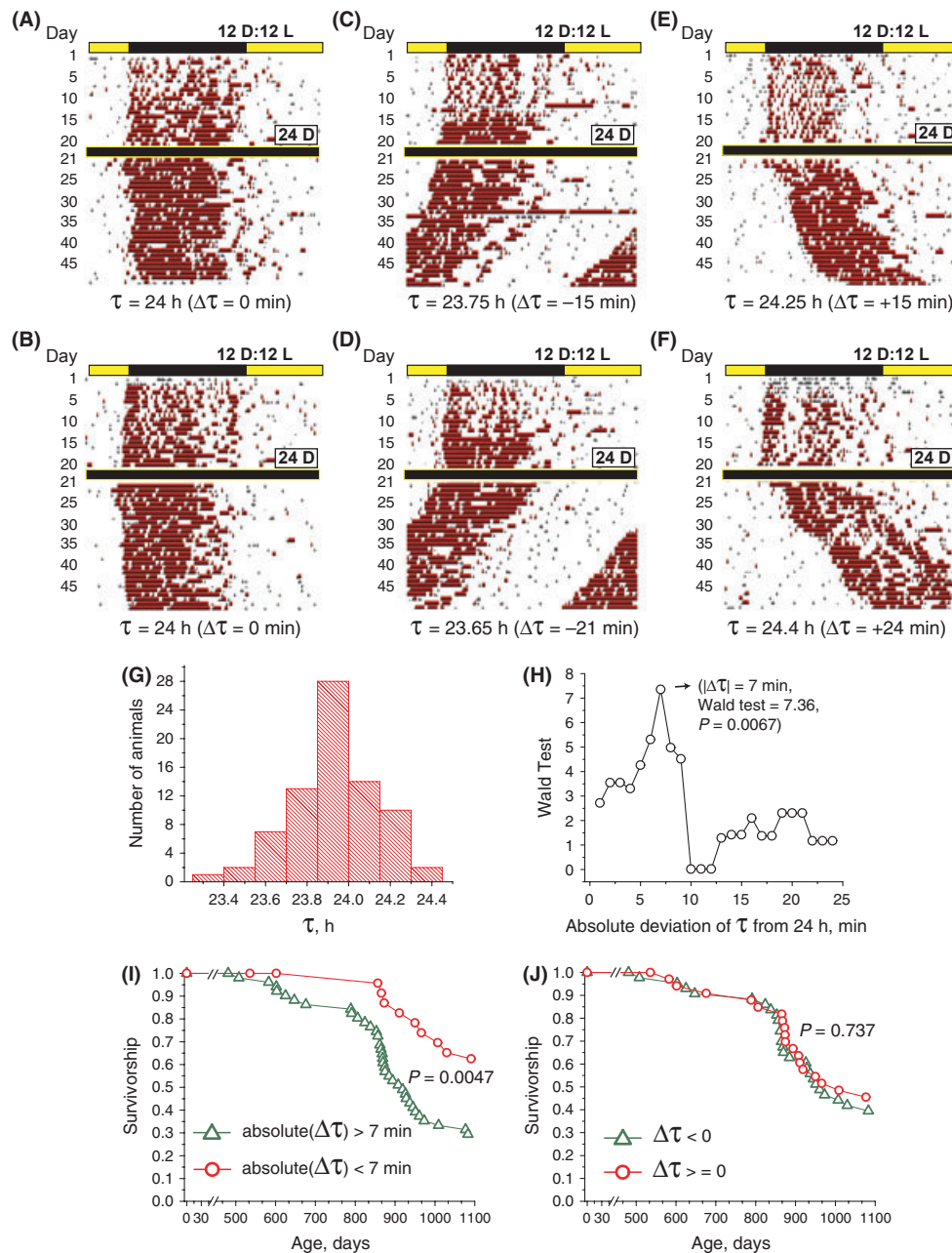
Fig. 1 Mice with innate circadian rhythm close to 24 h have reduced mortality. (A–F) Typical actograms of animal's activity are shown (each panel represents one animal). Running activity of each animal was binned in 10-min intervals and plotted against the time of the day (one line represents 24 h) for 50 consecutive days. Red bars indicate continuous running activity for at least 20 min with an average speed >2 feet per minute. (A,B) Typical actograms of animals with innate circadian period of 24 h. (C,D) Typical actograms of animals with innate circadian period shorter than 24 h. (E,F) Typical actograms of animals with innate circadian period longer than 24 h. (G) The histogram of the distribution of the innate circadian period for the tested cohort of mice is shown. The distribution has symmetrical bell-like shape. (H) Deviation of the innate circadian period (τ) by more than 7 min negatively impacts longevity of animals. Absolute deviation of the innate period ($\Delta\tau$) was assigned to each animal. Animals were grouped into two categories, those with $\Delta\tau$ less than particular cutoff value and those with $\Delta\tau$ greater than that value. Statistical significance (using Wald test) of survival difference between these two groups of animals is plotted against $\Delta\tau$ cutoff values ranging from 1 to 24 min. $\Delta\tau$ of 7 min yields the maximum difference in survivorship. (I) Animals with innate circadian rhythm close to 24 h ($\pm$7 min, red survivorship curve, $N = 24$) enjoy longer life span ($P = 0.0047$) than animals with circadian rhythm significantly longer or shorter than 24 h (greater deviation than 7 min, green curve, $N = 52$, see also Fig. S1 and Table S1). (J) Longevity of animals with longer vs. shorter circadian rhythm does not differ. Survival curves for two groups are shown. $N_{red} = 33$ (animals with circadian periods $\geq$24 h), $N_{green} = 43$ (animals with circadian periods <24 h), $P = 0.737$.

could not synchronize their activity with the external 12:12 light/dark illumination and their rest-activity patterns relied solely on their internal clock. In this cohort, we calculated each individual's innate period (τ) using the Lomb–Scargle periodogram (LSP) procedure (Van Dongen

et al., 1999), and we identified individuals with a near 24-h circadian period (typical actograms are presented in Fig. 1A,B), individuals with innate circadian period shorter than 24 h (typical actograms are presented in Fig. 1C,D) and individuals with innate circadian period
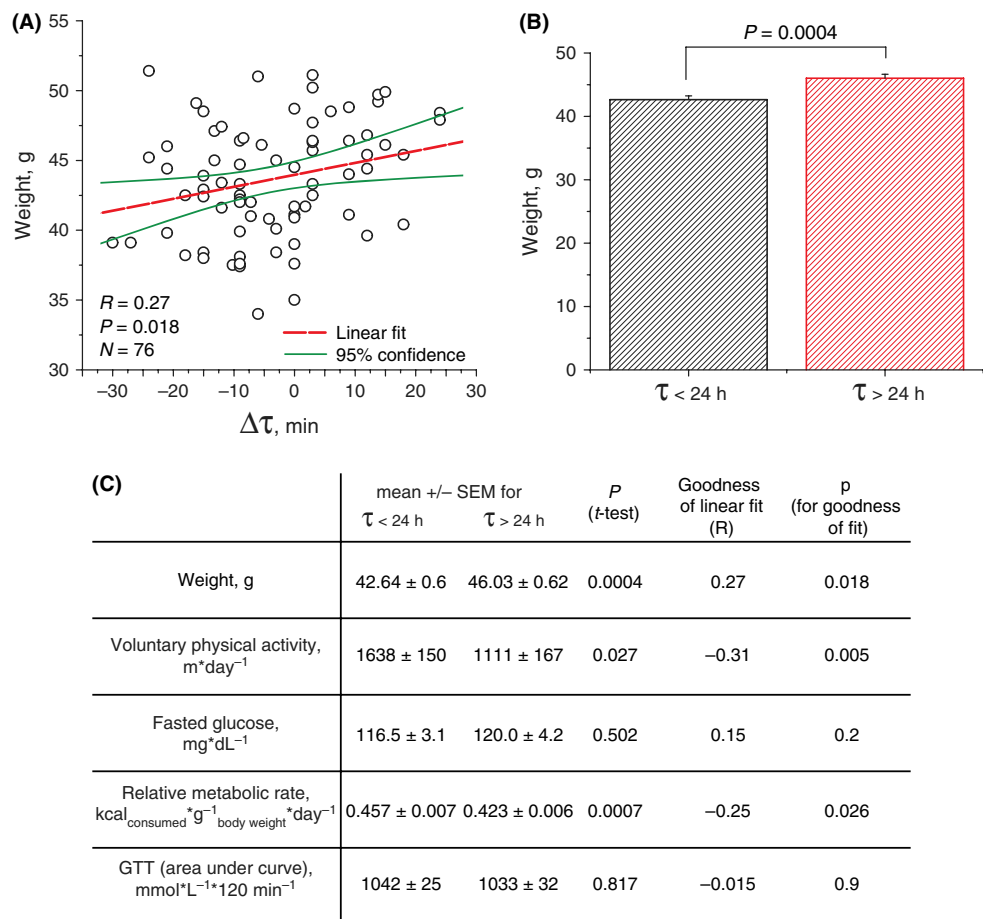
(A)

Y-axis: Weight, g; X-axis: Δτ, min

$R = 0.27$
$P = 0.018$
$N = 76$

- - - Linear fit
——— 95% confidence

(B)

$P = 0.0004$

Y-axis: Weight, g

τ < 24 h   τ > 24 h

(C)

	mean +/− SEM for		P (t-test)	Goodness of linear fit (R)	p (for goodness of fit)
	τ < 24 h	τ > 24 h			
Weight, g	42.64 ± 0.6	46.03 ± 0.62	0.0004	0.27	0.018
Voluntary physical activity, $m*day^{-1}$	1638 ± 150	1111 ± 167	0.027	−0.31	0.005
Fasted glucose, $mg*dL^{-1}$	116.5 ± 3.1	120.0 ± 4.2	0.502	0.15	0.2
Relative metabolic rate, $kcal_{consumed}*g^{-1}_{body\ weight}*day^{-1}$	0.457 ± 0.007	0.423 ± 0.006	0.0007	−0.25	0.026
GTT (area under curve), $mmol*L^{-1}*120\ min^{-1}$	1042 ± 25	1033 ± 32	0.817	−0.015	0.9

Fig. 2 Innate circadian rhythm correlates with certain physical parameters of individual animals. (A) Higher weight of animals correlates with longer innate circadian period (τ). Scatter plot for individual animals is shown. Weight of animals is plotted against deviation of innate circadian rhythm from 24 h (Δτ). Linear regression ($R = 0.27$, $P = 0.018$) reveals weak positive correlation between weight and τ. (B) Animals with innate circadian period τ >24 h on average weigh 8% more than animals with τ <24 h ($P = 0.0004$). (C) The table presents the summary of correlations between individual's weight, physical activity, glucose levels, metabolic rate, and glucose handling with their innate circadian period. Weight has positive correlation τ (also see A,B), physical activity and metabolic rate have negative correlation with τ, and glucose handling parameters do not show any correlation.
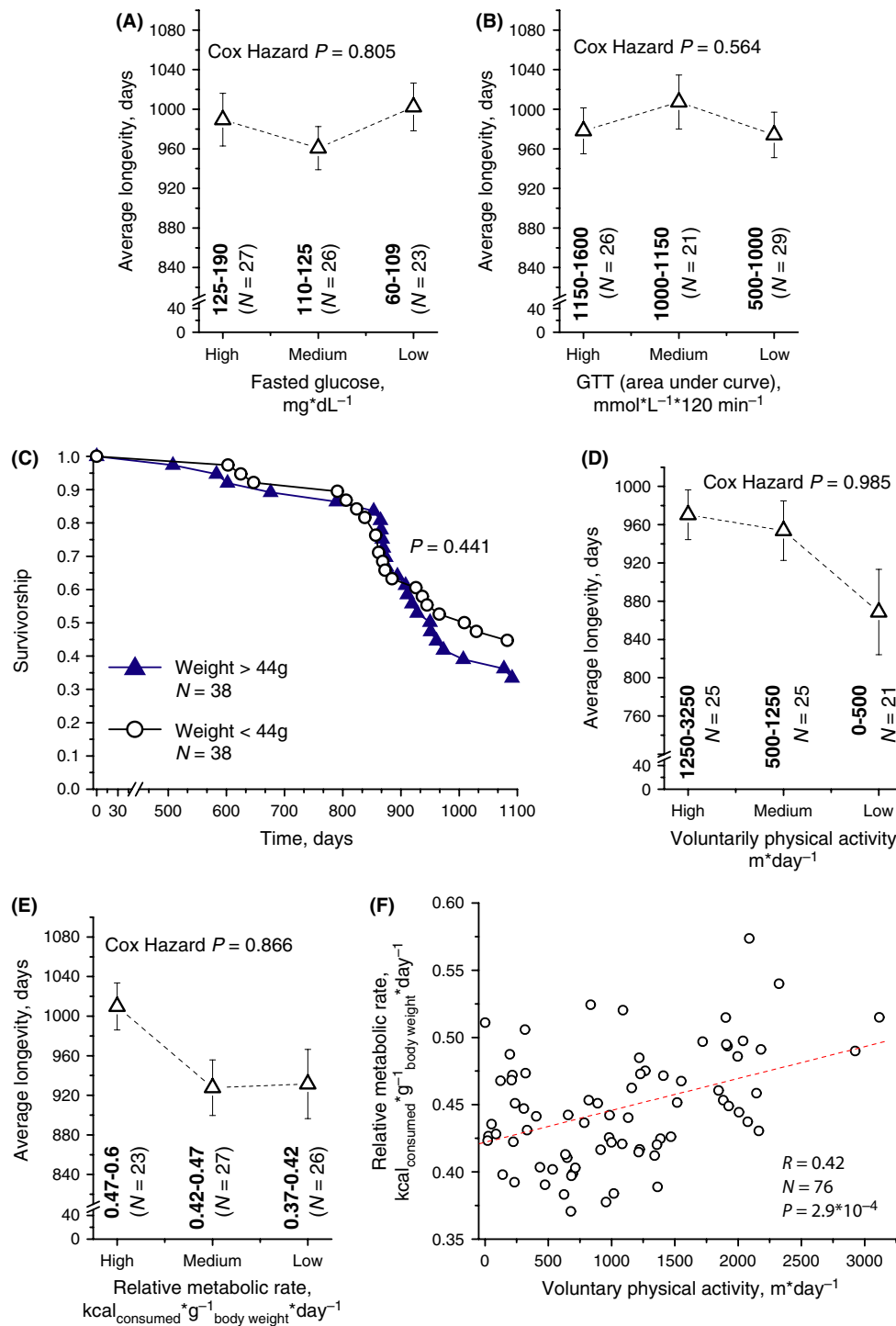
longer than 24 h (typical actograms are presented in Fig. 1E,F). The distribution of τ in this cohort of mice is rather broad and has a bell-like shape with its peak close to 24 h (Fig. 1G). After 30 days of circadian activity measurements, the light regime was returned to 12 h light/12 h dark, running wheels were removed from cages, and life spans of mice were monitored.

Fig. 3 Voluntary physical activity correlates with metabolic rate. (A) Fasted glucose levels do not correlate with longevity. We separated all the animals into three groups, according to their levels of blood glucose: high (125–190 mg dL^{-1}), medium (110–125 mg dL^{-1}), and low (60–109 mg dL^{-1}). Average longevity for these three groups is shown (±SEM). See also Fig. S2 for statistical analysis via Cox proportional hazard model. (B) Glucose tolerance does not correlate with longevity. We separated all the animals into three groups, according to their ability to absorb glucose in glucose tolerance test (GTT) as measured by the calculated area under the curve. Groups of animals with high area under the GTT curve (1150–1600 mM per 120 min), medium (1000–1150 mM per 120 min), and low (500–1000 mM per 120 min) were identified. Average longevity for these three groups is shown (±SEM). There are no statistically significant differences via *t*-test for all the possible pairwise combinations or ANOVA ($P = 0.62$). See also Fig. S2 for statistical analysis via Cox proportional hazard model. (C) Body weight of the animals does not strongly correlate with their life span. Median body weight of animals in our cohort was 44 g (body weight was measured at 1 year of age after overnight fasting). Survival curves for two halves are shown. Open circles represent lighter half of the cohort (body weight < 44 g, N = 38), and filled triangles represent heavier half of the cohort (body weight > 44 g, N = 38). There is no statistically significant difference in survival between these two groups ($P = 0.441$). (D) Animals with low voluntary physical activity tend to have shorter life spans. We separated the animals into three groups, according to their free-running activity: highly active individuals travelled from 1.25 to 3.25 km per day, individuals with moderate activity from 500 to 1250 m per day, and low activity from 0 to 500 m per day. Animals with higher activity had on average longer life spans ($P = 0.041$). However, Cox proportional hazard analysis did not reveal linear relation between physical activity and longevity (see also Fig. S2). (E) Animals with higher metabolic rate (as measured by calories consumed per gram of body weight per day) tend to have longer life spans ($P = 0.030$). We separated all the animals into three groups, according to their metabolic rate. Individuals with high metabolic rate (those that consumed from 0.47 to 0.6 kcal per gram of body weight per day), moderate metabolic rate (0.42–0.47), and low metabolic rate (0.37–0.42). Average longevity for these three groups is shown (±SEM). Animals with high metabolic rate had on average greater longevity then moderate or low metabolic rate animals. However, Cox proportional hazard analysis did not reveal a linear relation between metabolic rate and longevity (see also Fig. S2). (F) There is a strong correlation between voluntary physical activity and metabolic rate. Individuals with higher relative metabolic rate also tend to be the individuals with higher voluntary physical activity ($P = 2.9 \times 10^{-4}$). Scatter plot for these two values is shown. Each point represents an individual animal. This correlation shows that longevity increases shown on panels (D and E) are linked.

Ample data from simpler organisms suggested that deviation of the circadian cycle from 24 h might be detrimental for health and longevity. We decided to test this hypothesis. For each individual in our study, we computed a $\Delta\tau$ value, which is the deviation of that individual's innate circadian period from 24 h. We then asked whether animals with larger absolute deviations experienced significantly reduced life span compared to animals with deviations closer to zero. We did this

by splitting our single experimental cohort into two sub-cohorts based on a specific cutoff value of $|\Delta\tau|$. The first sub-cohort would be comprised of individuals whose innate circadian cycle deviated slightly from 24 h ($|\Delta\tau|$ < cutoff value), and the second sub-cohort was comprised of individuals whose innate circadian cycle exhibited greater deviation from 24 h ($|\Delta\tau|$ > cutoff value). Rather than choose our cutoff value *a priori*, we systematically assessed the difference in longevity between

these two sub-cohorts using a range of different cutoff values, where Cox Regression and Wald statistics were applied to quantify the statistical significance of the longevity difference (Fig. 1H). We found that a cutoff value of 7 min resulted in the two cohorts with the largest difference in life span and greatest Wald statistic. Indeed, the survivorship curves from these two sub-cohorts are strikingly divergent and reveal a statistically significant effect of tau deviation ($P = 0.0047$, Fig. 1H,I, and Table S1). Note that dividing the animals into two groups with shorter or longer than 24-h periods showed no significant differences in longevities between groups (Fig. 1J and Table S2). To test the relationship between $|\Delta\tau|$ and survivorship in greater detail, we treated $|\Delta\tau|$ as a continuous variable and performed Cox proportional hazard model analysis (Fig. S1). We found that $\Delta\tau_3$ was a significant predictor of mortality risk ($P = 0.00054$), while the linear and quadratic coefficients were not significant. These relationships were essentially unchanged following the inclusion of other physiological traits measured in this study as additional covariates in multi-covariate Cox hazard model (Fig. S1).

These data show that mice with innate periods close to 24 h enjoy greater longevity, and that epigenetic modifications or other processes that influence innate circadian rhythm may also affect the rate of aging.

It was of interest to determine whether other physiological parameters were impacted by the deviation of innate circadian period from 24 h ($\Delta\tau$). We performed this analysis in two ways. First, we used regression analysis to examine the linear relationship between $\Delta\tau$ and the value of interest, for example weight (Fig. 2A). This analysis revealed that heavier individuals tended to have longer innate circadian periods, or greater $\Delta\tau$ ($R = 0.27$ for linear fit, $P = 0.018$). Indeed, the sub-cohort consisting of individuals with $\Delta\tau < 0$ (i.e., $\tau < 24$ h) has a higher average weight than the sub-cohort that consists of individuals with $\Delta\tau > 0$ (i.e., $\tau > 24$ h, Fig. 2B, $P = 0.0004$). A summary of the regression and binning analyses for all of the parameters measured in this study reveals significant association between τ and weight as well as between τ and metabolic rate. The association between τ and physical activity was modestly significant, while we found no association with either fasted glucose levels or response to a glucose tolerance test (GTT) (Fig. 2C).

Second, we investigated the impact of other individual characteristics, which were previously reported to be associated with longevity, at the population level. Alterations in insulin signaling and glucose homeostasis have been associated with longevity in numerous species, including mammals (Bartke, 2011). We measured fasted glucose levels of individual animals as well as their response to glucose injection (GTT) and found no correlation between longevity and fasted glucose levels (Fig. 3A), or between longevity and the ability to handle glucose in a GTT (Fig. 3B). These factors also had little effect on organism life span, as assessed by Cox proportional hazard analysis (Fig. S2).

Across different species, animals with larger body size tend to live longer (Speakman, 2005), perhaps because they have smaller surface to volume ratio, which can support lower metabolic rates. There are a number of outliers for this observation, such as bats, which despite extremely high metabolic rate, enjoy lengthy life span. Paradoxically, within a species, smaller individuals tend to have enhanced longevity, perhaps because of decreased levels of IGF and growth hormone (Bartke, 2000; Harper *et al.*, 2006). We analyzed the effect of body weight on life span of individual animals in our cohort and found no significant difference (Fig. 3C and Table S3), although a trend for increased longevity of lighter animals was evident. Specifically, the average longevity of the lightest 38 animals (~50th percentile) was not statistically different from the heavier half of the cohort ($P = 0.439$). Cox propor-

tional hazard analysis did not support a significant impact of weight on longevity (Fig. S2).

It is generally agreed that exercise improves health and has the ability to extend longevity. Rats that had the ability to exercise enjoyed extended longevity when compared to their sedentary littermates (Holloszy & Schechtman, 1991). Recently, it has been shown that physical exercise is correlated with reduced mortality and extended longevity in humans (Wen *et al.*, 2011). However, despite numerous improvements of health characteristics, exercise did not extend life span in mice (Samorajski *et al.*, 1985). While we found a trend in the association between voluntary physical activity and longevity, individuals with high voluntary physical activity (1.25–3.25 km day^{-1}) benefited from longer life spans than their less active (0–0.5 km day^{-1}) siblings (Fig. 3D), Cox proportional hazard model did not support a statistically significant impact of physical activity on longevity (Fig. S2).

Likewise, whereas there was only a trend of correlation between higher metabolic rates and increased longevity (Fig. 3E), there was also a strong correlation (Fig. 3F, $R = 0.42$, $P = 2.9 \times 10^{-4}$) between the ability of the animal to exercise and their metabolic rate, indicating that high metabolic rates and heightened ability to exercise are not independent parameters.

Discussion

In summary, we investigated the longevity variation within a genetically homogeneous cohort of mice and found that individual life spans correlate with animal's ability to maintain a circadian period close to 24 h. This observation adds to our knowledge base relating the circadian clock to the rate of aging in mammals and extends observations previously made in simpler organisms. Our findings also show trends for correlations between physical activity, metabolic rates and longevity, but these are not nearly as strong as that between longevity and innate circadian period.

Why do animals with innate periods close to 24 h have longer life spans? An intriguing possibility is that synchrony between the intrinsic circadian period and the externally cued light cycle is advantageous. Animals with intrinsic periods significantly different from 24 h must reset their clocks daily in accord with the light cycle. This resetting itself may impose a metabolic stress that increases the rate of aging. An interesting prediction of this model is that placing individuals in an all-dark environment for the duration of their lives would normalize the differences in life spans.

Another interesting question is whether animals with a circadian period longer than 24 h and those with circadian period shorter than 24 h have shortened life spans for similar reasons. Our data suggest that reasons for shorter longevities are different for these two groups of animals. A number of physiological parameters, such as body weight, appear to be different between these two groups of animals. For instance, animals with $\tau > 24$ h weigh on average more than animals with $\tau < 24$ h (Fig. 2A,B). One can imagine that high body weight in animals with long periods might be deleterious to health and longevity because of obesity-induced inflammation. Conversely, low body weight in animals with short periods might be a signal of a health problem or require a very high metabolic rate to sustain normal temperature thereby taxing the organism with high ROS production.

It will be intriguing to determine whether any of our findings in these F1 hybrid mice apply to humans, which might suggest that strategies to match innate and extrinsic circadian periods would slow aging in at least some people. In this regard, it is interesting to note that melatonin, a drug that resets circadian rhythm (often used to alleviate jet lag), is also shown to increase longevity in simpler organisms (Bonilla *et al.*, 2002) and

mice (Anisimov *et al.*, 2001). This is one of many possibilities of how adjustment of innate circadian rhythm could be beneficial for improving health and longevity.

Experimental procedures

Animals

All procedures were performed according to guidelines and under supervision of Committee for Animal Care (CAC) of Massachusetts Institute of Technology.

Only males were used in this study. Seventy-six CB6F1 (F1 progeny of BALB/cBy and C57BL/6 cross) 1-year-old males were purchased from NIA, NIH (http://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook/available-strains). Animals were individually housed for the duration of life.

Diet

Ad libitum standard mouse chow (AIN-93G standard diet) and drinking water were provided for the animals, except 1 day before GTT (described below) and 1 day before 1-year-old body weight measurement (described in main text).

Running-wheel activity

At 14 months of age, low profile running wheels (Med Associates Inc., St. Albans, VT, USA) were introduced into each cage of singly housed mice. Each wheel was equipped with magnetic rotation counter, which was electronically monitored by a computer using PCI Input/output board (PCI-DIO-120; Access I/O products Inc., San Diego, CA, USA). Activity of mice was monitored for 20 days on 12 h dark: 12 h light regimes, after which activity of mice was monitored for 30 days at 24-h dark. During dark phase of the experiment, double black-out doors were used, so that veterinary personal could enter the mouse housing facilities without introducing ambient light. All mouse handling (during cage changes) was done using dim (<15 lux as measured by handheld light flux meter, VWR) deep red light (rodents have dichromatic vision and do not see red light, Szel *et al.*, 1992). All the procedures that required humans to enter mouse facility were scheduled at random times of the day, so that no pattern could be established by experimental animals. After 50 days, running wheels were removed from the cages and lighting was returned to 12:12 schedule.

Statistical analysis

Free-running period (tau, or τ) was estimated using LSP procedure (Van Dongen *et al.*, 1999) using script written by Dr. Refinetti (ver. 2.7, http://www.circadian.org/main.html). Statistical analyses were conducted in R (version 2.13.1) using the 'survival' package.

Glucose tolerance test

Glucose tolerance test was performed as described (Andrikopoulos *et al.*, 2008) at 14 months of age. Briefly, after overnight, fast basal glucose was determined by using a glucometer (Lifescan; Jonson & Jonson, New Brunswick, NJ, USA) in blood obtained by removing the tip of the tail. Glucose (Sigma-Aldrich, St. Louis, MO, USA) was injected i.p. at 2 mg kg^{-1} of body weight. Blood was subsequently sampled at 15, 30, 60, 90 and 120 min thereafter for glucose measurements.

Relative metabolic rate

Relative metabolic rate is a value calculated from individual's food consumption and weight. It reflects the amount of calories consumed per gram of weight per unit of time, when food is provided *ad libitum*.

Longevity

Animals in this longevity study were checked daily for health and survival and were minimally handled for cage changes and measurements described in this study. Animals that appeared to be near death (listless, unable to walk, and cold to the touch) or had large bleeding tumors or neoplastic growth approaching 10% of body weight were euthanized, and the date of euthanasia was considered the date of death.

Author contributions

S.L., M.S.B., and L.G. designed the experiments; S.L., M.S.B., and K.P. performed the experiments; S.L., M.S.B., and S.D.P. analyzed the data; S.L., M.S.B., S.D.P., and L.G. wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Deviation of the innate circadian period Dτ correlates with reduced longevity of mice.

Fig. S2 Metabolic rate, physical activity, weight, and glucose handling parameters do not linearly related to the hazard via Cox proportional model.

Table S1. Raw data for the survival experiment on main Fig. 1I is presented.

Table S2. Raw data for the survival experiment on main Fig. 1J is presented.

Table S3. Raw data for the survival experiment on main Fig. 3C is presented.

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