The Circadian Clock Gates the Intestinal Stem Cell Regenerative State

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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 (Borges 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 (Borges 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 the intestine (Pan and Hussain, 2009; Saito et al., 1976; Scheving, 2000), and per mutation hastens tumorigenesis in Wnt pathway-driven colorectal cancer in mice (Wood et al., 2008). Finally, the degree of intestinal mucositis displays time-of-day variability 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 RNA 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 (Amcheslavsky 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

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**Figure 1. PER Cycles and Functions in the Damaged Intestine**

(A) The ISC lineage. ISC, intestinal stem cell; EB, enteroblast; ee, enteroendocrine cell; EC, enteroocyte.

(B) per RNA expression (qPCR) in the intestine over ZT, with ZT0 denoting when lights are turned on. The ry506 control normally shows circadian rhythms, but these are absent in per01 mutants. Graph shows the average of two separate experiments (n = 15 guts/genotype/time point, expression normalized to ry506 ZT15, relative to GAPDH RNA; error bars = SEM).

(C) PER staining (red) shows nuclear accumulation in intestinal cells in the morning (ZT0) versus the evening (ZT12). Fibrillarin (green) marks the nucleolus, where PER is weaker.

(D) PER protein levels are rhythmic in ISCs (arrows) labeled with Delta (Dl, red).

(E) When flies are maintained in LD conditions (see Figure S1C for schematic), control (ry506) intestinal mitoses peak at ZT0, in contrast to per01. A UAS-per rescue construct expressed in ISCs using esg-Gal4 rescues this effect partially in the per01 background.

(F) Rhythms are present in Luciferase (esg > Luc RNAi is esg-Gal4/+; UAS-dcr2/UAS-Luc RNAi) controls, but PER knockdown in ISCs (esg > per RNAi is esg-Gal4/+; UAS-dcr2/UAS-per RNAi) phenocopies per01.

(G) PER knockdown in ECs also disrupts circadian mitotic rhythms (genotypes as above but with myo1A-Gal4/+).

See also Figures S1, S2, S3, and S5.
Drosophila intestine divide (Ohlstein and Spradling, 2006), and mitotic ISCs were scored by phosphorylated histone H3 positivity. Control (y^P05) ISCs show a peak in mitoses occurring at dawn (Figure 1E, ZT0), the transition between night and day when PER accumulates. This peak is absent in per^P11 intestines, which show reduced mitoses at all time points (Figure 1E). A UAS-per transgene, which restores circadian rhythms behaviorally when expressed in pacemaker neurons (Figure S2), partially restored the mitotic peak in per^P1 when expressed in ISCs using esg-Gal4 (Amcheslavsky et al., 2009), but not in ECs using myo1A-Gal4 (Jiang et al., 2009; Figure 1E). Importantly, the esg-Gal4 and myo1A-Gal4 drivers are not expressed in pacemaker neurons, and do not rescue per^P1 arrhythmic behavior when driving UAS-per (Figure S2). A characteristic of circadian rhythms is their free-running nature (Hardin, 2011), which we tested by shifting flies to constant darkness (DD) after LD entrainment. PER expression rhythms and intestinal mitotic rhythms perpetuate in DD, demonstrating their circadian nature (see Figures S1F and S5A–S5C). Together, these results show that ISCs divide according to a circadian rhythm in response to damage, and that this response is per dependent.

Undamaged per^P1 intestines do not show obvious deficiencies in epithelial cell types (Figures S3A and S3B) or rhythmic mitoses (see Figure 4C). Both ISCs and ECs participate in regeneration (Biteau et al., 2011), raising the question as to which cells are responsible for the inability of per^P1 intestines to display mitotic rhythms. A second important question is whether mitotic rhythms in response to damage are linked to behavioral activity or feeding (Xu et al., 2008). We validated a UAS-per RNAi construct for its ability to reduce PER expression and abolish circadian behavior rhythms (Figure S2). PER knockdown in ISCs phenocopied the arrhythmic per^P1 intestine (Figure 1F) and, strikingly, PER depletion in ECs also abolished ISC proliferation rhythms (Figure 1G). These phenotypes are not correlated with circadian behavior (Figure S2) or feeding (Figures S3C and S3D), which are rhythm (although we do note an ~1 hr circadian period lengthening in the esg-Gal4 driver). Since only ISCs divide in this tissue, per RNAi disruption in ISCs (Figure 1F) accounts for the per^P1 phenotype (Figure 1E), whereas per RNAi in ECs simply abolishes a peak at ZT0 (Figure 1G). These results suggest that PER is required separately in both ISCs and ECs to produce intestinal mitotic rhythms, and that these rhythms are separate from feeding and behavioral rhythms.

Next, we generated per-deficient mutant clones to test whether the defect associated with PER loss was cell autonomous. Following damage, per^P1 and per RNAi clones are slightly smaller (Figure S4) and show reduced size over long periods of time in the absence of acute damage. This suggests that PER has a weaker ISC-autonomous role in initiating or boosting proliferation following damage or stress, but that overall a stronger nonautonomous role is predominant.

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^c and tim^c loss-of-function mutants are also viable, and also display altered intestinal mitotic rhythms in response to damage (Figures 2A and 2B). The expression of a UAS-cyc transgene in ISCs (esg-Gal4) in the cyc^c background was able to partially rescue this phenotype, but expression in ECs (myo1A-Gal4) did not (Figure 2A). Although the cyc^c phenotype is the opposite of the per^P1 phenotype, we note that the tim^c phenotype is not the same as that of per^P1, suggesting that tim may have additional functions in this tissue. It is also possible that genetic background plays a role in the level of mitoses observed in these conditions. We tested the epistatic relationships between these genes. The per^P1;tim^c double mutant displays the per^P1 phenotype (Figure 2C), and the cyc^c;per^P1 double mutant displays the cyc^c phenotype (Figure 2D), as would be predicted from the circadian clock transcriptional feedback loop, which undergoes circadian rhythms in this tissue (Figure S1). We further tested the requirement of CYC in the regenerative process by expressing a functionally validated UAS-cyc RNAi construct (Figures S2 and S3) in ISCs and ECs. CYC is required in both of these cell types to produce mitotic rhythms, 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). Together, 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^c and per^P1 mutant flies show phenotypes similar to those observed under DSS (Figure 2G). The cyc^c mutants exhibit reduced survival on Bleocin (Figure 2H) or DSS (Figure S5), and per^P1 and tim^c show similar reduced survival (Figures 2H, 2I, and S5). The knockdown of CYC or PER within ISCs or ECs results in reduced survival on Bleocin (Figures 2J and 2K). Hence, the disruption of the circadian clock either throughout the body or only in ISCs or ECs negatively impacts the survival of animals when the intestine is damaged.

Clock-Deficient ISCs Lag in the Cell Cycle during Regeneration

The accumulation of mitotic cyc^c ISCs (Figure 2A) suggests that loss of cyc throughout the animal causes ISCs to overproliferate or stalls these cells in mitosis. An EdU uptake assay, which measures cells in S phase, revealed that control (y^P05) ISCs show a peak in S phase at ZT6. The cyc^c and per^P1 mutants do not exhibit any peaks, and cyc^c mutants do not exhibit increased S phase (Figures 3A and 3B). Hence, it is unlikely that cyc^c ISCs overproliferate, and cyc RNAi clones also did not show an overproliferation phenotype (Figure S4).

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 Dl+. 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 (ry<sup>506</sup> and y,w) intestinal mitoses peak at ZT0, in contrast to cyc<sup>0</sup> and tim<sup>0</sup> mutants. A UAS-cyc construct expressed in ISCs (esg-Gal4) partially restores this rhythm in the cyc<sup>0</sup> background. ry<sup>506</sup> data are duplicated from Figure 1E.

(C and D) per<sup>01</sup>; tim<sup>0</sup> double-mutant intestines resemble the per<sup>01</sup> mutant phenotype. per<sup>01</sup>; cyc<sup>0</sup> double-mutant intestines resemble the cyc<sup>0</sup> mutant phenotype. Control and mutant data are duplicated from Figures 1E, 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 1F 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 (ry<sup>506</sup>) intestinal mitoses peak at ZT0, in contrast to per<sup>01</sup> and cyc<sup>0</sup>, 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.
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 RNAi 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, and 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...
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 Kmr1, 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 (Abruzzi 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 (Matsumu 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 disrupted systemically or in all ISCs/ECs by RNAi. This suggests that the clock predominantly regulates nonautonomous functions and may be involved in the synchronization of cell states across this tissue during the damage response. Indeed, because esg-Gal4 is expressed in both ISCs and their immediate progeny (the EBs) for some time while they differentiate, it is possible that the clock regulates EB-to-ISC signaling. Intriguingly, disruption of the circadian clock in different cells leads to the accumulation of ISCs in different cell states; for instance, the cyc0 mutant stalls during mitosis when CYC is absent systemically (Figure 2A), whereas it stalls during G1 if CYC is depleted in all ISCs (Figures 3C and 3D). This G1 lag explains why cyc RNAi ISCs show reduced mitoses compared with the cyc0 mutant; however, given that the mechanisms underlying these processes are unresolved, it is possible that these differences are due to genetic background. At present, we thus conclude that rhythmic cell proliferation normally occurs in the damaged intestine and that this is dependent on the clock. We also note that forced expression of per or cyc in ISCs is able to partially restore rhythmic divisions in their respective mutant backgrounds (Figures 1E and 2A), whereas disruption of these genes in only ECs perturbs ISC rhythmic division (Figures 1G and 2F). This highlights the complexity of clock-regulated processes and suggests that desynchrony between ISCs and their surrounding cells (Figures S1G and S1H) can have different outcomes.

Circadian rhythms occur in many intertwined processes, including metabolism (Sahar and Sassone-Corsi, 2009), posttranscriptional regulation (Koike et al., 2012), and oxidation-reduction cycles (O’Neill and Reddy, 2011). The rhythmic expression of Cka, which brings together kinases and transcription factors to transduce JNK signal (Chen et al., 2002), and Ipk2, which may boost the activity of cytokines involved in regeneration (Müller et al., 2005), suggests that the clock sensitizes the intestine to engage the regenerative response at specific times. For instance, several of the genes that exhibit circadian rhythms during regeneration also show these rhythms prior to damage (Figure 4D). An emergent function of the clock could be to coordinate stem cell states according to either local niche signals or systemic signals, each of which would be under autonomous circadian control (Figure 4E).

Although per mutation increases cancer incidence (Borgs et al., 2009; Fu et al., 2002; Wood et al., 2008) and cancer cell proliferation (Borgs et al., 2009; Janich et al., 2011), our work suggests it is not simply a tumor suppressor. Recently, the circadian clock was shown to influence mammalian blood and hair stem cell biology (Janich et al., 2011; Méndez-Ferrer et al., 2008). In particular, hair stem cells are strikingly heterogeneous 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 5% w/v DSS (MP Biomedicals) or 25 μg/mL Bleocin (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
- ry006
- y, w
- cyc0, ry006
- perF1; ry006
- perF1; tim0; ry006
- perF1; cyc0, ry006
- y, w; tim0
- UAS-per16
- UAS-cyc6
- esg-Gal4
- esg-Gal4, UAS-eGFP, tub-Gal80TSA
- myo1A-Gal4
- tim-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-H-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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REFERENCES


Supplemental Information

EXTENDED EXPERIMENTAL PROCEDURES

Housing
Animals were maintained at 25°C, 12 hr light and 12 hr dark (LD) conditions, under constant humidity. For damage, a 5% w/v sucrose (Sigma) + water solution containing either 5% w/v DSS (MP Biomedicals) or 25 ug/mL Bleocin (Calbiochem) were applied for 2 days prior to timeseries analysis (2–3 days total exposure). Chemicals were refreshed daily, and flies were maintained on LD conditions as before, with the exception of experiments where the lights were changed to complete darkness or complete light (see Figures S1 and S5 for experimental schematics). Female flies of < 14 days of age were used in all experiments, except in the mosaic analysis where guts were obtained from flies 23 days following clone induction.

Dissection and Staining
Guts were dissected in 1X PBS (GIBCO) and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) diluted with 1X PBS. Samples were washed 3X with PBS, then blocked for 30 min in 1X PBS, 1% BSA (Sigma), 0.2% Triton X-100 (Sigma). The following antibodies were used: Rabbit anti-Period, Mouse anti-Delta (Developmental Studies Hybridoma Bank), Mouse anti-Prospero (Developmental Studies Hybridoma Bank), Mouse anti-Fibrillarin (Encore Biotechnology), Rabbit anti-phospho-Histone3 (Millipore). Samples were washed 3X with PBS, and stained with secondary antibodies: Donkey anti-mouse Alexa 555 (Molecular Probes), Donkey anti-mouse Alexa 647 (Molecular Probes), Goat anti-rabbit Alexa 488 (Molecular Probes), Donkey anti-rabbit Alexa 555 (Molecular Probes). EdU exposure was carried out for 45 min on dissected guts according to the manufacturer’s instructions, labeling was also performed according to the Click-IT EdU Alexa Fluor 555 Imaging Kit (Invitrogen) instructions. All samples were counterstained with DAPI (Molecular Probes) and mounted using Vectashield (Vector). For brain dissection, 2–5 day post-eclosion flies were entrained 3 days under LD conditions, and were stained as described in Zhang et al. (Zhang et al., 2010).

RNAi Screen
We performed a genetic screen for transcription factors required in Drosophila ISCs during regeneration, by expressing UAS-RNAi constructs (Ni et al., 2009) to suppress transcription factors/regulators, using a temperature-inducible esg-Gal4, tub-Gal80TS driver. Flies were raised at 18°C to prevent the expression of UAS-RNAi, then, at 2–5 days post-eclosion, shifted to 29°C for 8 days to induce expression in ISCs. Flies were exposed to 5% DSS for an additional 3 days, and guts were dissected and stained for phospho-Histone3 as described above. Total mitoses per gut were scored for each RNAi and compared to Luc RNAi controls, whose division is increased 10-20 fold from baseline in the presence of DSS. Two separate RNAi constructs for per were recovered as strong hits in the screen, suppressing division by at least 80%.

RNA Purification and qRT-PCR
15–20 midguts from each genotype were collected in RNAlater reagent (QIAGEN; n = 3 biological replicates). mRNA was isolated using a Oligotex Direct mRNA Mini Kit and/or RNA using the RNEasy Mini Kit (QIAGEN), and the iScript cDNA Synthesis Kit (Biorad) was used to transcribe cDNA. qPCR was then carried out on a CFX96 Real-Time System / C1000 Thermal Cycler (Biorad) with IQ SYBR Green Supermix (Biorad). Expression was normalized to GAPDH control transcript, then normalized relative to the appropriate control at ZT15. qPCR primers:

GAPDH1-F: CCAATGTCTCCGGTGGGA
GAPDH1-R: TCAGGTGAGCCAGAGGATT
per-F: TCATCCAGAAGCGTTGCTACG
per-R: CCTGAAAGACCGATGGGT
tim-F: CAGGATAGTTCAAAAGCAG
tim-R: GCAGGTGCGAAACTGTGTATG
Cka-F: aaggatatgcacagcaga
Cka-R: gcacatcatctctgattcacc
Kmn1-F: tcgctatgaagaacagtctt
Kmn1-R: cctcgtcctcgcagcagtta
Ipk2-F: atggcgcctccagcaggt
Ipk2-R: atgcagggcggttagtgt

Microarray Analysis
n = 20 midguts, in duplicate, were collected every 4h over 24h following Bleocin damage. RNA was purified as above, then processed and hybridized on Affymetrix Drosophila Genome 2.0 chips, according to the manufacturer’s instructions, at the Microarray Core, Dana-Farber Cancer Institute. Data were normalized and analyzed as described in Xu et al. (2011).
Survival Assays
Flies were maintained under light/dark conditions as above. Flies were fed either 5% or 10% w/v DSS (MP Biomedicals) or 25 ug/mL Bleocin (Calbiochem), mixed in a solution of 5% w/v sucrose (Sigma). Approximately 15 flies were loaded per vial (n = 3 vials per genotype), and solutions were refreshed daily. Survival was scored each day and assays were performed on 2 separate occasions.

CAFÉ and Blue-Dye Assays
Flies were maintained under light/dark conditions as above. Blue dye assay was performed as reported in Xu et al. (2008) For the CAFÉ assay, 5 mL of 1% Drosophila Type II Agar (Apex BioResearch Products) was solidified in one vial to provide hydration. 5-10 flies were loaded into each of these vials and a capillary of feeding solution (5% w/v sucrose and 2.5% w/v yeast extract) was presented in each. Flies were maintained for one day, to acclimatize to feeding from capillaries, before the start of the assay. Capillaries were refreshed once every 3 hr, and the solution levels consumed in each capillary noted.

Behavior Assay
Adult flies (2–5 days old) were entrained for 3 days under LD (500 lux intensity), and then released into constant darkness for at least 5 days. Single fly locomotor activity was measured with TriKinetics Activity Monitors (Waltham, MA) in I36-LL Percival Incubators. Data analysis was performed using the FAAS-X software (Grima et al., 2002).

Mosaic Analysis
Flies were maintained on a 12–12 light/dark cycle, as above, and transferred to fresh media every 1–2 days. For MARCM clones two 1 hr 37°C heat shocks were applied at 2–3 days following eclosion, while for RNAi clones one 12 min 37°C heat shock was applied at 2–3 days following eclosion. A region of leaky GFP expression was noted in the RNAi clone stock, and this was omitted from these analyses. Clones were scored as clusters of 2 or more directly adjacent cells.

Imaging and Data analysis
Confocal microscopy was done using the Zeiss LSM 780 with the Zen LSM software package (Zeiss). Images were processed in Photoshop CS5.1 (Adobe). The percentage of cells was calculated by determining the number of (+) cells of interest divided by the total DAPI+ nuclei in one FOV from posterior midgut using a 40X objective. ~300–400 cells were scored per FOV per midgut, and > 5 midguts were analyzed per genotype. The same approach was used to quantify the percentage of (+) cells using the FUCCI cell cycle reporter and EdU, but in these cases, the total was that of the total Dl+ or total diploid cell nuclei present, respectively. Statistics (either paired or unpaired t tests, or ANOVA with Dunnett’s post-test to compare to controls, as appropriate) were carried out using Graphpad Prism 5.0.
Figure S1. PER/TIM Expression in the Gut, Related to Figures 1 and 2

(A) per RNA expression (qPCR) over Zeitgeber Time (ZT) normally shows circadian rhythms in the intestine (ry506 control), which are absent in per01 (ry506 and per01 data are the same as in Figure 1B). The per01 mutation is a nonsense mutation at exon 4 that produces a truncated non-functional product. per expression is very low in cyc0, because per is a direct transcriptional target of CLK/CYC.

(B) tim is also a clock target and fluctuates in the same manner as per. tim RNA remains high in the per01 mutant (qPCR), and the cyc0 mutant shows very low tim RNA level. As per/tim are transcribed by CLK/CYC they increase in level and eventually repress their own transcription. Degradation of PER/TIM eventually frees CLK/CYC, thus completing the feedback loop. Graphs a-b show average of 2 separate experiments (n = 15 guts/genotype/time point, p < 0.05 by ANOVA, expression normalized to ry506 ZT15, relative to GAPDH control RNA, error bars ± SEM).

(C) Schematic shows synchronization of flies on 12-12 LD cycle (for at least 3 days), followed by DSS (or Bleocin) application and 24 hr analysis 2 days later. See Extended Experimental Procedures for full description.

(D) PER staining (green) shows nuclear accumulation in intestinal epithelial cells in the morning (ZT0) versus the evening (ZT12), ECs can be recognized by their large polyploid nuclei.

(E) At ZT0, PER protein is expressed in ISC (arrows) labeled with Delta (Dl, red). PER staining under DD conditions similarly shows accumulation and reduction over 24h rhythms.

(G) PER protein does not accumulate in ECs at ZT0 when per RNAi is expressed in ECs using myo1A-Gal4.

(H) Similarly PER is not present in ECs, but is present in ISCs and EBs when the PER rescue construct is forcibly expressed using esg-Gal4 in the per01 mutant background. Note that PER is present at much higher levels using the rescue construct (compare with Figure 1D and S1E). This suggests the ISCs and ECs are desynchronized in these two experiments.

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Figure S2. Validation of RNAi and Rescue Reagents, Related to Figures 1, 2, 3, and 4

(A) The esg-Gal4 driver (esg > GFP is esg-Gal4, UAS-GFP) is expressed in several neurons in the brain, but it is not expressed in pacemaker neurons stained for PER protein (blue). Image shows a single confocal plane. Similarly, myo1A-Gal4 was not colocalized with pacemaker neurons (not shown).

(B and C) Tables show periods of activity in male and female flies in constant darkness, following light/dark synchronization. Flies normally exhibit characteristic peaks of activity in the early morning and late evening which oscillate in a circadian manner (see for instance w1118 and Luc RNAi controls). Expression of UAS-per RNAi in pacemaker neurons using tim-Gal4 completely disrupts behavior rhythms with UAS-dcr2, and increases the period of rhythms without UAS-dcr2. Similarly, UAS-cyc RNAi in pacemaker neurons completely disrupts behavior rhythms with and without UAS-dcr2. In contrast behavior rhythms persist when the same constructs are expressed in ISCs using esg-Gal4, or in ECs using myo1A-Gal4. We note, however, that the period of behavior rhythms is slightly (~1h) longer than 24h in esg > Luc RNAi (24.9h for males, 24.3 for females), esg > cyc RNAi (25.1 for males, 25.8 for females), and esg > per RNAi (24.5h for males, 25.1 for females). We further tested the possibility that the esg-Gal4 and myo1A-Gal4 drivers were expressed in the brain, by using them to express a rescue construct, UAS-per16, in the per16 mutant background. per16 flies are completely arrhythmic, but tim-Gal4 driven expression of UAS-per16 effectively restores behavior rhythms. In contrast, neither esg-Gal4 or myo1A-Gal4 restore rhythms to per16. The tubulin-Gal4 driver knocks down cyc mRNA (tub-Gal4/+;UAS-cyc RNAi/+ to 20% of wild-type cyc levels at ZT3, and per mRNA (tub-Gal4/+;UAS-per RNAi/+ to 25% of wild-type per levels at ZT15 (peak of per expression). These data support the use of the esg-Gal4 and myo1A-Gal4 drivers and RNAi constructs to disrupt circadian rhythms in ISCs or ECs, respectively, without affecting circadian behavior.

**Table B**

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<tr>
<th>Genotype (males)</th>
<th>Number</th>
<th>Rhythmicity</th>
<th>TauSem</th>
<th>PerSem</th>
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<tr>
<td>myo1A-Gal4/+; Per RNAi/+</td>
<td>14</td>
<td>75.6%</td>
<td>25.2±0.07</td>
<td>54.0±8.23</td>
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<td>esg-Gal4/+; Per RNAi/+</td>
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<td>93.3%</td>
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<td>64.0±3.79</td>
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<td>Tim-Gal4/+; Per RNAi/+</td>
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<td>27.5±0.40</td>
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<td>Tim-Gal4, UAS-dcr2/+; Per RNAi/+</td>
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<td>myo1A-Gal4/+; Cyc RNAi/+</td>
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<td>78.6%</td>
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<td>esg-Gal4/+; Cyc RNAi/+</td>
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<td>86.7%</td>
<td>25.1±0.13</td>
<td>60.1±4.59</td>
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<tr>
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<td>myo1A-Gal4/+; Luc RNAi/+</td>
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<td>86.7%</td>
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<td>w¹¹¹</td>
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**Table C**

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<td>myo1A-Gal4/+; Per RNAi/+; UAS-dcr2</td>
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<td>64.5±4.29</td>
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<td>myo1A-Gal4/+; Cyc RNAi/+; UAS-dcr2</td>
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<td>myo1A-Gal4/+; Luc RNAi/+; UAS-dcr2</td>
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<td>23.9±0.11</td>
<td>55.3±4.23</td>
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<tr>
<td>esg-Gal4/+; Luc RNAi/+; UAS-dcr2</td>
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<td>33.3%</td>
<td>24.3±0.13</td>
<td>25.1±6.20</td>
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<tr>
<td>Tim-Gal4/+; Luc RNAi/+; UAS-dcr2</td>
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<td>98.3%</td>
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**Table D**

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<td>15</td>
<td>0%</td>
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</tbody>
</table>

---

Figure S2A. Validation of RNAi and Rescue Reagents, Related to Figures 1, 2, 3, and 4

(A) The esg-Gal4 driver (esg > GFP is esg-Gal4, UAS-GFP) is expressed in several neurons in the brain, but it is not expressed in pacemaker neurons stained for PER protein (blue). Image shows a single confocal plane. Similarly, myo1A-Gal4 was not colocalized with pacemaker neurons (not shown).

(B and C) Tables show periods of activity in male and female flies in constant darkness, following light/dark synchronization. Flies normally exhibit characteristic peaks of activity in the early morning and late evening which oscillate in a circadian manner (see for instance w1118 and Luc RNAi controls). Expression of UAS-per RNAi in pacemaker neurons using tim-Gal4 completely disrupts behavior rhythms with UAS-dcr2, and increases the period of rhythms without UAS-dcr2. Similarly, UAS-cyc RNAi in pacemaker neurons completely disrupts behavior rhythms with and without UAS-dcr2. In contrast behavior rhythms persist when the same constructs are expressed in ISCs using esg-Gal4, or in ECs using myo1A-Gal4. We note, however, that the period of behavior rhythms is slightly (~1h) longer than 24h in esg > Luc RNAi (24.9h for males, 24.3 for females), esg > cyc RNAi (25.1 for males, 25.8 for females), and esg > per RNAi (24.5h for males, 25.1 for females). We further tested the possibility that the esg-Gal4 and myo1A-Gal4 drivers were expressed in the brain, by using them to express a rescue construct, UAS-per16, in the per¹¹¹ mutant background. per¹¹¹ flies are completely arrhythmic, but tim-Gal4 driven expression of UAS-per16 effectively restores behavior rhythms. In contrast, neither esg-Gal4 or myo1A-Gal4 restore rhythms to per¹¹¹. The tubulin-Gal4 driver knocks down cyc mRNA (tub-Gal4/+;UAS-cyc RNAi/+ to 20% of wild-type cyc levels at ZT3, and per mRNA (tub-Gal4/+;UAS-per RNAi/+ to 25% of wild-type per levels at ZT15 (peak of per expression). These data support the use of the esg-Gal4 and myo1A-Gal4 drivers and RNAi constructs to disrupt circadian rhythms in ISCs or ECs, respectively, without affecting circadian behavior.
Figure S3. Circadian Clock Mutants Show No Obvious Intestinal Cell Deficiencies, and Rhythmic Mitoses in the Intestine Are Not Dependent on Feeding, Related to Figures 1, 2, 3, and 4

(A) ISCs, labeled for Delta (D, red) are evident in both per01 and tim0 mutant intestines.

(B) The proportion of ISCs (%Delta+), ECs (%polyploid nuclei), or ees (%Prospero+) does not significantly vary between per01 and tim0 mutant, and ry506 or y,w control intestines. cyc0 mutant intestines showed a similar proportion of these cell types (not shown).

(C) If perturbation of circadian rhythms affected feeding, the reduction and arrhythmic intestinal mitoses in the per01 mutant or the esg > per RNAi could be simply a result of insufficient uptake of DSS. It has been previously shown that circadian clock genes regulate the timing of feeding rhythms, but that mutants do not feed less over a 24h period (Xu et al., 2008). Although we did not observe any mitotic rhythms in the intestine of the mutants tested, the CAFE assay, was applied to test the amount of food consumed by flies over 24 hr. The volume of media consumed was measured at 3h intervals, where knockdown of CYC or PER in either the ISCs or ECs had no effect on feeding. Because mitoses peak at ZT0, and the highest level of food consumed is from ZT0-ZT3, this suggests that mitoses peak before feeding levels. In addition, the amount of food consumed over the entire 24h period was equivalent between all of these genotypes (not shown). For instance, esg > per RNAi shows very low and arrhythmic mitoses at all time points (Figure 1F), but feeds equivalently to the esg > Luc RNAi control, whose mitoses are higher and rhythmic over the same time points. Finally, we tested feeding levels using a blue-dye uptake assay and found that ry006, per01, cyc0 and tim0 animals fed equivalently over 24 hr (not shown).

(D) Flies were entrained on LD conditions as before but food was presented from ZT12-ZT0 only, and flies were shifted onto 5% agar/H2O from ZT0-ZT12. Following 2 days DSS exposure (ZT12-ZT0 only), mitotic rhythms still peak at ZT0 further suggesting that ISC division is independent from the timing of DSS consumption. We note, however, that mitoses are reduced in these conditions, perhaps as a result of the restricted availability of DSS.
Figure S4. ISC-Autonomous Proliferation Is Decreased when CYC or PER Is Lost, Related to Results and Discussion

(A) The frequency of proliferating clones (≥2 cells in size) in the whole gut was measured over 23 days following clonal induction (n = 10 guts/genotype/time point, error bars ± SEM).

(B and C) 14 days following induction, control (FRT19A is FRT19A/hsFlp,FRT19A,Tub1-Gal80; act > y+ > Gal4, UAS-GFP/) clones, under 2 days DSS damage, are larger than per mutant clones (per01 FRT19A is per01, FRT19A/hsFlp,FRT19A,Tub1-Gal80; act > y+ > Gal4, UAS-GFP/). Note that Dl+ cells are present in per01 clones, indicating that the ISCs are not lost. per01 clones show a different distribution of sizes, brackets indicate number of clones examined.

(D) 14 days following induction, per01 are the same size as wild-type, however upon damage per01 clones are slightly but significantly smaller. Damaged clones are same as those reported in Figure S4 C. Brackets indicate number of clones examined, *significance by t test (p < 0.05).

(E) At 4 days, the size of per01 clones is similar to controls. Brackets indicate number clones examined.

(F) 4 day per01 clones, exposed to DSS for 2 days, show a different distribution of sizes. Brackets indicate number of clones examined.

(G) 4 days following induction, per01 are the same as wild-type, however upon damage per01 clones are slightly but significantly smaller. Damaged clones are the same as those reported in Figure S4F. Brackets indicate number of clones examined, *significance by t test (p < 0.05).

(H) At 23 days, in the absence of damage, per01 clones show a different distribution of sizes. Brackets indicate number of clones examined.

(I) The average size of per01 clones at 23 days following induction, even in the absence of damage, is significantly smaller than controls. Clones are the same as those in Figure S4H. Brackets indicate number of clones examined, *significance by t test (p < 0.05).

(J) The same assay was undertaken using RNAi clones (for instance the control flpout > Luc RNAi refers to hsFlp/+; act > CD2 > Gal4, UAS-nlsGFP/UAS-dcr2; UAS-Luciferase RNAi/+). All genotypes are only different in the RNAi transgene on chromosome 3 (n = 10 guts/genotype/time point, error bars ± SEM).

(K) 14 day cyc RNAi and per RNAi clones, exposed to DSS for 2 days, show a different distribution of sizes. Brackets indicate number of clones examined.

(L) cyc RNAi and per RNAi clones are slightly but significantly smaller. Clones are the same as those reported in Figure S4K. Brackets indicate number of clones examined, *significance by t test (p < 0.05).

(M) At 4 days, the size of cyc RNAi and per RNAi clones is similar to controls. Brackets indicate number clones examined.

(N) At 23 days, with no damage, both cyc RNAi and per RNAi clones show different size distributions compared to Luc RNAi controls. Brackets indicate number clones examined.

(O) Both cyc RNAi and per RNAi 23 day clones are slightly but significantly smaller. Clones are the same as those reported in Figure S4N. Brackets indicate number of clones examined, *significance by t test (p < 0.05).
Figure S5. Mitotic Rhythms Are True Circadian Rhythms and Are Required for Survival following Gut Damage, Related to Figures 1, 2, and 4

(A) If lights are turned off, after light/dark synchronization (see schematic below), rhythms in mitoses persist 2 days later in ry506 but not in per01.

(B) If lights are turned off, after light/dark synchronization, rhythms in mitoses also persist 3 days later in ry506. The peak in mitoses seen at 2–3 days DD thus repeats itself 24h later, at 3–4 days, in the absence of light cues.

(C) cyc0 mutant intestines were examined when flies were shifted from 12-12 light/dark conditions to complete darkness (as in schematic below). The circadian rhythms in controls are perpetuated in these conditions (ry506 data is taken from Figure S5A), but cyc0 intestines remain arrhythmic and at same levels as in Figure 2A. Graphs show n = 10 guts/genotype/time point (error bars ± SEM).

(D) Schematic shows synchronization of flies on 12-12 Light/Dark cycle (for at least 3 days), followed by DSS application, shifting to complete darkness, and 24 hr analysis 2 days later. See Extended Experimental Procedures for full description.

(E and F) When light/dark synchronized flies are exposed to constant light (as in schematic below), mitotic rhythms in the intestine are no longer observed (n = 10 guts/genotype/time point, error bars ± SEM). Neither ry506 nor y,w control flies exhibit peaks of mitoses at ZT0 under constant light (compare with Figures 1E, 2A, and 2B), rather these show high mitoses at all times.

(G) Schematic shows synchronization of flies on 12-12 Light/Dark cycle (for at least 3 days), followed by DSS application, shifting to complete light, and 24 hr analysis 2 days later. See Extended Experimental Procedures for full description.

(H and I) Survival of circadian mutant animals compared to their respective controls on 5% DSS (black lines), cyc0 and tim0 mutants show reduced survival on 5% DSS.

(J and K) Survival assays of same genotypes on 10% DSS, where cyc0, tim0, and per01 mutants show reduced survival as they do on Bleocin (Figures 2H and 2I). Graphs show representative experiments (n = 3 vials/15 flies per vial, genotypes are as above).