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REVIEW

Time after time: circadian clock regulation of intestinal stem cells

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Abstract

Daily fluctuations in animal physiology, known as circadian rhythms, are orchestrated by a conserved molecular timekeeper, known as the circadian clock. The circadian clock forms a transcription–translation feedback loop that has emerged as a central biological regulator of many 24-h processes. Early studies of the intestine discovered that many digestive functions have a daily rhythm and that intestinal cell production was similarly time-dependent. As genetic methods in model organisms have become available, it has become apparent that the circadian clock regulates many basic cellular functions, including growth, proliferation, and differentiation, as well as cell signalling and stem cell self-renewal. Recent connections between circadian rhythms and immune system function, and between circadian rhythms and microbiome dynamics, have also been revealed in the intestine. These processes are highly relevant in understanding intestinal stem cell biology. Here we describe the circadian clock regulation of intestinal stem cells primarily in two model organisms: *Drosophila melanogaster* and mice. Like all cells in the body, intestinal stem cells are subject to circadian timing, and both cell-intrinsic and cell-extrinsic circadian processes contribute to their function.

Keywords Stem cells · Digestive tract · Cellular signalling · Immunity · Cell cycle

Abbreviations

Apc	Adenomatous polyposis complex
Ascl2	Achaete scute-like homolog 2
Bmal1	Brain and muscle Aryl hydrocarbon receptor nuclear translocator like
Bmi1	B-cell-specific moloney murine leukemia virus integration site 1
Bmp	Bone morphogenic protein
CBC	Crypt base columnar cell
ChIP	Chromatin immunoprecipitation
Chk	Checkpoint kinase
Ck1	Casein kinase 1
Clk	Clock
Clock	Circadian locomotor output cycles kaput
Cry	Cryptochrome
Cxcl12	C-X-C chemokine ligand 12
Cxcr4	C-X-C chemokine receptor type 4
Cyc	Cycle
Gsk3	Glycogen synthase kinase 3
Hopx	Homeoboxdomain-only protein
IBD	Inflammatory bowel disease
IL	Interleukin
ISC	Intestinal stem cell
Jak/Stat	Janus kinase/signal transducers and activators of transcription
Klf9	Kruppel-like factor 9
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
Lrig1	Leucine-rich repeats and immunoglobin-like domains-protein 1
Lrp	Low density lipoprotein receptor
Mapk	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
Olfm4	Olfactomedin 4
Per	Period
Ror	Retinoic acid receptor-related orphan receptors
S6K	S6 kinase
Sirt1	Sirtuin-1
Tert1	Telomerase reverse transcriptase
Tim	Timeless
Tnf	Tumour necrosis factor
Upd	Unpaired
Vri	Vrille
Pdp1	PAR domain protein-1

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Wee1	Wee1 G2 checkpoint kinase
Xpa	*Xeroderma pigmentosum* group A

Circadian rhythms

Circadian rhythms are 24-h recurring physiological processes such as daily sleep/wake cycles, feeding/fasting cycles, daily changes in body temperature, hormone levels, and cardiovascular function [1–3]. The word Circadian is derived from Latin "*circa*" (meaning "about") and "*diem*" (meaning "day"). Circadian Rhythms have four main characteristics: (1) an approximately 24-h period, corresponding to the 24-h rotation of the Earth's axis; (2) temperature-compensation, meaning they are maintained as a 24-h process under a wide range of environmental temperatures; (3) the ability to be synchronized by external cues such as light and feeding (called "zeitgebers" from German meaning "time giver"), to synchronize 24-h internal timekeeping with 24-h changes in environment; (4) the ability to persist in the absence of external cues (called "free-running"), which means the physiological changes arising from circadian clock function reflect ongoing internal cellular and molecular timing rather than a simple response to the cues [1, 2]. These characteristics differentiate circadian clock and other environmental-responsive processes, including diurnal rhythms whose daily repetition may be determined by light responses, and ultradian and infradian rhythms whose period is less or more than 24-h, respectively. Hence, circadian rhythms are 24-h oscillatory networks of molecular and cellular activity, present in animals to maximize their health and fitness under constant 24-h planetary change.

The observation that biology is synchronized to the 24-h day–night cycle and persists in the absence of external cues was first supported by experimental evidence in 1729 when French scientist Jean-Jacques d'Ortous de Mairan kept a plant in a windowless room and observed that the cyclic behaviour of leaf opening continued in complete darkness [4]. Rhythmic behaviours were documented in a wide variety of organisms, in the conidiation of fungi [5], the eclosion of fruit flies [6–9], and the locomotor activity of finches [10]. This research eventually led to the discovery of an endogenous genetic timekeeper: the study of eclosion rhythms in fruit flies, *Drosophila melanogaster*, unearthed three genetic mutants with disrupted rhythms, the *Period* (*Per*) mutants [9]. Mapped to the X-chromosome of *Drosophila*, the *Per* gene linked circadian rhythms to a molecular timekeeper, the circadian clock [11], and was the first demonstration that animal behaviour could be attributed to the function of a single gene.

Recent studies have implicated the working of the circadian clock system with Intestinal Stem Cell (ISC) function [12–16]. This review will explore the role of the circadian clock in regulating processes relevant to intestinal and ISC biology, in mice and *Drosophila*. As this field is still in its infancy, connections between the circadian clock and molecular processes important in ISCs, but not yet linked with circadian function, will be highlighted as possible directions for future work.

The circadian clock

Circadian rhythms are maintained by a cell-intrinsic molecular transcription–translation feedback cycle called the circadian clock. This molecular system influences the expression of many genes that contribute to 24-h cycles of cellular function. The circadian clock is conserved from flies to mammals and has been shown to regulate a total of ~ 40% of genes in the mouse [17], and possibly an even higher number in primates [18]. These genes vary between different tissues, suggesting circadian functions are specific depending on cell type [17, 19]. Due to the tremendous number of cellular processes it can regulate, it is perhaps not surprising that the circadian clock has been linked to many diseases [20, 21] including cancer [22–24], diabetes [25], inflammatory bowel disease [26, 27] and obesity [28, 29]. The mechanisms of how the clock fully impacts intestinal health remain to be elucidated.

The components of the circadian clock in animals are highly conserved between *Drosophila* and mice. In *Drosophila*, the circadian clock is a transcription–translation feedback loop in which protein heterodimers, Per and Timeless (Tim), repress their own transcriptional activators, Clock (Clk) and Cycle (Cyc) (Fig. 1a). In the beginning of the day, the repressors are active, however, over the day, light-induced degradation of Tim by the photoreceptive protein Cryptochrome (Cry) releases Per-Tim inhibition. These repressors are degraded, allowing the Clk/Cyc transcriptional activators to bind to E-box (5′-CACGTG-3′) regions all over in the genome, including that of their repressors, starting the next cycle [30–34]. This process is free-running: in the absence of environmental cues, phosphorylation and degradation of Per reset the circadian clock to maintain its approximately 24-h period [35–37]. A secondary feedback loop consists of transcription factors *Vrille (Vri)* and *PAR-domain protein 1 (Pdp1)* that are transcribed by Clk/Cyc and modify *Clk* expression; Vri represses *Clk* and Pdp1 activates *Clk* in turn [38–41]. This second transcription/translation system is thought to confer greater robustness to the clock since expression of both the repressors and activators oscillate in opposite phases. The mechanism for temperature compensation remains unknown, although in plants the ratio of clock components may buffer rhythms in varying temperatures [42].

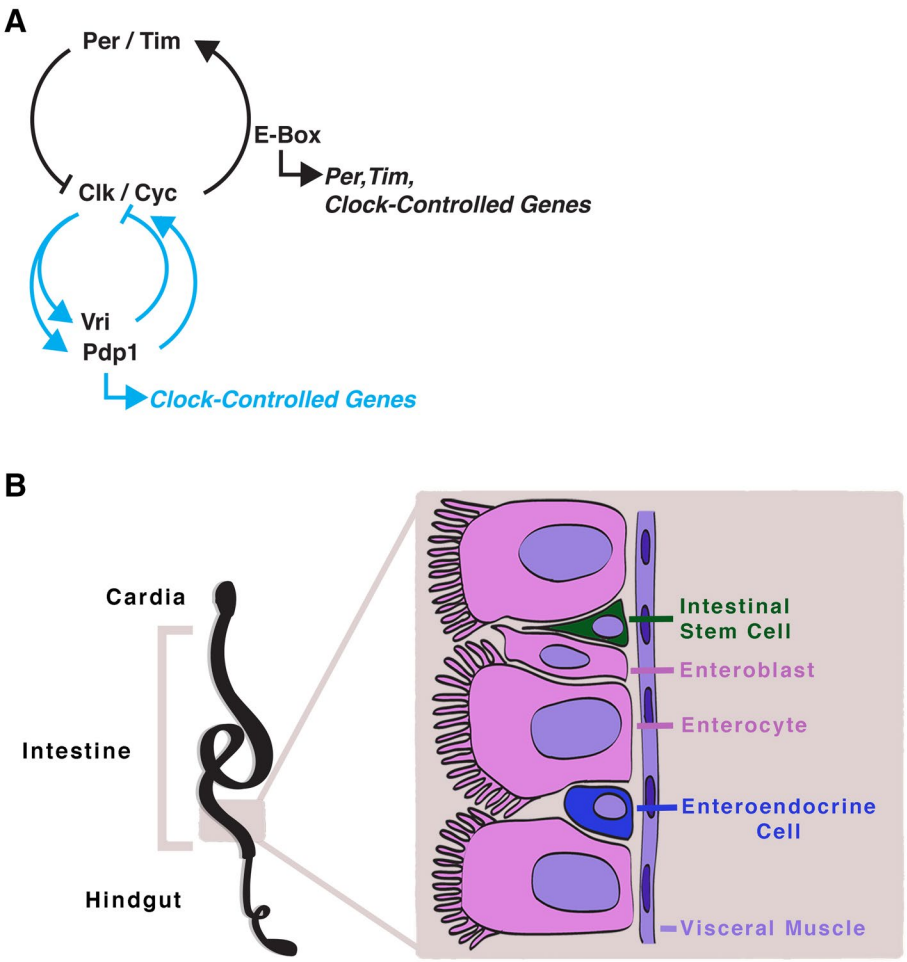

Fig. 1 The *Drosophila* circadian clock, and intestine. **a** The circadian clock in *Drosophila* consists of the basic-helix–loop–helix (bHLH) transcription factors Clk and Cyc which bind to E-box regions in the genome driving the transcription of clock-controlled genes. The core circadian clock genes *Period* (*Per*) and *Timeless* (*Tim*) form a protein heterodimer that represses Clk/Cyc activity. A secondary stabilizing loop involving Pdp1 and Vri activates or represses Clk, respectively. Pdp1 and/or Vri could themselves regulate target genes in a rhythmic fashion as well, contributing to the overall clock-controlled gene rhythm. **b** The intestine in *Drosophila* is a pseudostratified epithelium consisting of basally located intestinal stem cells (ISC) which either self-renew to maintain the stem cell pool or divide into progenitor cells, called enteroblasts. The different epithelial cells as well as the visceral muscle are thought to serve as a niche for the ISCs in this simplified epithelium

In mice, Brain and Muscle Aryl hydrocarbon receptor nuclear translocator Like protein 1 (Bmal1) and Circadian Locomotor Output Cycles Kaput (Clock) proteins similarly dimerize and transcriptionally activate *Per* and *Cry*, which in turn repress Clock/Bmal1 activity (Fig. 2a). In mammals, *Tim* is not part of the central clock mechanism, it is substituted by *Cry* itself and the transcription–translation feedback loop is otherwise similar. The mammalian clock has additional genetic redundancy, as it consists of multiple paralogs of clock components: Bmal1-2 [43], Clk1-2 or Npas2 [44–46], Per1-3 [47–51] and Cry1-2 [52–54]. As in *Drosophila*, the activity of Clock/Bmal1 drives its own repression, and a secondary stabilizing loop is also present, but consisting of *Reverbα* (also known as *Nuclear Receptor Subfamily 1 Group D Member 1*, *Nr1d1*), or *Reverbβ (Nr1d2)* [55], and multiple isotypes of *Retinoic Acid Receptor-Related Orphan Receptor* (*Rorα*, also known as *Nr1f1*, as well as *Rorβ* and *Rorγ*, with several isoforms of each of these) [56, 57], which feedback in a tissue-specific manner to drive a second opposite-phased system. Post-transcriptional and post-translational modifications of circadian clock components also play a role in regulating circadian gene expression [58]. The mechanisms of both *Drosophila* and mammalian circadian clocks are well-established due to decades of research [1, 2]. It is also important to mention that non-transcriptional mechanisms can establish circadian rhythms, although in this review we will primarily consider the canonical transcription–translation circadian clock system.

Entrainment of the circadian clock

A major question in circadian biology is understanding how the clock is entrained by environmental cues to synchronize its activity with 24-h cycles. Light, temperature and feeding are three cues that have been shown to synchronize circadian clocks. In many animals, the circadian system is hierarchical, being composed of a central pacemaker driving the synchronization of peripheral pacemakers located inside cells throughout tissues of the body [59]. Photoperiod changes, the periods of light and darkness, are sensed by retinal cells of the eye, then sent to the central pacemaker in the brain. This central pacemaker, located in the

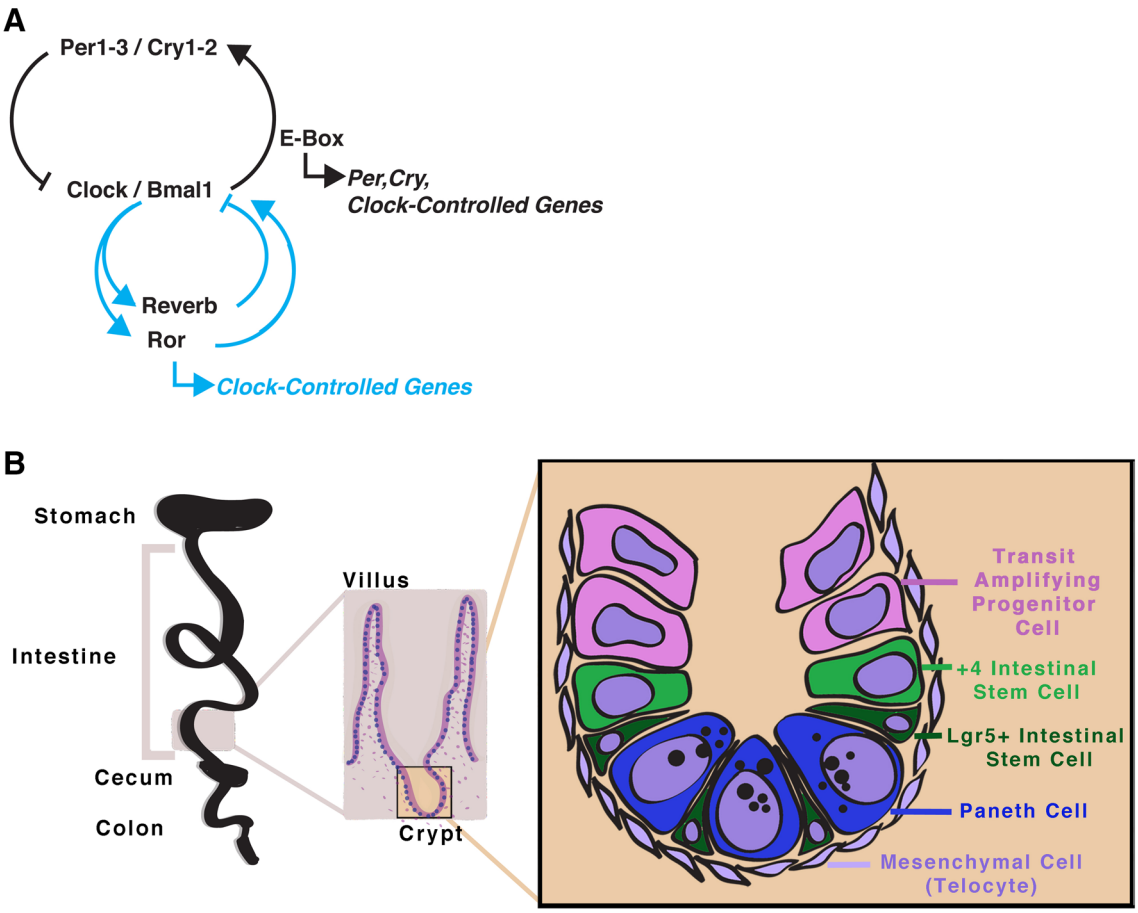

Fig. 2 The mouse circadian clock, and the intestinal crypt. **a** The circadian clock in mice consists of basic-helix-loop-helix (bHLH) transcription factors clock Clock (or Npas2) and Bmal1 which bind to E-box regions in the genome driving the transcription of clock-controlled genes. These genes include the core circadian clock genes *Per1-3* and *Cry1-2* which form protein heterodimers that represses Clock/Bmal1 activity. A secondary stabilizing loop involving *ROR*s and *Reverb*s (and their respective isotypes) activate or repress *Bmal1*, respectively. Both of these nuclear receptors could regulate their own clock-controlled target genes as well. **b** The intestine in mice is folded into crypts and projections into the lumen (villi). Differentiation occurs as progenitor cells move upward to the villi, and old cells are shed from the tips of the villi. The epithelium consists of rapidly dividing LGR5+ intestinal stem cells (ISC) located at the base of the crypt as well as quiescent +4 ISCs. which either self-renew to maintain the stem cell pool or divide into progenitor cells, called transit amplifying progenitor cells. Both mesenchymal cells, in particular telocytes, and Paneth cells serve as a Wnt pathway niche for the ISCs

suprachiasmatic nucleus of the hypothalamus, is thought to coordinate these peripheral clocks through neuronal signals, and the hypothalamo-pituitary-adrenal axis [60–65]. Hormones sent via this system, time the peripheral pacemakers in distant tissues, that work together to establish circadian rhythms in the body [66, 67]. Each peripheral timekeeper maintains rhythmic gene expression patterns relative to the central pacemaker [66, 68–70]. This complex and intimately connected system allows the body to overall coordinate the timing of physiological functions to anticipate the demands of the organism based on 24-h time.

Photoperiod light is sensed by opsins in mice [52, 71–75], and Cry in *Drosophila* [76–80]. While in mammals a hierarchical system conveys photoperiod information from the retina to peripheral tissue clocks, in *Drosophila*, photoperiod cues can synchronize clocks in tissues directly and independently [81, 82]. Although the mechanism for 24-h period temperature compensation is unknown, in mammalian cells [83] and in *Drosophila* [84] temperature changes can synchronize the circadian clock in peripheral tissues. Feeding has been shown to be an important entrainment cue in peripheral tissues in mice, such as the liver, kidney, and heart [85–88], as well as their equivalents in *Drosophila* [89–91]. Indeed, peripheral clocks are highly dependent on feeding time. In mice it was shown that feeding can uncouple peripheral and central clocks, as the former is directly entrained by the time of food intake while the latter is entrained by photoperiod. This likely reflects the hierarchical system of clock timing in mammals, where the central clock of the suprachiasmatic nucleus receives timing cues from light hitting

the retina, and the peripheral clocks receive hormonal cues from the central clock. In vitro, serum shock [92], the glucocorticoid dexamethasone [93], forskolin [94], and insulin [95] can be used to synchronize the clock, underscoring the importance of hormones and cellular signaling in entraining clock timing. In *Drosophila*, hormonal inter-organ communication is not so clear, however, studies have revealed elements of circadian time-setting [90, 91], including food synchronization of peripheral tissues [16, 91, 96].

Intestinal stem cells

The intestine is a dynamic tissue that undergoes nearly continuous cellular turnover. This is most likely due to the harsh processes of nutrient digestion, that can damage intestinal cells needed for nutrient absorption, hormone release, and pathogen defense. In addition to its role in digestion, the intestine is also a barrier to the outside environment that is evolved to cope with constant bombardment of pathogens and harmful chemicals. Nearly the entire intestinal epithelium is renewed weekly in mammals, and its dynamic turnover is supported by a population of ISCs [97–99]. In animals, the intestinal epithelium consists of several cell types: (1) ISCs; (2) progenitor cells (transit amplifying cells in mice, or enteroblasts in *Drosophila*); (3) absorptive cells (enterocytes); (4) enteroendocrine cells; (5) secretory cells (Paneth cells, goblet cells). Secretory cells have not been found in *Drosophila*, whose epithelium and immune system is simplified in its cellular diversity. However, the *Drosophila* intestine resembles and is regulated by the same pathways of its mammalian counterpart, making this system an attractive model for basic research [100, 101].

Drosophila intestinal stem cells

The *Drosophila* intestine (also known as the midgut) is a pseudostratified epithelium with ISCs located near the base and differentiated cells facing the lumen (Fig. 1b). ISCs divide into daughter ISCs or enteroblasts [102–104] that differentiate into enterocytes or enteroendocrine cells [105, 106]. *Drosophila* ISCs are marked by the Notch pathway ligand Delta, that is involved in signaling differentiation to ISC daughter cells as they are produced [104]. The stem cell niche is well defined in the *Drosophila* intestine, consisting of important signals for ISC maintenance that are provided by the stem and progenitor cells themselves [107–110], as well as the visceral muscle [108, 111–117]. The Bone Morphogenic Protein (Bmp) and Wnt signaling pathways present in this system, further highlight the similarity between the *Drosophila* ISC system and that of mammals [101, 115, 118–122].

Mammalian intestinal stem cells

In mammals, the intestinal epithelium is arranged in a crypt-villus structure with stem cells located at the base of the crypts of Lieberkühn, and differentiating daughter cells moving upward to the tip of the villus [123] (Fig. 2b). The progenitor cells differentiate as they travel up the crypt to the villus, although differentiated Paneth cells will migrate back down to the crypt where they reside with the stem cells at the base. In mice, at least two distinct populations of stem cells are known: the +4 ISCs (sometimes also called label-retaining cells) and the crypt base columnar (CBC) cells. The CBCs are a population of quick-cycling ISCs in which Wnt signaling is important for stem cell proliferation and differentiation. The +4 ISCs, or ISCs at approximately that position in the crypt, have been separately identified by the markers *Bmi1* [124], *Tert* [125], *Hopx* [126], *Lrig1* [127], and most recently by *Clusterin* [128], however, the precise relationships between cells bearing these markers is an area of ongoing research. Some of these markers may be also expressed by CBCs [129], and the quick-cycling CBCs are specifically marked by *Leucine-rich repeat-containing G-protein coupled receptor* (*Lgr5*) [130], *Olfm4* [131] and *Ascl2* [132, 133]. Complex and not fully understood lineage relationships exist between these different ISC populations in this tissue, but it is thought that during baseline, uninjured conditions the Lgr5 + ISCs divide once every 1–2 days to produce the differentiated cells of the intestinal epithelium [130, 134, 135]. The +4 cells on the other hand seem to play a role during stress and regeneration. At the base of the crypt, mesenchymal cells and Paneth cells form the Lgr5+ ISC niche, by secreting Wnt pathway ligands that create a zone of high Wnt activity (described in detail below). ISCs in both *Drosophila* and mice have proven to be excellent, and mutually beneficial systems to inform our knowledge of basic stem cell biology.

Circadian regulation of the intestine

Nearly all cells of the body are thought to harbor circadian clock activity, and the intestine is no exception. In both *Drosophila* and mammals, clock gene transcriptional rhythms are present in the intestine [12, 16, 136, 137]. Experiments in vivo in *Drosophila* and mice, and recently in vitro using intestinal organoids, 3-dimensional cell culture models, further confirm that the circadian clock is present in the intestinal epithelium [12, 14–16]. Analysis of 24-h changes in transcript abundance has been a central test of circadian output in many different contexts, including the digestive tract. In wildtype mice, > 1000 transcripts are thought to oscillate under photoperiod [138], although free-running conditions and circadian clock mutants have not

yet been tested to confirm these are a result of *bona fide* clock activity [138–140]. In *Drosophila*, over 400 genes show circadian expression rhythms, that are absent when the clock is non-functional [12]. Furthermore, one of the most highly expressed transcription factors in *Drosophila* ISCs is the circadian clock gene *Cyc* [107], the ortholog of *Bmal1*, which suggests that at least this one core clock gene plays an important role in these cells. The clock appears to be robust in the intestine, functioning under different diets and physiological contexts [138–140]. However, it is important to note that the transcriptional analyses thus far represent an average of all the different cell types present, and not necessarily information about cell-specific circadian rhythms in this tissue. In addition, these studies have been carried out under constant light/dark photoperiod, meaning that they have not addressed the free-running nature of the circadian clock system, and cannot completely discriminate between circadian clock target genes and light-response genes. Determining the precise functions of the clock in the intestinal epithelial cells, as well as their non-epithelial neighbors, is a problem for future research.

Despite a large number of potential transcripts, the functions of the circadian rhythms in the intestine remain poorly understood. The intestine of clock mutant *Drosophila* or mice has no obvious size or morphological phenotypes [12, 15, 141], yet daily rhythms in the expression and activity of enzymes and transporters for carbohydrates, peptides, and fats are very well-established [140, 142–147]. This highlights that one of the primary functions of the intestine, to absorb and digest nutrients, is probably under circadian regulation. This would be a significant role for the circadian system, likely connecting this tissue to the circadian metabolic control of the whole animal. The role of circadian rhythms in governing intestinal absorption and digestion functions have been addressed in previous reviews [148–150].

Circadian regulation of stem cells

Do stem cells themselves have a circadian clock? Early observations of circadian rhythms in mitosis and apoptosis were indeed attributed to epidermal stem cells in tongue, skin and intestinal epithelium [151]. However, studies showing circadian rhythms in stem cells, specifically, occurred later when the molecular tools needed to identify and study these cells became available. Diurnal variation in stem cell characteristics was initially shown in the haematopoetic stem cells [152, 153] and subsequently in epidermal stem cells [154, 155]. Clock genes are expressed by haematopoetic stem cells, but it is not yet clear whether circadian transcriptional cycles are present [156]. However, in the body the circadian clock regulates oscillation of the chemokine *Cxcl12* and its receptor, *Cxcr4* [152, 153]. These regulate extrinsic stem cell signaling, causing daily patterns of migration and homing of haematopoietic stem cells from the bone marrow to the bloodstream. Recent reports have further implicated circadian rhythms with the cell-intrinsic proliferation and self-renewal of haematopoietic stem cells, and leukemia stem cells [157, 158]. Per2 expression is increased in aged haematopoietic stem cells where it increases apoptosis and DNA-stress response [158]. In the case of leukemia stem cells, *Clock* and *Bmal1* are required for sustained proliferation and the maintenance of the undifferentiated state [157]. In these blood cells, it is not yet clear if these are due to circadian functions, or non-circadian functions of these clock genes in different cellular pathways. In the epidermis, the role of the clock is complex since many different cell types participate in regeneration, including epidermal stem cells, and the output of the circadian system may be different depending on cell type. Hence, connecting circadian regeneration rhythms to actual stem cell activity is a challenge. As in the intestine, circadian rhythms in the mitosis of epidermal cells are present and are clock-dependent [159], and two genes, *P21* and *Klf9* have both been implicated as mechanisms connecting the clock to cellular proliferation [113, 160]. Migration of fibroblasts during wound closing also has a circadian regulatory role [161], suggesting several stages of skin wound healing would be time-dependent. The epidermal stem cells themselves, exhibit circadian clock function, and the loss of *Bmal1* and *Per1/2* increases and decreases proliferation, respectively [154]. Overall, it is clear that circadian rhythms exist in the blood and skin, and are the product of circadian clock output in many different cell types. The role of the circadian clock in stem cells in the haematopoietic and epidermal tissues has been reviewed [162–167], raising many interesting areas for future study.

In the previous examples, stem cells can be regulated by cell-extrinsic clock mechanisms, as well as cell-intrinsic ones. This raises the question of the relative contribution of extrinsic versus intrinsic mechanisms in stem cell biology, and whether stem cells need to have intrinsic circadian clock function to display time of day changes in their behaviour. Adult tissue stem cells represent a relatively undifferentiated population, that give rise to a lineage of increasingly differentiated progeny. When does the clock arise during development? The notion that the circadian system emerges as a result of differentiation is a compelling framework. In early mouse embryonic stem cells the circadian clock is completely absent [168], arising at later stages during embryonic development [169]. In the adult, however, hair follicle stem cells and muscle stem cells both have circadian clock function [154, 170]. It has been documented that adult ISCs have weak to no circadian clock activity in vitro [14], suggesting these would resemble embryonic stem cells rather than the tissue stem cells found in skin or muscle. However, in the adult *Drosophila* intestine, circadian clock function is

present in ISCs [12, 16]. It is possible that these differences are simply species-specific, or that the physiological state of the environmental milieu, which is different in vivo than in vitro, regulates clock activity in ISCs. How cell-intrinsic circadian activity in intestinal cell lineage emerges during differentiation is an important question for future research.

Circadian regulation of intestinal stem cells

Are any ISC-related processes under similar circadian control? Tissue stem cells' primary function is to replace the surrounding differentiated cells to maintain tissue homeostasis throughout adulthood. In the circadian field, it is generally accepted that proliferation of intestinal precursors follows 24-h cycles, based on seminal studies by Sigdestad and Potten nearly 50 years ago [148, 151, 171, 172]. These studies found that mitoses show diurnal rhythms in rodents, peaking in the early morning when the nocturnal animal begins to slow its activity and return to sleep. However, in the stem cell field, daily proliferation rhythms do not receive much attention. Indeed, early studies of intestinal tissue renewal including one by the pioneer of ISC biology, Charles Leblond, found that intestinal precursor proliferation was constant and showed no time of day dependence [97, 173, 174]. A recent study has addressed this discrepancy, showing that prior to stress, rhythms in proliferation are weak, however, under regenerative conditions where inflammation is high, cell proliferation follows clear daily rhythms [12, 15]. Together these studies hint that stem cell output is gated by the circadian clock, and modern cellular and genetic tools can now be applied to resolve the contribution of a fundamental cellular timekeeper to ISC biology.

Like many adult tissue stem cells, ISCs are located in regions of the intestine called the niche, a localized zone of cellular signaling that determines their undifferentiated status and proliferative capacity. In the mammalian intestine, homeostasis is chiefly maintained through Wnt and Bmp that regulate a balance between ISC self-renewal and differentiation [123]. In *Drosophila*, these same pathways contribute to ISC regenerative activity, and the overall system shares many conserved features, albeit not the exact same details [101]. For instance, Bmp can promote *Drosophila* ISC proliferation, while in mice it inhibits proliferation through differentiation of ISCs. Here, we will consider primarily conserved cellular processes that might be controlled by the clock in the ISC niche in both systems. In both *Drosophila* and mammals, during baseline, undamaged conditions, ISCs proliferate to renew the epithelium constantly, and during regenerative, post-damaged conditions ISCs are thought to further increase this regeneration. As mentioned above, certain subpopulations of ISCs in the mammalian intestinal crypt are thought to form complex lineage relationships, and these are particularly susceptible to changes from baseline to regeneration [175]. Intestinal epithelial mitoses in the *Drosophila* and mouse intestine shows circadian rhythms during regeneration [12, 15], which suggests that pathways which regulate ISC proliferation are regulated by the circadian clock. Since the study of circadian regulation of ISCs is a new area of research, five different possibilities that could mechanistically explain these rhythms will be discussed below, based on studies of the circadian clock in the intestine and other systems (Table 1). These five possibilities are highly relevant to both colorectal cancer and IBD pathology as well.

Cell cycle control of stem cell proliferation

Many cells divide approximately once per day, and a link between the circadian clock and the cell cycle has been actively researched [176]. Initial studies indicated that the cell cycle is likely to be coupled to the circadian clock in fibroblasts and in the liver [177, 178]. Live-imaging of circadian clock activity in immortalized fibroblasts revealed that the clock functions in dividing cells and helps to establish the timing of cell cycle progression, while at the same time being affected by the cell cycle during mitosis, which causes a delay in circadian clock progression [178]. The first explanation for the link between the circadian clock and the cell cycle is that, like Huygen's pendulum, the circadian cycle of transcription/translation and the cell cycle of growth/division could exist as two oscillating processes that impact each other's activity, in essence synchronizing them relative to one another over time in the same cell [179, 180]. This is an important concept, and the coupling of oscillators in the same cell could be a general principle linking many recurring processes with daily circadian activity. However, it is important to note that these cycles can be decoupled, and it has been shown that the cell cycle is not under any obligatory circadian clock control [181].

The regulation of the cell cycle by the clock in a top–down fashion is a second, parsimonious explanation for daily rhythms that have been observed in tissue regeneration. In the liver, *Wee1* (a G2/M cell cycle checkpoint regulator) is directly controlled by the circadian clock thereby regulating the proliferation of liver cells during regeneration [177]. In hepatocytes the cell cycle inhibitor, *P21*, is also a circadian clock target that establishes the proliferative timing of cells [182]. This means that in the liver, multiple phases of the cell cycle appear to be downstream of the clock, thus clock mutants have abnormal regenerative output. In other tissues, other cell cycle checkpoint regulators are under circadian control, including *P16*, thereby influencing the timing of fibroblast division during wound healing in mice [183]. In the skin, the role of the clock is very complex. For instance, it regulates growth and the cell cycle in epidermal stem cells

Table 1 Regulatory mechanisms

Process	Tissue/cell	Role of circadian clock	References
1. Cell cycle/DNA repair	Brain	DNA repair by *Xpa*	[193]
	Epidermal stem cells	Regulation of cell cycle (*p21*, *Cdk4*, etc.)	[154]
	Fibroblasts	Regulation of *p16* through *NONO*	[183]
	Intestinal Stem Cells	Regulation of cell proliferation (via cell signaling)	[12, 14, 15]
	Intestine	Regulation of mitosis	[151, 171]
	Kidney	Regulation of *Chk1*	[187]
	Liver	Regulation of *Wee1*, *p21*, DNA repair by *Xpa*	[177, 182, 192]
	Skin	Timing of DNA repair	[190, 191]
2. Self renewal (Wnt signaling)	Bone	Regulation of osteogenesis	[210]
	Brain	Regulation of *c-Myc*	[216–218]
	Fat	Regulation of adipogenesis	[211]
	Intestinal stem cells	Regulation of *Wnt3A* in Paneth cells	[14]
	Intestine	Regulation of *β-Catenin*	[212]
	Liver	Regulation of *c-Myc*	[215]
	Muscle progenitor cells	Regulation of myogenesis	[208, 209]
3. Differentiation	Epidermal stem cells	Regulation of Notch, Bmp signalling	[154, 155]
	Fat, Tendon	Regulation of Bmp signalling	[236, 237]
	Intestinal stem cells	Loss of Notch signalling disrupts circadian clock	[16]
	Keratinocytes	Regulation of *Klf9*	[160]
4. Feeding and growth (insulin signaling)	Brain, liver, fibroblasts	mTOR/Bmal1 regulated protein translation	[251]
5. Immune response	White blood cells	Proliferation, circulation, regulation of inflammatory response	[258, 259, 261–270, 277]
	Intestine	Microbiome, regulation of inflammatory response (*Toll-like receptors*, *Tnf*, etc.)	[12, 15, 274–276, 279–284]
	Colon	Regulation of inflammatory response, and inflammasome (*Nlrp3*)	[26, 277, 278]

A list of five potential regulatory mechanisms through which the circadian clock controls ISC function. References listed correspond to the papers in the text, and studies carried out on stem cell populations are emphasized

[154, 155, 170] that determines the propensity of epidermal stem cells to activate during regeneration or to remain dormant [154]. Interestingly, during youth the circadian clock targets genes involved in regulating epidermal cell proliferation, but during age shifts to genes involved in DNA repair and stress, indicating that the circadian program is mutable [170]. In addition to stem cells, cell cycle checkpoints in other proliferating skin cells such as hair follicle precursors are regulated by the circadian clock [113, 159], thereby influencing the growth of hair. Together, these elegant studies have shown that the clock plays cell-specific roles in diverse cells of the skin. Since the intestinal crypt of mammals houses a complex population of stem cells, as well as transit-amplifying progenitors and committed progenitors, the clock could play a highly complex role in the intestinal epithelium. In the intestine it is not yet clear if regulators such as *Wee1*, *P21*, or *P16* cause circadian rhythms in proliferation, and whether these act at the levels of ISCs, or within other dividing intestinal precursors [15, 184]. Future studies will reveal the precise contributions of the circadian clock in different intestinal cells, and how or whether these interact to drive daily rhythms in overall cell cycle.

A third possibility is that the clock might not regulate the cell cycle directly, but through processes such as DNA repair that stall proliferation to correct errors. Several links between DNA damage and repair pathways and the circadian clock pathway have been established, and reviewed elsewhere [185, 186]. For instance, the mouse ortholog of the core clock gene, *Tim*, links DNA repair checkpoint with the circadian clock through Chk1 [187], and similarly, both Per1 and Per3 interact with Chk2 [188, 189]. This would mean that components of the circadian clock, whose levels oscillate during the day, would restrict both single and double-strand DNA repair to enable cell cycle progression at particular times of day. Although these connections have not been tested in the intestine yet, such processes could impact the timing of cell proliferation in tissues like the intestine, that undergo frequent oxidative stresses and subsequent

DNA damage. Indeed, the mouse epidermis that is subject to ultraviolet light-induced DNA stress, shows time of day dependent DNA damage responses [190]. In this case, both the outcomes of DNA damage and the timing of cell proliferation that results have daily rhythms and are clock-dependent. Indeed, *Xpa*, a gene that mediates the excision repair of DNA nucleotides is under circadian clock control in multiple tissues, suggesting DNA repair is a fundamentally clock-regulated process [191–193]. The DNA damage response in the intestine is an interesting potential area of research that could be quite relevant in the intestine where a high metabolic rate, and frequent inflammatory events, can lead to DNA damage. However, it is important to note that the links between cell cycle control and the circadian clock have not been studied in ISCs per se, but rather other proliferating cell types, so it is not yet clear if these connections are relevant to ISCs. Indeed, in intestinal epithelial cells, a definitive link between cell cycle control and the circadian clock has not been observed to date, and it has been proposed that the correlation between the proliferation of intestinal cells and 24-h rhythms is due to other cellular processes [12, 14, 15].

Stem cell self-renewal: Wnt signaling

Wnt signaling is intimately connected to ISC biology and, through ISCs, plays a critical role in the mammalian intestine [194]. The canonical Wnt pathway proceeds from the secretion of Wnt ligand which binds to the *Frizzled* receptor [195], activating the coreceptors *Lrp* [196] and *Dishevelled* [197]. This inactivates a destruction complex composed of Axin, Apc, and Gsk3 to stabilize β-catenin and activate the transcription of genes including *Lgr5*, *Axin2* and *c-Myc*. These target genes are thought to be present in Wnt-receiving cells, including ISCs. In the mouse intestine, Wnt signaling is boosted by R-spondins and their receptor Lgr5 to promote self-renewal of Lgr+ stem cells [198], and intestinal crypt growth and proliferation [199, 200]. In mice, a major source of Wnt ligands are telocytes, a type of mesenchymal cell [201], although Paneth cells and other cell types near the crypt also express redundant Wnt sources [194, 202–205]. In *Drosophila*, the ISC niche also contains redundant Wnt signals originating from the visceral muscles [206, 207] and intestinal progenitors [120]. The Wnt pathway is a conserved ISC self-renewal mechanism.

Several lines of evidence indicate that the circadian clock may regulate aspects of this important signaling pathway. A recent paper by Matsu-Ura et al. used intestinal organoids to reveal a mechanism for how Wnt signaling can be regulated by the circadian clock in the intestinal epithelium [14]. Intestinal organoids are 3-dimensional stem-cell-based cultures that recapitulate aspects of intestinal physiology, such as regeneration and differentiation of epithelial cells [200]. Using an elegant combination of the FUCCI cell cycle reporter (Fluorescence Ubiquitination-based Cell Cycle Indicator—which labels cells with green and red fluorescence depending on their stage in the cell cycle), and the TOP-FLASH Wnt reporter (which reports TCF/Lef transcriptional activity via Luciferase), Matsu-Ura et al. determined that the circadian clock regulates production of Wnt3A ligand by Paneth cells, resulting in 24-h oscillations of signaling activity that couple circadian rhythms to proliferation [14]. Although Matsu-Ura et al. did not find that ISCs themselves exhibited circadian clock activity, clock control of Wnt signaling ISC niche propagates rhythms to these cells extrinsically. Along these lines, in the epidermis, ChIP analysis has shown that Bmal1 binds rhythmically to Wnt pathway related gene promoters suggesting that the clock may modify sensitivity to Wnt signals [154]. In muscle, fat, and mesenchymal cells, the clock components Bmal1 and Reverb also have been shown to transcriptionally target various components of the Wnt pathway [208–211]. Although these studies were not carried out on stem cells, they raise interesting possibilities of how the circadian clock can regulate ISC behaviour by driving intrinsic cellular processes. The intestinal organoid system provides a means to investigate these processes and is amenable to studies of circadian clock function [13, 15]. Of note, *Per2* and *β-catenin* have been suggested to be mutually inhibitory in the intestinal epithelium, with Per2 normally downregulating β-catenin [212], while high β-catenin levels tipping the balance to result in Per2 loss [213]. The mutual negative regulation of key components of the Wnt and circadian clock pathways in the same cell, once again underscores possible bidirectionality of cellular signaling processes and circadian clock function.

Downstream components of the Wnt signaling pathway have been shown to be regulated by the circadian clock, including target genes such as *c-Myc*. *C-Myc* is a transcription factor that regulates ISC biology [214] and shows circadian rhythms in its expression in several other tissues [215, 216]. *C-myc* is also interconnected with the circadian clock, like Clock/Bmal1, c-Myc binds to E-boxes in the genome where it dampens Bmal1-driven rhythms of *Reverb* expression [217], and/or opposes Bmal1/Clock at E-boxes [218, 219]. Of note, the circadian clock can also influence *c-Myc* expression through Bmal1 binding of the *c-Myc* promoter [218]. Overall, these studies highlight the possibility that c-Myc and the clock could oppose one another's activity, and notably in the intestine *c-Myc* is highly expressed by proliferating crypt cells [220]. It is thus possible that circadian clock activity is weaker in the base of the crypt where Wnt signaling is highest, where ISCs are themselves located, in line with reports that stem cells do not have clock function [14, 168]. A bidirectional interaction between the circadian clock and c-Myc may enable a balance between circadian

clock control and stem cell driven processes [221], to coordinate environmental signals with intestinal epithelial tissue renewal. Another link may exist between the Wnt pathway regulator, Gsk3b, which inhibits Wnt signal transduction but also regulates the phase of clock activity [222, 223]. However, again it is important to note that these ideas remain to be tested directly in ISCs, rather than other cell types. Circadian regulation of Wnt signaling in ISCs is a relatively unexplored area with many opportunities for future study.

Control of intestinal differentiation: a new role for the circadian clock?

The ISC niche maintains intestinal precursors in an undifferentiated and proliferative state; outside the niche, cell signaling processes cause the progeny of ISCs to differentiate. This delicate balance of ISC differentiation could be another mechanism through which the circadian clock regulates ISCs, and early studies indicated that crypt cell number exhibits daily variation [224, 225]. The Bmp pathway plays a role in intestinal morphogenesis [226–228] and is a positive regulator of differentiation that opposes Wnt signaling during ISC niche formation [229, 230]. Loss of function mutations in *Bmp* have been shown to increase intestinal cell proliferation in mice [231] and *Drosophila* [115]. In mammals, Bmp ligands are found primarily in the villus region, where differentiated intestinal cells reside, whereas the Bmp antagonist, Noggin, is found near the crypt base, where ISCs are present [228, 232–234]. In the simplified niche of *Drosophila*, Bmp signaling is found primarily in the visceral muscle where it regulates stem cell division following regeneration [115, 235]. Is Bmp signaling regulated by the clock? Bmp pathway components are transcriptionally targeted by Bmal1 in epidermal stem cells in mice [154, 155]. Indeed the circadian regulation of the Bmp pathway to control differentiation has been documented in both fat and tendon tissues [236, 237]. Although it has not been studied in the intestinal crypt thus far, circadian clock regulation of the Bmp pathway is a potential mechanism.

Other pathways including the Mitogen-Activated Protein Kinase (Mapk/Erk) pathway, and the Notch pathway, also influence the balance between differentiation and proliferation in the intestinal epithelium. While it is beyond the scope of this review to fully cover these, in the *Drosophila* intestine, Mapk/Erk signaling is well-established to regulate ISCs proliferation and rapid differentiation of progeny during the stress response [114, 238–240]. In the mouse intestine this same signaling pathway also promotes proliferation [200, 205]. The Mapk/Erk pathway interacts with the circadian clock in many systems [241], and has been recently shown to promote *Tim* expression, and regulate the proliferation of cancer cells [242]. Its role in integrating environmental stresses in ISCs, as well as its role in modifying the circadian clock, warrants examining whether the Mapk/Erk pathway is regulated by the circadian clock in ISCs.

The Notch pathway has a complex role in the differentiation of ISC progeny in both mammals and *Drosophila*, overall pushing cells toward the enterocyte cell fate in the intestine [101, 123]. Human epidermal stem cells have daily rhythms in the expression of Notch pathway components [155], and *Per3* overexpression in cancer cells decreases Notch signaling components [243]. This suggests that Notch-driven ISC differentiation also could be regulated by the circadian clock. However, as is the case with Wnt signaling, these pathways can be bidirectional, as disruption of Notch signaling in *Drosophila* ISCs results in arrhythmic clock activity [16]. Overall, the connections between ISC differentiation, clock activity, and the regulation of cell fate by the clock are worthwhile to consider. Indeed in the epidermis, the transcription factor *Klf9* establishes a precedent, as it functions downstream of the clock to drive rhythms in the differentiation of skin cells [160].

Feeding and the regulation of cell growth

The intestine receives food to digest and absorb, and it is highly sensitive to the timing of food intake. Normally the central pacemaker in the suprachiasmatic nucleus and peripheral pacemakers in the digestive tract work in concert together, with periods of activity determining the time of food intake and subsequent changes in hormone levels. However, when food is presented during periods of inactivity, peripheral digestive system clocks are synchronized independently from the central clock [85]. This is in part due to the levels of circulating insulin, which is elevated during feeding, and which is a strong entrainment cue for peripheral tissue cell clocks [93, 95]. Strikingly, it was recently shown that daily changes in proliferation of colon cells could be restored in *Clock* mutant mice under a restricted feeding paradigm [244]. Thus, the time of food intake can set into motion circadian-dependent signaling processes that could affect ISC biology. Insulin was recently shown to directly synchronize intestinal organoid cultures [95], making it a highly relevant candidate mechanism of how the circadian clock impact ISC activity.

In *Drosophila* insulin induces ISCs to grow and proliferate, because it is a signal of overall nutrient levels in the body that guides tissue size expansion [107, 245–247]. Tissue growth is particularly important during development and regeneration, and insulin signaling is critical to increase intestinal cell production at these times [245, 247]. However, in mammals the role of insulin signaling is not altogether clear. Insulin can affect mammalian ISC function, both directly and indirectly, through activation of the insulin pathway component *mammalian Target of Rapamycin* (*mTOR*). During caloric restriction, Paneth cells, that are

an important part of the ISC niche, activate mTOR to augment ISC number [248]. In ISCs, mTOR can also cooperate with SIRT1 to enhance mTOR activity cell-intrinsically during calorie restriction [249]. In both of these cases, calorie restriction (and thus presumably lower insulin) paradoxically increases ISC number but not necessarily tissue size [248]. Yet another phenomenon can also occur in this complex system during another physiological context: regeneration following acute fasting. In this case, a different population of non-Lgr5+ ISCs is activated to drive proliferation through mTOR to presumably restore tissue to its normal size following a period of fasting [250]. The precise role of the insulin pathway in mammalian ISCs is not wholly consistent between *Drosophila* and mice, but taken together the pathway appears to regulate the ISCs of the intestinal epithelium in a context-specific fashion, and its connection to the circadian clock, feeding behaviour, and digestive tract physiology, make it an attractive candidate for future studies. Indeed, the finding that Bmal1 is phosphorylated by the mTOR target S6K, to act as a positive regulator of translation, implicates the circadian clock system with the timing of protein production from mRNA [251]. This fundamental process means that the clock not only drives rhythms in transcript abundance but, in cooperation with insulin signaling, boosts the production of proteins from these transcripts.

Inflammation and the immune system

The immune system is highly active in the digestive tract, where many types of white blood cells interact with the microbiome of the intestinal lumen. Detection of pathogens by immune system cells stimulates an inflammatory response, that subsequently affects the intestinal epithelium. In *Drosophila*, bacterial infection results in large changes in epithelial gene expression [252] and activation of the Janus Kinase/Signal Transducers and Activators of Transcription (Jak/Stat) pathway [253]. It is now well accepted that the Jak/Stat pathway functions in *Drosophila* ISCs to both promote differentiation of enterocytes [254] and rapid ISC proliferation during an inflammatory response [111, 112, 245, 253]. In this system, the cytokines Upd1-3 function to activate Stat in ISCs, coordinating the stress/infection response to ISC proliferation to replace damaged epithelial cells. In mammals, the Jak/Stat pathway is conserved, where the cytokines IL-6, IL-22, and the transcription factor Stat3 play a similar role in driving the proliferation of Lgr5+ ISCs during inflammation [255–257]. These studies reveal that the intestinal epithelium receives and translates pro-inflammatory signals into a potent ISC regenerative response.

In this context, it is important to note that many immune system functions have circadian effects including white blood cell proliferation [258] and circulation [259], and the susceptibility to infections [260, 261]. In *Drosophila*, the time of pathogen infection dictates survival and the immune system response downstream of the circadian clock [262, 263]. In mice, monocytes [264], macrophages [265, 266], natural killer cells [267] and T cells [268, 269] have all been shown to possess cell-intrinsic circadian clocks. This is particularly well-established in macrophages, where approximately 8% of protein-coding genes including immune response, cytokine transcription and cytokine stability have circadian rhythmicity [265]. Pro-inflammatory interleukins, such as IL-6, in macrophages mediate time-of-day-dependent inflammatory responses that determine the body's response to infection [270]. Circadian timing of the immune system output has emerged as an important physiological mechanism, that adjusts inflammatory timing to maintain homeostasis effectively [271–273].

Several studies have recently addressed the role of inflammation in digestive tract circadian clocks. Immune system factors involved in the detection and clearance of infections, including *Toll-like receptors* and *Cryptidins*, display daily rhythms in the intestine [274, 275]. Furthermore, inflammation and cytokine expression are affected by the time of infection [276], and direct the stress response of the damaged intestinal epithelium. This results in different outcomes when mice are infected by gastrointestinal bacteria at different times of day, highlighting the circadian-dependence of the inflammatory response. Two recent studies have determined that the inflammasome, a sensor of bacterial or damage-related stresses, is regulated by the circadian clock through the inflammasome component, *Nlrp3*. In the liver, circadian regulation of the inflammasome in macrophages primes the sensitivity of the tissue to acute toxic insults [277], and in the colon, acute colitis is worsened due to inappropriate *Nlrp3* expression [278]. In both of these cases, *Nlrp3* is a direct transcriptional target of Reverb, that is itself rhythmically expressed by the core circadian clock. This means that in peripheral tissues like the intestine, the circadian clock adjusts sensitivity to pathogenic responses according to time of day. Indeed, other components of the innate immune system in the intestine have been shown to oscillate under Clock/Bmal1 according to time of day [279], and cytokines such as Tnf are also under circadian clock regulation to drive daily rhythms in intestinal epithelial proliferation [15]. This means that the intestine is likely to time the stress response as well as metabolic processes, such as digestion and feeding, although it is not clear whether to coordinate or separate these two functions. In the nearby colon, the regenerative response to acute inflammatory bowel disease has a clock-dependent phenotype as well [26]. In this case, a mouse model indicates that *Per1/2* circadian clock mutants are highly susceptible to acute colitis, and have a poor regenerative response, consistent with *Reverb/Nlrp3* studies [278]. Importantly, mice simply exposed to an altered photoperiod resembling shift-work

show these same effects, suggesting this is in fact a *bona fide* circadian phenomenon rather than a non-clock role for circadian clock genes [280]. Although these studies have not yet examined the inflammatory responses of ISCs directly, it is likely that these cells respond to the timing of cytokine release from the surrounding immune system cells.

Another possible mechanism that might regulate ISCs in the intestinal epithelium, is the microbiome, a topic of growing interest in the circadian biology field. The intestinal lumen contains a tremendous population of diverse microbiota which interacts with overall animal physiology [281, 282]. Recent research has shown that intestinal microbiome composition varies over the course of the day and the microbiome can influence the host intestinal transcriptome [281]. This is in part because all animals exhibit circadian rhythms in food intake, carried out when they are active, that provide sustenance to the microbiome resident in the digestive tract. The microbiome in their turn influence host response and physiology. However, in the intestinal epithelium the clock target gene *Nfil3*, coordinates rhythms in the absorption of lipid nutrients, but requires microbiota to be present, revealing that microbiota is an active player in regulating circadian physiological rhythms [283]. Indeed, there are both evidence that the microbiota are required for normal circadian transcription rhythms in the intestine [284], and evidence that the clock functions independently of microbiota [139]. Many questions in this area need to be resolved, as the circadian timing of microbiotal activity clearly influences transcription and subsequent physiological responses in the intestinal epithelium [281]. A recent study has shown that microbiota is a source of important sex-specific signals, that affect male versus female daily microbial rhythms and sex-specific growth hormone production [139]. The role of the microbiome in regulating organism physiology is an exciting area of research. The precise mechanisms connecting the microbiome and animal physiology are poorly understood, and how these may affect ISC function are an open question.

Circadian rhythms and gastrointestinal disease

Human beings experience both photoperiod and food-induced circadian clock entrainment. In modern society, human circadian clocks are subject to socio-economic factors such as shiftwork, travel across time-zones, and the use of artificial light sources, that influence their synchronization. In shift workers, who regularly experience disrupted circadian rhythms, meta-analyses of clinical studies have revealed that there is an increase in various gastrointestinal disease symptoms such as pain, and inflammation [285]. In particular, two specific diseases increased in shift workers that are relevant to stem cell biology include colorectal cancer [286, 287], and inflammatory bowel disease (IBD) [288].

Colorectal cancer incidence has been examined extensively in the nursing profession, and a significant increase in cancer risk has been found in nurses that perform shift work for > 15 years [286, 289]. However, no significant trends were observed in nurses that perform sporadic shift work, or fewer years of shift-work, suggesting that only long-term circadian disruption elicits these effects. In colorectal cancer cells themselves, circadian clock genes have also been found to be dysregulated [23] with reduced *Per1* levels [290–292], and mutations in *Per1* [293], a core genetic component of the circadian clock. *Rorα*, which regulates *Bmal1* expression, has also been correlated with increased risk of colorectal cancer [294]. In addition, there are reports regarding the expression of the clock genes *Bmal1*, *Cry1* and *Cry2* in colorectal cancer. Decreased *Bmal1*, *Per1-3* and *Cry2* levels have been observed in some studies [291], while others report decreased *Per1* and *Per3* levels and increased *Clock* expression [292], or increased *Cry1* expression [295]. It is not clear why certain cancers show either increases or decreases in the expression of various clock gene alleles. This seemingly conflicting data could be simply due to heterogeneity within tumours, or variation between patients. It may reflect either clock gene mutation as a promoting factor in colorectal tumorigenesis during disease progression or as a passenger mutation effect of genetically unstable cancer cells. However, it is important to consider circadian dysfunction as a contributing factor to cancer growth, because the outputs of the circadian system can have multiple pro-growth and pro-metabolic effects on proliferating cells. The connection between many types of cancer and the circadian clock has been reviewed extensively, and both epidemiological and basic research suggests aberrant clock function is likely to be pro-tumorigenic [296, 297].

IBD involves the chronic inflammation of the gastrointestinal tract, that is present in two subtypes of IBD: Crohn's disease and Ulcerative Colitis. A large (> 1000 people) occupational risk study analyzing health benefits in Germany has linked occupations with irregular shift-work to higher rates of IBD [288]. Socio-economic factors that alter circadian rhythms, such as poor sleeping habits on weekends, poor food timing, and sleep debt, have also been linked to augmented IBD symptoms in a study of 115 IBD patients [298]. This suggests that disruption of circadian rhythms is one of the many factors that may influence IBD. Transcript analysis suggests that circadian clock genes, including *Per1*, *Npas2*, *Cry1*, and *Rorα*, have altered expression in intestinal mucosa biopsies from IBD patients [299, 300], as well as lower expression of many clock genes in their peripheral blood cells [300]. Young, untreated patients recently diagnosed with IBD also show low clock gene expression in the intestinal mucosa and white blood cells [301]. Taken together, this

indicates that circadian dysfunction in white blood cells is a feature of IBD, and it remains to be determined whether the same is true for the different cells of the epithelium, and surrounding lamina propria. The precise mechanisms linking circadian clock function to IBD remain to be tested, but given its role in regulating the immune system it is highly plausible that circadian disruption would increase inflammation in IBD patients [302–305]. Taken together, many studies suggest a role for circadian rhythms in gastrointestinal health and disease.

Conclusion

A healthy digestive tract is central to overall health. The high cellular turnover of the intestine in response to daily damage depends on a population of ISCs, that are highly sensitive to cell signaling pathways activated according to physiological need. Many of these pathways have shown circadian oscillations in *Drosophila* and mice which supports a role for the circadian clock in modulating the responsiveness of ISCs according to time of day. As well, the circadian clock has been shown to regulate a number of intrinsic cellular processes including growth, proliferation, differentiation, and DNA damage, which are likely to be, in part, downstream of cellular signals. In essence, the circadian clock regulates both external physiological processes and internal processes in cells that establishes temporal connections between a specific cell type and the body. Although a number of studies have addressed these ideas in other cell types, much work needs to be done specifically in ISCs to test these relationships. Further research will explore these possible connections, and provide critical information about the molecular details of the circadian biology of ISCs.

This work is highly relevant in understanding how the circadian clock impacts the health of the intestine, in biology, medicine, and evolution. Why does the body coordinate the timing of intestinal processes? One possibility is to increase certain physiological mechanisms to occur at optimal times of day. For instance, coordinating the time of cell growth and division with the time of activity and feeding, so that nutrients are readily available when growth is highest. Another possibility is that the circadian system coordinates the anti-phasic timing of incompatible processes. For instance, separating the timing of cell growth and division from the timing of inflammation and DNA repair. These possibilities are not mutually exclusive and speak for a role of the circadian clock in optimizing health and fitness according to a 24-h schedule that is the result of millions of years of evolution. In this sense, the study of circadian clock regulation of ISCs can be an excellent model system to address fundamental questions about the biology of circadian systems in general. These same ISC-related processes are also relevant to our understanding the connections between ISCs and diseases such as colorectal cancer and IBD.

It is clear that these studies are highly impactful in both research and medicine. Circadian biology has aptly demonstrated that, in the life sciences, the time of sample collection is a critical variable. As a gene of interest expression and activity oscillates according to time of day, interactions that occurred at one time may not be present at another. Circadian rhythms may function to synergize or separate incompatible biological processes within the same cell, or between cells of the body. As this field matures, it is important that such a fundamental system is considered in many if not most life sciences research. For medicine, these concepts provide important information for both the prevention of disease and the treatment of disease. The concept of chronotherapy refers to the application of medical treatments, such as the timing of drugs, according to an optimal physiological schedule. This has been supported experimentally [17, 306], and is an important consideration in medical practice. In addition, knowing that human activities such as shift-work, travel across time zones, and social jetlag (the weekly shifting of sleep/wake schedules from weekday to weekend) affect circadian rhythms, implies that these activities are unhealthy. This adds circadian disruption to a list of avoidable social behaviors, especially in individuals that might have a genetic propensity in developing certain diseases shown to have a circadian component. The role of the circadian clock in gastrointestinal research and health is an exciting area for future research with tremendous potential.

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