The Circadian Clock Gates the Intestinal Stem Cell Regenerative State

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http://dx.doi.org/10.1016/j.celrep.2013.03.016

SUMMARY

The intestine has evolved under constant environmental stresses, because an animal may ingest harmful pathogens or chemicals at any time during its lifespan. Following damage, intestinal stem cells (ISCs) regenerate the intestine by proliferating to replace dying cells. ISCs from diverse animals are remarkably similar, and the Wnt, Notch, and Hippo signaling pathways, important regulators of mammalian ISCs, are conserved from flies to humans. Unexpectedly, we identified the transcription factor *period*, a component of the circadian clock, to be critical for regeneration, which itself follows a circadian rhythm. We discovered hundreds of transcripts that are regulated by the clock during intestinal regeneration, including components of stress response and regeneration pathways. Disruption of clock components leads to arrhythmic ISC divisions, revealing their underappreciated role in the healing process.

INTRODUCTION

Although many pathways that are required for healing have been discovered, little is known about how or whether healing is synchronized with general processes that regulate an animal’s homeostasis and behavior. The circadian clock is an ancient molecular pathway that synchronizes organisms with daily environmental cues (zeitgebers) such as light intensity and temperature oscillations (Borgs et al., 2009; Hardin, 2011). Circadian rhythms are repeated over a 24 hr cycle, yet this chronological aspect of cell state has received little attention in the field of regenerative biology. For instance, many of the pathways that regulate intestinal regeneration and intestinal stem cells (ISCs) have been the subject of important studies (Biteau et al., 2011; Casali and Batlle, 2009), but most of these studies did not consider whether results obtained during one part of the day occur at all times.

Circadian rhythms are thought to influence the cell cycle (Borgs et al., 2009), and there is some evidence that the clock plays a role in regeneration and proliferation. Hepatocyte cell division exhibits rhythms and is delayed following hepatectomy if circadian rhythms are disrupted (Matsuo et al., 2003). Earlier studies in the intestine indeed found a daily rhythmicity in cell number and villus length (Qiu et al., 1994; Stevenson et al., 1979), as well as proliferation (Al-Nafussi and Wright, 1982; Potten et al., 1977), although clock mutants were not examined and ISCs were not specifically identified in those reports. Further, it was reported that metabolic processes display time-of-day variation in cancer patients treated by radiation (Shukla et al., 2010). This suggests that circadian rhythms may influence the intestine’s regenerative response, although the reasons for this remain a mystery.

RESULTS

The *Drosophila* Intestine Has a Circadian Clock

The intestinal biology of *Drosophila* parallels that of mammals (Biteau et al., 2011; Casali and Batlle, 2009) and allows for functional in vivo analyses to elucidate regenerative processes. *Drosophila* ISCs divide to produce progenitors called enteroblasts (EBs) that differentiate directly into absorptive enterocytes (ECs) or secretory enteroendocrine cells (Figure 1A). We performed a transgenic RNAi screen for transcription factors required in *Drosophila* ISCs during regeneration (see Experimental Procedures). It was previously shown that after damage occurs, ISCs regenerate the intestine by proliferating to replace dying cells (Biteau et al., 2011; Medema and Vermeulen, 2011). Here we discovered that among the ~600 genes tested, *period* (*per*) was required for proliferation of adult ISCs following damage by dextran-sodium sulfate (DSS), a chemical that models inflammatory bowel diseases in flies and mice (Amchislavsky et al., 2009).

The *Drosophila* circadian pacemaker comprises the transcription factor partners *clock* (*clk*) and *cycle* (*cyc*), which are negatively regulated by *per* and *timeless* (*tim*; Hardin, 2011). One transcriptional target of CLK/CYC is *per* itself, which represses its own production and causes the cyclical transcriptional rhythms that underlie circadian rhythms. The existence of independent clocks throughout *Drosophila* tissues is known (Plautz et al., 1997), and we confirmed the cyclical accumulation and loss of *per* in the intestine when flies were kept on a 12 hr light/12 hr dark (LD) regimen (all of the experiments described below were performed under LD and chemical damage unless...
otherwise noted). Quantitative RT-PCR (qRT-PCR) confirmed that per mRNA accumulates in the early evening (zeitgeber time 12–18 [ZT12-18]; Figures 1B and S1A), and staining for PER confirmed its nuclear accumulation in the late night/early morning (Figure 1C, ZT0). PER is expressed in the epithelial cells of this tissue (the polyploid ECs as well as the diploid ISCs; Figures 1D and S1).

**The Clock Gene per Regulates Rhythmic Intestinal Regeneration**

The per01 allele is a loss-of-function nonsense mutation (Hardin et al., 1990). Although they are viable, per01 mutant animals do not exhibit circadian gene expression or behavioral rhythmicity (Figures 1B, S1, and S2). We assayed the regenerative response of per01 ISCs following damage by DSS. Only the ISCs in the
Drosophila intestine divide (Ohstein and Spradling, 2006), and mitotic ISCs were scored by phosphorylated histone H3 positivity. Control (ry

A UAS-per transgene, which restores circadian rhythms behaviorally when expressed in pacemaker neurons (Figure S2), partially restored the mitotic peak in per

ISCs divide according to a circadian rhythm in response to damage (Figures 2A and 2B). The expression of a UAS-cyc transgene in ISCs (esg-Gal4) in the cyc

When flies are exposed to light-only (LL) conditions, the rhythmic rhythms observed. Light levels entrain the circadian clock, and the loss of CYC in either ISCs (Figure 2E) or ECs (Figure 2F) abolished any rhythms observed. Light levels entrain the circadian clock, and when flies are exposed to light-only (LL) conditions, the rhythmic nature of mitoses is abolished and remains constant at all time points (Figures S5E–S5G). Altogether, these data confirm that the circadian clock is required in both ISCs and their EC neighbors for mitotic rhythms.

Bleocin is a potent DNA-damaging chemical that causes apoptosis in the intestine (Amcheslavsky et al., 2009), and it was applied to investigate the outcome of a circadian-deficient damage response. Following Bleocin-induced damage, mitoses in control versus cyc

Next, we generated per-deficient mutant clones to test whether the defect associated with PER loss was cell autonomous. Following damage, per

The Core Clock Functions during Intestinal Regeneration

Because per and tim work together to inhibit clk/cyc, the outcomes of CYC activity would be expected to oppose those of PER. The cyc

damage (Figures 2A and 2B). The expression of a UAS-cyc transgene in ISCs (esg-Gal4) in the cyc

The accumulation of mitotic cyc

We applied the FUCCI cell-cycle reporter (Nakajima et al., 2011; Sakae-Sawano et al., 2008), which accumulates mAG-Geminin during S/G2/M phases (Azami Green positive), to determine cell-cycle states when circadian rhythms are absent in ISCs. We expressed the FUCCI reporter along with cyc RNAi or per RNAi with esg-Gal4, and identified ISCs using Di+. The control RNAi lines show a gradual accumulation of S/G2/M-phase-positive ISCs up to ZT18, when these cells divide.
Figure 2. The Circadian Clock Is Required in the Damaged Intestine

(A and B) When flies are maintained in LD conditions, control (ry506 and y,w) intestinal mitoses peak at ZT0, in contrast to cyc0 and tim0 mutants. A UAS-cyc construct expressed in ISCs (esg-Gal4) partially restores this rhythm in the cyc0 background. ry506 data are duplicated from Figure 1 E.

(C and D) per01; tim0 double-mutant intestines resemble the per01 mutant phenotype. per01; cyc0 double-mutant intestines resemble the cyc0 mutant phenotype. Control and mutant data are duplicated from Figures 1 E, 2A, and 2B.

(E and F) CYC knockdown in ISCs (esg > cyc RNAi is esg-Gal4/+; UAS-dcr2/UAS-cyc RNAi) or in ECs (myo1A > cyc RNAi is myo1A-Gal4/+; UAS-dcr2/UAS-cyc RNAi) disrupts circadian mitotic rhythms. Control data are from Figures 1 F and 1G. All graphs show the average of two separate experiments (n = 10 guts/genotype/time point, error bars ± SEM, *p < 0.05 at ZT0).

(G) Following Bleocin exposure, control (ry506) intestinal mitoses peak at ZT0, in contrast to per01 and cyc0, similarly to what happens following DSS damage.

(H and K) The survival rates of all circadian clock mutants as well as animals in which PER or CYC was knocked down by RNAi in either ISCs or ECs are reduced compared with controls on Bleocin (black lines). Graphs show representative experiments (n = 3 vials, 15 flies per vial; genotypes as above). See also Figures S1, S2, S3, and S5.
(Figures 3C and 3D). However, not all ISCs are in S/G2/M phases, indicating that a significant reserve population of ISCs exists at all times. Irrespective of time, nearly all cyc RNAi ISCs are S/G2/M phase negative, whereas nearly all per RNAi ISCs are positive. Because its loss causes ISCs to accumulate in G1 (or G0), these results suggest that CYC promotes the G1 to S phase transition. Conversely, when PER is lost, movement through G1 is unopposed, but ISCs accumulate after S phase entry without entering mitosis (see Figure 1F). Thus, we propose that the circadian clock regulates the G1 to S phase transition in ISCs following damage.

The Clock Regulates the Transcription of Hundreds of Genes in the Intestine

More than 10% of all mammalian genes are regulated in a circadian fashion (Panda et al., 2002), and components of the clock directly regulate transcription in a tissue-specific manner (Abruzzi et al., 2011; Akhtar et al., 2002), suggesting that a tremendous variety of cell states are outcomes of circadian processes. Since per RNA and protein oscillate in the midgut, and per was identified in our screen, we performed genome-wide expression analysis on ry506 control intestines and cyc0 mutants over 24 hr following damage (Figure 4A; Tables S1, S2, and S3). We reasoned that clock target genes would show 24 hr rhythms and would be perturbed if CLK/CYC were disrupted. We found that 433 genes were rhythmic in controls, like per, but arrhythmic in cyc0, indicating that they are under clock regulation in this tissue (Table S1). For instance, Connector of kinase to AP-1 (Cka), a scaffold protein required for signal transduction of the JNK stress-response pathway (Chen et al., 2002), peaks at ZT15 (Figure 4B). Direct CLK/CYC targets would be expected to be strongly reduced in cyc0 mutants, yet only 21 of 433 genes (including per and tim) fit this profile (Table S2); hence, most rhythmic genes are likely to be indirectly regulated. Two hundred rhythmic genes showed the opposite phase to that of per, suggesting they are regulated by the transcription factors vrille or
**Figure 4. The Clock Regulates the Expression of Diverse Transcripts**

(A) All genomic transcripts were interrogated for rhythmic expression during regeneration. Heat maps reveal 433 genes with circadian rhythms in \( ry^{006} \) controls but not in \( cyc^{0} \) mutants.

(B) \( Cka \), \( Ipk2 \), and \( Kmn1 \) RNA expression (qPCR) in the intestine over 24 hr. \( Cka \) shows per-like rhythms, whereas \( Ipk2 \) exhibits antiphase rhythms. \( Kmn1 \) displays no circadian rhythmicity but is significantly downregulated in the \( cyc^{0} \) mutant. Graphs are reported as in Figure 1B.

(C) Flies maintained in LD conditions on regular media do not show a mitotic peak at ZT0, in contrast to when the intestine is damaged. Under these conditions the mitotic index is similar between \( ry^{006} \) controls and \( cyc^{0} \) or \( per^{01} \) mutants.

(D) In the absence of damage, the expression of \( Cka \) and \( Ipk2 \) (qPCR) is rhythmic, similar to what is observed during regeneration. \( Kmn1 \) (qPCR) also shows lower expression both before and after damage.

(E) A model of how the clock synchronizes ISC division: CYC is important for the transition through G1, and the clock also initiates systemic signals and local niche signals originating from ECs. Together, these signals activate ISC divisions, most likely through nonautonomous mechanisms.

See also Figures S2, S3, and S5.

**Pdp1**, which are part of the clock and together generate antiphase transcript rhythms that peak in the early day (Hardin, 2011; Table S1). One of these, \( Ipk2 \), is an inositol phosphate kinase and a positive regulator of Jak/STAT signaling (Müller et al., 2005), a pathway that is critical during intestinal regeneration (Figure 4B). Another one of these genes, bazooka, was recently
reported to polarize ISCs (Goulas et al., 2012), suggesting that the clock also regulates cell polarity. An additional 205 genes showed low expression in cyc0 mutants but did not display rhythms (Table S3). This includes Kmn1, which enables chromosome segregation during anaphase (Venkei et al., 2011), suggesting that mitosis could be disrupted (Figure 4B). Overall, a great diversity of intestinal transcripts are thus influenced by the clock.

**DISCUSSION**

Circadian pathway mutants are viable and their cells readily proliferate during development. Unlike other tissues (Abruuzzi et al., 2011; Borgs et al., 2009), cell-cycle regulators do not seem to be clock targets in the intestine (Table S1). Although they are readily detected, neither cyclins nor regulators such as Wee1 (Matsuo et al., 2003) exhibit circadian rhythms in this tissue. In the absence of acute damage, clock mutant ISCs divide normally (Figure 4C) and have no ISC-autonomous phenotypes (Figure S4). So it is quite surprising that PER and CYC are critical for adult ISC division during regeneration.

The ISC-autonomous phenotypes that occur during regeneration are modest compared with those that arise when the clock is cyc0 of ISCs in different cell states; for instance, the circadian clock in different cells leads to the accumulation of the clock regulates EB-to-ISC signaling. Intriguingly, disruption in their circadian rhythm activity (Janich et al., 2011), for unknown reasons. The coordination of proliferation, by synchronizing internal with external rhythms, may thus represent an important difference between normal stem cells and neoplastic cells.

**EXPERIMENTAL PROCEDURES**

Animals were maintained at 25°C under LD conditions and damaged by being fed SSS (MP Biomedicals) or 25 μg/mL Bleomycin (Calbiochem). The flies were maintained under LD conditions as before, except for experiments in which the light conditions were changed to complete darkness or complete light. Female flies < 14 days of age were used in all experiments, with the exception of the mosaic analysis. The following Drosophila lines were used:

- OreR
- ry06
- y, w
- cyc0, ry06
- per01, ry06
- per01; tim0; ry06
- per01; cyc0, ry06
- y, w; tim0
- UAS-per16
- UAS-cycl6
- esg-Gal4
- esg-Gal4, UAS-eGFP, tub-Gal80TS
- myo1A-Gal4
- laim-Gal4
- hsFlp, FRT19A, tub-Gal80; act < y; < Gal4, UAS-GFP / CyO hsFlp; act < CD2 > Gal4, UAS-nlsGFP / CyO w; UAS-dcr2 (II)
- w; UAS-dcr2 (III)
- UAS-S/G2/M-Green / CyO
cyc RNAi (National Institute of Genetics #8727R-1, Mishima, Shizuoka, Japan)
- per RNAi (TRIP #JF01226, Harvard Medical School, Boston, USA)
- Luc RNAi (TRIP #JF01355, Harvard Medical School, Boston, USA).

Full details regarding the procedures are provided in Extended Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures, three tables, and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.03.016.

**LICENSING INFORMATION**

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ACKNOWLEDGMENTS

Stocks and antibodies were provided by Drs. Paul Hardin, Michael Rosbash, and Stephen Hou, and the Bloomington Drosophila Stock Center. We thank members of the Perrimon laboratory, particularly Richard Binari and Akhila Rajan, for their assistance. This work was supported by the Human Frontier Science Program (P.K.) and the Harvard Stem Cell Institute. P.E. is supported by NIH grants GM66777 and GM79182. N.P. is an investigator of the Howard Hughes Medical Institute.

Received: November 21, 2012
Revised: March 11, 2013
Accepted: March 12, 2013
Published: April 11, 2013

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